Prion2006 Abstracts

Welcome Address

Dear colleagues:

on behalf of the European Network of Excellence NeuroPrion and the Italian prion researchers, we are happy to welcome you in Torino to the international conference “Prion 2006: strategies, advances and trends towards protection of society”.

The aims of the European Network of TSE researchers are to integrate and coordinate the research efforts of their members in prevention, treatment, control and management of prion diseases and to avoid future crises related to prions.

On the heels of Paris 2004 and Düsseldorf 2005, Prion 2006 is a further step in the better co-ordination and reinforcement of international research activities.

It was your contribution that enabled us to put together a programme of exceptional scientific excellence which will be equally attractive in the different areas of prion research. The scientific programme of “Prion 2006” includes 5 plenary lectures, more than 50 oral presentations selected from almost 400 abstracts, and poster sessions on the classical themes of the NeuroPrion network (i.e., prevention, control, treatment, management and risk analysis of prion diseases). This event provides a great opportunity for scientists from all over the world to share their thoughts, findings and progress in this attractive and interesting setting.

The meeting is also an opportunity to know one of the nicer town in Italy. Torino was the first Italian capital and a lot of beautiful memories passed on through buildings, royal residences and gardens in the heart of the city. Torino is a treasure of historic cafes, located throughout the twelve kilometers of arcades that wind through the center’s streets and squares.

We would like to thank the Italian Ministry of Health, the Regione Piemonte, the City of Torino, the CRT Foundation, the European Commission and a number of industrial sponsors for their generous funding of this great event.

We wish you all a pleasurable and inspiring time in Torino!

Maria Caramelli, Torino
Gianluigi Forloni and Fabrizio Tagliavini, Milano
Jean-Phillippe Deslys and Jens Schell, Paris

Content

NEUROPRION – A JOINT FORCE OF EXCELLENCE:

Constituted in September 2003, NeuroPrion is a self-managed network of prion researchers from 52 public research organisations in 20 European countries. With more than 120 individual research groups the network covers more than 90% of the leading research groups in Europe. Aiming at coordinating and integrating the research efforts of the different teams, NeuroPrion constitutes a first approach towards a new European Research Area open to collaboration with prion researchers from all over the world.

PRION CRISES – FROM REACTION TO ANTICIPATION

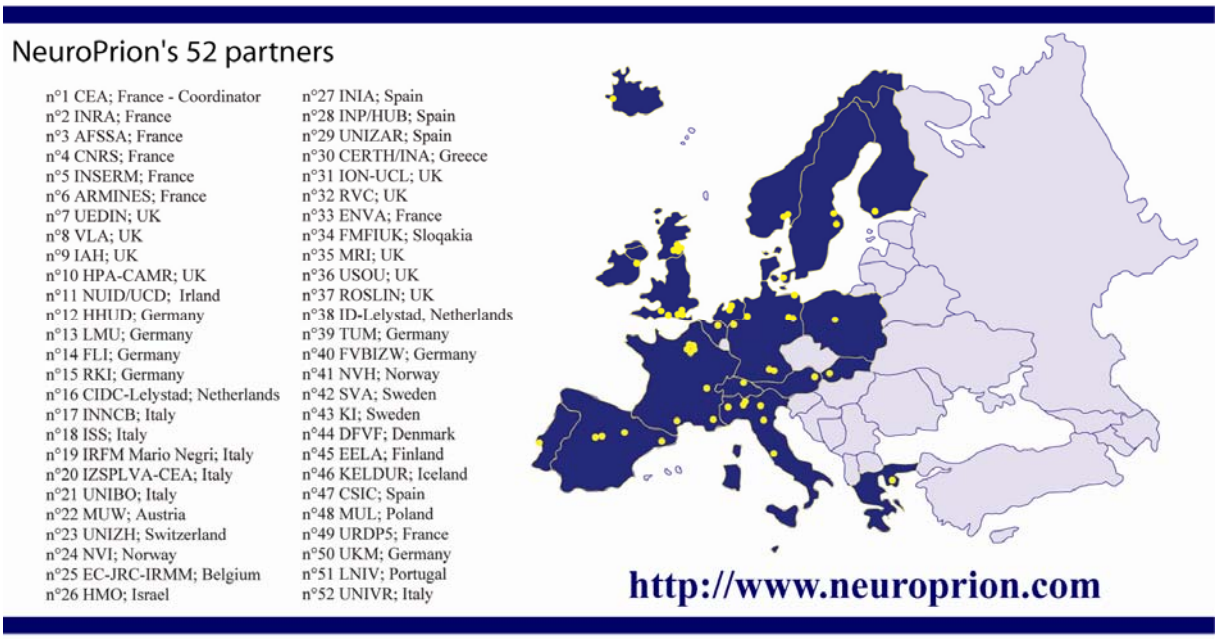
Since the appearance of the first cases of 'mad cow disease' in the 1980s, prion diseases have become a major problem for society both in Europe and worldwide, with important health and economic consequences. The total cost of the BSE crises in Europe is estimated to be more than €90 billion since 1996, while just a fraction of this sum invested into research. The recent and continuing BSE crisis in Canada has resulted in an estimated loss of more than $12 billion since 2003. In the last decades Prion research has led to major advances in the understanding of these diseases, enabling European and National decision makers to properly manage the crisis and to reduce the negative impact on society. The efficient cooperation between science and regulatory authorities has resulted in better consumer protection and traceability of animal products. Thus, in parallel to a decrease in the total numbers of cattle and human cases , the public perception of the risk related to

prions has also declined . Today, however, many questions remain. Fundamental research is crucial to understanding the processes of infection, replication and pathogenesis of these lethal diseases, which infect humans and animals with transmissible agents that are resistant to almost all classical decontamination procedures .

The recent discovery of previously unrecognised atypical cases of scrapie in sheep and atypical BSE in cattle in Europe and the US has raised concerns that previously unidentified strains of prions may pose new risks for public health. Furthermore, the compelling evidence that prion diseases are transmissible through blood transfusion, indicates that research on prion disease must continue to progress in order to ensure the safety of public health.

Today, thanks to NeuroPrion, the combined know-how of the main prion research teams constitutes a unique core of expertise putting science at the service of society in order to prevent new crises. However, to improve the level of protection needed by society the time frame of research, which is very different than that of the media and/or politics, must be taken into account,. The development of an adequate knowledge base and provision of concrete solutions to society's needs will require continuing research support for at least the next 15 years. In prion diseases, history has already demonstrated that anticipation is more effective and less cost-intensive than only reacti to crises.

Furthermore prion research, with its original models and approaches, has already started to influence related research in pathologies such as Alzheimer's disease and major discoveries will be presented at NeuroPrion's next congress with implications for public health, which will need careful assessment.

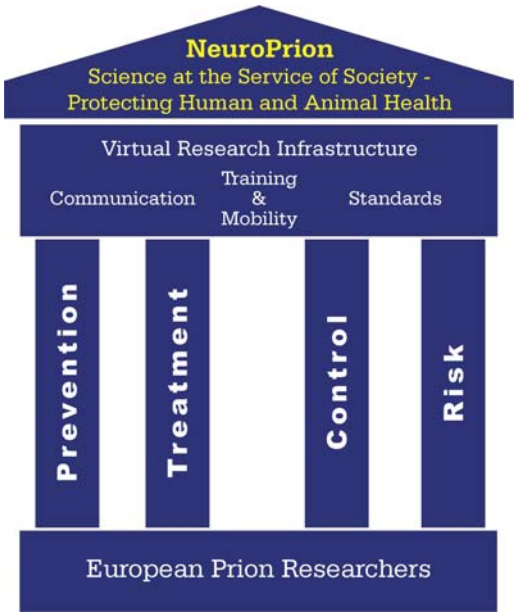


NEUROPRION – MORE THAN A FLEXIBLE STRUCTURE AND MORE THAN RESEARCH

Prion research is at the heart of the NeuroPrion consortium.. However, research and research policy are changing in the global context and researchers must adapt to new developments. NeuroPrion has identified areas where greater coordination of research would be beneficial and others where novel, applied research is needed. Based on these assessments, NeuroPrion has implemented a joint programme of activity, which is built on the following four research pillars:

PREVENTION:
Decontamination
Diagnosis
Differentiation of strains

TREATMENT:
Development of New Drugs
Definition of New Targets



CONTROL:
human and animal Surveillance
human and animal Tissues Bank

RISK:
Communication & Management
Assessment & Factors

For efficient management of these research activities, NeuroPrion has created a new virtual research infrastructure to facilitate collaborations, exchange of results, know-how and samples. Together the members of the network are able to respond efficiently to the requirements of society. The excellence of the Network is also enhanced by joined training and mobility activities in order to render prion research more attractive and to help new young researchers.

Another main priority of NeuroPrion is communication not only to scientists, but also to authorities, stakeholders, industry and citizens; NeuroPrion's annual congresses have become a major forum for knowledge dissemination and cover an increasing range of scientific issues.. Together in Neuroprion, we contribute to the protection of public and animal health, to the avoidance of future prion crises, and to putting Science at the service of Society.

Oral Presentations

ORAL-01

VARIANT CJD: 10 YEARS AFTER

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The identification of variant Creutzfeldt-Jakob disease (vCJD) depended on the systematic characterisation of suspected cases of human prion disease throughout the UK and comparison of data with other European countries in which systematic surveillance of CJD was also being undertaken. The collaborative study of CJD has been funded by the EU since 1993 and this has allowed accurate international data on vCJD to be provided to the scientific community, policy makers and the general public.

This has been important because vCJD is a fatal zoonosis caused by infection with the agent of bovine spongiform encephalopathy. The implications for public health have been significant because of the long interval between exposure and disease expression, the probable transmission route through the human food chain and the probability of transfusion transmission of infection. Concerns have lessened in the past few years because of the decline in BSE epidemics in most countries and the relatively limited and declining outbreak of vCJD in the UK. An important question is whether or not it is appropriate that public health concerns regarding vCJD may also be in decline.

There are many remaining scientific uncertainties. Why is there a mismatch between the extensive human exposure to the BSE agent, the estimated prevalence of infection and the actual observed number of cases of vCJD? Will there be further outbreaks related to variation in human *PRNP* genetic background? Will other mechanisms of transmission be identified, for example via contaminated surgical instruments or via a maternal route? Will further countries be affected and have cases caused be exposure to non-UK indigenous BSE yet been observed? Is there a possibility that newly identified forms of animal disease such as BASE or atypical scrapie have implications for human health?

These important questions should be addressed before there is complacency about the potential public health implications of prion diseases and continuing international collaboration may still have important contributions to make in the study of natural diseases.

ORAL-02

BSE IN THE WORLD

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Few months ago a press release was issued by the UN Food and Agriculture Organization (FAO) with an encouraging title: “Mad cow disease on the wane worldwide”. The FAO focussed on the period between 2003 and 2005 when the BSE epidemic was dropping at the rate of 50 percent a year. It holds also for the current year with only about 200 cases confirmed so far.

Those figures are the result of a huge number of animals recruited by the international surveillance despite large differences among nations. For instance in the US since June 2004 some 785,000 cattle, most of them fallen stock, have been tested; the effort in the European Union (EU) has been tremendous involving more than 21 million cows in the last 2 years.

To gain a throughout insight in the spread and recent trend of BSE, many sources of data are available and have been looked up for this review. The European Commission has issued annual reports of the monitoring activity since the implementation of the EU-wide active surveillance in 2001 and similar data are available for extra-EU countries. A parallel work of data collection involving 64 countries of the five continents has been carried out to assess the geographical risk of BSE (GBR): it provides fundamental information on the spread of the disease, in particular where few or any surveillance data were made available. Meanwhile the GBR methodology is being reviewed and new assessments are ongoing.

At the moment, comparisons are not easy to carry out and the researcher must be cautious. Data provided by the countries are heterogeneous in their quality, the enforced surveillance systems differ each other both in term of criteria applied and efficacy (even within the EU!), only unadjusted prevalence/incidence rates are available and finally population data often are missing.

What we are observing is a real overall declining trend associated with a decrease in the prevalence in the youngest birth cohorts. However some alarming exceptions are evident and the major risk is in the ongoing will of softening part of the measures of known efficacy.

ORAL-03

THE EPIDEMIOLOGY OF SCRAPIE

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Whether a sheep can acquire scrapie infection or, following infection, proceed to clinical disease, is affected by different alleles of the sheep *PrP* gene. An aspect of scrapie genetics that has attracted relatively little attention is the different patterns across breeds. First, the five major PrP alleles are present at very different frequencies within different breeds. The origins of these differences are unknown, but may reflect the different breed origins (phylogeny or founder effects), genetic drift following reproductive isolation or, perhaps, are a response to different scrapie histories.

There is also variation between breeds in the genotypes that succumb to scrapie, with an unknown biological basis. They may reflect a variety of strains of scrapie, with different genotype-tropisms, circulating in different breeds; alternatively, they may indicate certain innate differences between breeds in their responses to scrapie infection.

In this talk I present the results of an analysis of several UK-derived datasets on scrapie in different breeds of sheep and their underlying genotype frequencies. The purpose is not to provide explanations, but to identify the patterns that need explaining and measure their robustness.

Breed differences notwithstanding, the strong genetic control of sheep in susceptibility to classical scrapie has led to the possibility of its control by selective breeding. A two pronged-approach is now being applied across the EU and elsewhere: (1) culling of all sheep or all but the most genetically-resistant sheep, within scrapie-affected flocks; (2) culling of the most susceptible sheep within unaffected flocks of high genetic merit.

A genetic approach to control within scrapie-affected flocks is both appropriate and effective. The application of large-scale selective breeding to unaffected flocks may make them resistant to becoming newly infected by classical scrapie. However, this latter approach will also reduce the overall variability of the ovine *PrP* gene in our sheep populations and it is worth asking, therefore, why this variability arose in the first place and why it has persisted until now. One possibility is that the gene has evolved under 'negative frequency dependent selection'; ie, resistant genotypes are resistant to only some scrapie strains and as one 'resistant' genotype becomes more common, so it becomes the target of a novel strain. 'Atypical' scrapie may be one such novel strain, targeting the alleles that confer resistance to classical scrapie.

If this hypothesis is correct, current selective breeding programmes may provide a short-to-medium, but not a long-term solution for scrapie control in sheep. Accordingly, the preservation of variability at the ovine *PrP* gene may be worth increased consideration.

ORAL-04

EPIDEMIOLOGY OF CHRONIC WASTING DISEASE IN NORTH AMERICAN CERVIDS

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Chronic wasting disease (CWD) occurs naturally in North American deer (*Odocoileus* spp.), wapiti, and moose (collectively called "cervids"). CWD presently occurs in scattered foci throughout North America, both in the wild and in commercial facilities. CWD is contagious among its natural hosts, and epidemics can persist under both captive and free-ranging conditions, resulting in remarkably high infection rates. The precise mechanism of contagion remains unclear, although accumulations of disease-associated prion protein (PrP^{CWD}) in lymphatic tissues associated with the gastrointestinal tract suggest shedding via feces and perhaps saliva. Analyses of epidemic data suggest that indirect (animal–environment–animal) transmission may be the dominant force in epidemic dynamics, and the CWD agent has been shown to persist in environments contaminated by excreta or carcass remains for years. Variation in cellular prion protein appears to influence CWD pathogenesis, and may provide a biological mechanism for emergence of variant strains within and among the four naturally susceptible species. The long-term implications of CWD for public, livestock, and wildlife health remain uncertain. Unfortunately, limitations of existing technology available to combat prion diseases make control of CWD ineffective or infeasible under most conditions.

ORAL-05

EPIDEMIOLOGY OF HUMAN TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

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An international study of the epidemiologic characteristics of human transmissible spongiform encephalopathy (TSE) diseases was established in 1993 and included national registries in France, Germany, Italy, the Netherlands, Slovakia, and the United Kingdom. In 1997, the study was extended to Australia, Austria, Canada, Spain, and Switzerland. Data were pooled from all participating countries for the years 1993 to 2004 and included deaths from definite or probable TSE diseases of all etiologic subtypes. Five thousand six hundred fifteen cases were available for analysis and included 4,727 cases of sporadic Creutzfeldt-Jakob disease (CJD), 560 genetic cases, 170 iatrogenic cases, and 158 variant cases. The overall annual mortality rate between 1999 and 2004 was 1.68 per million for all cases and 1.42 per million for sporadic CJD. There was heterogeneity in the distribution of TSE cases by etiologic subtype.
Data for the analyses of predictors of survival were available in sporadic (n = 4618), iatrogenic (n = 163) and variant Creutzfeldt–Jakob disease (n = 158), and in cases associated with mutations of the prion protein gene (n=530). Overall, survival for each disease type was assessed by the Kaplan–Meier method and the multivariate analyses by the Cox proportional hazards model.

ORAL-06

TSE RISK ASSESSMENTS – AN OVERVIEW

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Risk Assessment as we now recognise it has its origins in the risk assessments carried out for the US nuclear power programme in the 1970s. It was taken up and developed as a tool to help manage major hazards in the process industries and has now become a ubiquitous part of our modern life. Risk assessment is firmly embedded in the approach taken by the European Union and other countries to a broad range of policy issues. Risk Assessment has been part of the approach to managing Bovine Spongiform Encephalopathy (BSE) since it was first identified as a new disease in cattle in 1986, but it was not until 1996, following the discovery of variant Creutzfeldt-Jacob Disease (vCJD) and its possible link to BSE, that quantitative risk assessment was used to assess the potential exposure of people to the BSE infective agent. Since 1996 there have been many risk assessments carried out as part of decision making processes on BSE and the other TSEs; for example: decisions on whether to ban beef on the bone in the UK (1997), the replacement of the over thirty month rule by BSE testing (2003), the age limit in cattle for the removal of SRM (2005). As the risk from BSE reduces in Europe it is expected that there will be a gradual relaxation of some of the control measures that have been put in place to protect human health as set out in the EC's TSE Roadmap published last year. It is more than likely that any significant change will need to be supported by a re-assessment of the risk so that such changes can be justified. In carrying out a TSE risk assessment it is important that the uncertainties and limitations of the data used are recognised and incorporated in the assessment as far as possible. This is complicated because some of the factors remain difficult to quantify and are still subject to scientific debate, e.g., the amount of infective material required to infect a human. Despite such limitations, quantitative risk assessment can still provide valuable inputs to the decision making process.

ORAL-07

RISK LINKED TO BLOOD

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Although Blood Services have taken a precautionary approach over the past 10 years in relation to the possibility that variant CVD may be transmissible by blood and tissues, emerging evidence that there may be a significant cohort of individuals with sub-clinical disease, along with three cases of transmission of variant CJD prions by blood components, has increased concern that blood transfusion and tissue transplantation may provide a route to extension of the outbreak of this disease. The exact level of risk remains difficult to assess because of continuing uncertainties around the prevalence of sub-clinical disease, the concentration and distribution of infectivity and the overall transmissibility of variant CJD by these routes. Similarly, the extent to which current risk reduction measures including donor selection and universal leucodepletion are effective in reducing the risk of transmission is unclear. Newer technologies including prion reduction filters and peripheral blood prion assays may provide a significant improvement in management of this risk, but there are problems in terms of validation, countervailing risks and ethical and social considerations.

ORAL-08

A QUANTITATIVE ASSESSMENT OF THE RESIDUAL BSE RISK IN BOVINE DERIVED PRODUCTS AND THE RISK MANAGEMENT ACTIONS IN EUROPE BASED ON THIS ASSESSMENT

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The European Food Safety Authority (EFSA) main tasks consist of issuing scientific opinions or conduct risk assessment on all matters linked to food and feed safety, including animal health and welfare, food-borne zoonoses and plant protection. It shares a responsibility on risk communication with the European Commission who acts as risk manager.
Concerning Bovine Spongiform Encephalopathy (BSE) in the EU, we currently have come to a stage that amendments of certain measures could be envisaged without endangering the health of the consumer or the policy to eradicate BSE. In that light EFSA was asked to assess the validity of the outcome of a previously conducted quantitative assessment of the residual BSE risk in bovine derived products, carried out for gelatine, tallow and dicalcium phosphate from bones, tallow from fat tissues and tallow from rendered mixtures of tissues, and for the presence of small amounts of meat-and-bone meal in feeding stuffs intended for ruminants.
A stochastic quantitative risk assessment model has been developed and was fed with relevant data and expert opinion. The description of the model and the opinions derived from the output can be found on the EFSA website.
We will discuss this quantitative assessment and focus on the assumptions and inputs of the model. Following this, the presentation of the results to the risk managers and the interactive nature of that process are highlighted including different difficulties encountered and the approaches used to make it clear for the risk manager.
Finally we discuss the risk management actions on European level that are or will be taken based on this assessment.

ORAL-09

THE OIE SYSTEM FOR THE EVALUATION OF COUNTRY STATUS FOR BOVINE SPONGIFORM ENCEPHALOPATHY

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The *Terrestrial Animal Health Code* of the World Organisation for Animal Health (OIE) (Chapter 2.3.13) makes provision for the BSE risk status of the cattle population in a country, *zone* or *compartment* to be determined on the basis of a risk assessment and other evaluation criteria described in the *Terrestrial Code*. Pending the outcome of the risk assessment the cattle population in a country, *zone* or *compartment* can be classified as a *negligible BSE risk*, a *controlled BSE risk* or an *undetermined BSE risk*. Applications of Member Countries are assessed using the information in the completed questionnaire for BSE status recognition, the requirements of Chapter 2.3.13 and the guideline for Surveillance for BSE (Appendix 3.8.4) of the *Terrestrial Code*. The applications of Member Countries are evaluated by an *ad hoc* Group of experts and recommendations made to the OIE Scientific Commission for Animal Diseases. Every year, during the General Session in May, the International Committee of the OIE composed of the Official delegates of the OIE Member Countries adopts a list by Resolution of countries recognised as a negligible BSE risk or a controlled BSE risk

The adoption of Resolution XXVII at the 74th General Session of the OIE in May 2006, giving recognition by the International Committee of the OIE to the bovine spongiform encephalopathy (BSE) categorisation status of several Member Countries, also signifies the acceptance by the European Commission, the EU Council and the EU Parliament of a process that would now be the sole responsibility and mandate of the OIE replacing the evaluation and classification process for the allocation of a GBR index in respect of BSE to Member States and third countries by the European Commission.

The Member Countries of the OIE that were listed following the adoption of Resolution XXVII, were evaluated in accordance with the 2004 edition of the Terrestrial Animal Health Code with the provision that any re-evaluations or new applications from Member Countries, will be done in accordance with the 2006 edition of the *Terrestrial Code* or the *Terrestrial Code* current at the date of evaluation and also using the questionnaire for BSE risk assessment that was adopted at the 74th General Session. In the Resolution four countries were listed as *BSE free* and four countries as *provisionally free* from BSE in accordance with the 2004 *Terrestrial Code* classification. The status allocated to these countries will be published by the OIE until May 2008.

ORAL-10

STRUCTURE, TRAFFICKING AND INHIBITION OF INFECTIOUS SCRAPIE PARTICLES

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The wide diversity of known or postulated PrP-res particle sizes and ultrastructures raises questions as to which are the most infectious and smallest infectious units. Size-based fractionations of partially fragmented 263K PrP-res aggregates in detergent showed that non-fibrillar particles, with masses equivalent to 14-28 PrP molecules, are by far the most infectious particles per unit protein, while particles smaller than PrP hexamers had no converting activity. As long as particles were above the minimum size, the infectivity levels appeared to be approximately proportional to particle concentration rather than PrP-res concentration. The proteinase K-resistance of PrP-res increased with particle size. To visualize the interaction between exogenous PrP-res and neuronal cells, fluorescently labeled, infectious PrP-res was used to infect cultured neurons. PrP-res aggregates were internalized into intracellular vesicles and transported along neurites to points of contact with other cells, apparently by a relatively non-specific pinocytosis or transcytosis mechanism. These experiments have visualized and characterized initial steps associated with scrapie infection and PrP-res transport within neuronal cells. To find treatments for TSE diseases, we have continued to seek new inhibitors of PrP-res formation. A variety of TSE-infected cell cultured have been employed to screen for such inhibitors, including a newly developed chronic wasting disease-infected mule deer cell line, MDB^{CWD}. Among the recently identified inhibitors are the phosphorothioate oligonucleotides (PS-ONs), which had been shown previously to have prophylactic anti-scrapie activity in the form of CpG PS-ONs. Although it had been hypothesized that the CpG PS-ONs act by stimulating innate immune mechanisms, we have found non-CpG PS-ONs can directly bind to PrP^{C}, potently inhibit PrP-res accumulation in TSE-infected cells, and triple the survival times of scrapie-infected mice.

ORAL-11

BIOLOGICAL PROPERTIES OF THE PRION STRAIN LINKED TO THE AMYLOIDOTIC FORM OF BOVINE SPONGIFORM ENCEPHALOPATHY (BASE)

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In the past few years, two atypical forms of bovine spongiform encephalopathy (BSE) have been recognized in different European countries, Japan and USA through active surveillance systems. One of these phenotypes has been identified in Italy and is distinguishable from classical BSE for remarkable differences in pattern of deposition and brain regional distribution of PrP^{Sc}, with presence of PrP-immunoreactive amyloid plaques and severe involvement of the olfactory system with relative sparing of the brainstem. The molecular signature of this “amyloidotic” form of bovine spongiform encephalopathy, named BASE, is a PrP^{Sc} type having a protease-resistant core of lower molecular mass than BSE-PrP^{Sc} with predominance of the monoglycosylated species. We carried out strain typing studies using transgenic mice expressing bovine PrP (Tg Bov mice) and inbred lines of non-transgenic mice, including SJL, C57Bl/6, RIII and VM mice. Both BSE and BASE transmitted readily to Tg Bov mice, and produced different clinical, neuropathological and molecular disease phenotypes indicating the propagation of two distinct prion strains. Conversely, all inbred mouse lines showed a substantial barrier to primary transmission of BASE. Unexpectedly, second-passage transmission of the BASE strain to non-transgenic mice induced a neuropathological and molecular disease phenotype indistinguishable from that of BSE-infected mice. The existence of more than one agent associated with prion disease in cattle and the ability of the BASE strain to convert into the BSE strain may have important implications with respect to the origin of BSE and spongiform encephalopathies in other species including humans.

ORAL-12

DIVERSITY AND COMPLEXITY OF NATURAL RUMINANT PRIONS

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Scrapie in small ruminant is the most common natural prion disease. Earlier studies in our laboratory have shown that the use of transgenic mice expressing ovine PrP^{c} (VRQ allele) greatly facilitates the sheep-to-mouse transmission of field isolates compared to conventional mice, thus providing a valuable tool to investigate natural variation of scrapie agent. Within a large panel of isolates from various European countries that were inoculated to one such line (tg338), all were found to successfully transmit disease. A notable strain-specific variation was observed based on the molecular profile and brain distribution of PrP^{res}, and on the survival time upon primary and subsequent transmission. The picture emerging is that the scrapie agent comprises not less than five major strain groups, including the Nor98-like group called atypical scrapie. No correlation could be evidenced between the strain phenotype identified in mice and the PrP genotype of the donor. Each group could be clearly differentiated from BSE agent from various sources, thus consolidating existing data obtained through biochemical typing.

One major group of isolates produced a quite complex pattern of transmission, notably accompanied by a shift in the PrP^{res} molecular profile and leading to the individualisation of phenotypically distinct strains. Strikingly, this phenomenon occurred also in a situation of homotypic transmission. Data from further transmission experiments provided strong evidence that this natural scrapie source consists of a mixture of strains. Of particular interest, the preferential amplification of one specific component appeared to be controlled by the level of PrP^{c} expression in the brain. Furthermore, these strain components exhibited markedly different tropisms for the nervous and lymphoid tissues, leading to the preferential propagation of one component depending on the inoculation route. Thus, co-propagation of distinct strain components within a same individual might account for the reported clinico-pathological heterogeneity between scrapie cases in addition to *Prnp* polymorphism and strain variation. Altogether these findings point to further complexity in the relationship between molecular properties of PrP^{sc} and prion disease phenotype, and are of possible relevance with the recent observations of co-occurrence of multiple PrPsc types in human TSE patients

Transmission to ovine and bovine PrP transgenic mice was also found useful to characterise the newly recognised bovine prions and to analyse their relationship with epizootic BSE agent.

ORAL-13

CHARACTERIZATION OF A NOVEL, SOLUBLE FORM OF MUTANT PrP FROM THE BRAINS OF TRANSGENIC MICE

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A nine-octapeptide insertion in the prion protein (PrP) gene is associated with an inherited form of prion disease. Transgenic (Tg) mice that express the mouse homologue of this mutation (designated PG14) accumulate in their brains an insoluble and weakly protease-resistant form of the mutant protein that resembles PrP^{Sc}. This mutant PrP is highly neurotoxic, but it is not infectious. In contrast, when Tg(PG14) mice are inoculated with the RML strain of scrapie, they accumulate a form of PG14 PrP that is highly protease resistant and infectious upon serial passages. This RML-seeded form of the protein ($PG14^{RML}$) displays different biological and molecular properties from the non-infectious form ($PG14^{Spon}$). Here, we describe the isolation and the characterization of a soluble form of PG14 (called $PG14^{Sol}$), which differs from $PG14^{Spon}$ and $PG14^{RML}$ in several biochemical features. $PG14^{Sol}$ was purified by immunoprecipitation followed by Cu2+-IMAC, while aggregated $PG14^{Spon}$ and $PG14^{RML}$ were isolated by differential centrifugation. $PG14^{Spon}$ and $PG14^{RML}$, like PrP^{Sc}, are insoluble in non-ionic detergents, protease-resistant, precipitated by PTA, and unable to bind Cu2+-IMAC. In contrast $PG14^{Sol}$, like PrP^{C}, is soluble, protease-sensitive, not-precipitated by PTA, and capable of binding Cu2+-IMAC. In addition, CDI experiments, which compare the accessibility of several different antibody epitopes, indicate that the conformation of $PG14^{Sol}$ resembles that of PrP^{C}, while $PG14^{Spon}$ and $PG14^{RML}$ are similar to PrP^{Sc}. Our data indicate that $PG14^{Sol}$ represents a common substrate for formation of both $PG14^{Spon}$ and $PG14^{RML}$. We are currently attempting to generate $PG14^{Spon}$ and $PG14^{RML}$ from $PG14^{Sol}$ substrate using *in vitro* conversion reactions. The identification of $PG14^{Sol}$ contributes to the definition of the molecular events responsible for the pathology observed in Tg(PG14) mice and raises the possibility that this soluble form may represent a toxic species of PG14.

ORAL-14

PROBING THE CONFORMATION OF THE PRION PROTEIN WITHIN A SINGLE AMYLOID FIBRIL USING A NOVEL IMMUNOCONFORMATIONAL ASSAY

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The coexistence of multiple strains or subtypes of the disease-related isoform of PrP in natural isolates, together with the observed conformational heterogeneity of PrP amyloid fibrils generated *in vitro*, indicates the importance of probing conformation of single particles within heterogeneous samples. Using an array of PrP-specific antibodies, we report the development of a novel immunoconformational assay. Uniquely, application of this new technology allows the conformation of multimeric PrP within a single fibril or particle to be probed without pretreatment of the sample with proteinase K. Using amyloid fibrils prepared from full-length recombinant PrP, we demonstrated the utility of this assay to define (i) PrP regions that are surface-exposed or buried, (ii) the susceptibility of defined PrP regions to GdnHCl-induced denaturation, and (iii) the conformational heterogeneity of PrP fibrils as measured for either the entire fibrillar population or for individual fibrils. Specifically, PrP regions 159-174 and 224-230 were shown to be buried, and were the most resistant to denaturation. The 132-156 segment of PrP was found to be cryptic under native conditions and solvent-exposed under partially denaturing conditions, whereas the region 95-105 was solvent-accessible regardless of the solvent conditions. Remarkably, a subfraction of fibrils showed immunoreactivity to PrP^{Sc}-specific antibodies designated as IgGs 89-112 and 136-158. The immunoreactivity of the conformational epitopes was reduced upon exposure to partially denaturing conditions. Unexpectedly, PrP^{Sc}-specific antibodies revealed conformational polymorphisms even within individual fibrils. Our studies provide valuable new insight into fibrillar substructure and offer a new tool for probing the conformation of single PrP fibrils.

ORAL-15

LESS STABLE PRIONS REPLICATE MORE RAPIDLY IN MICE

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Infectious synthetic prions obtained from purified recombinant prion protein of mouse residues 89 to 230 [MoPrP(89–230)] show increased conformational stability when inoculated into transgenic (Tg) mice expressing PrP of the same sequence. These novel Mo synthetic prion strains (MoSP) were also characterized by unique neuropathologic changes in inoculated Tg or non-Tg mice. On passaging of strain MoSP1, two strains emerged with incubation times differing by nearly 100 days. Using conformational-stability assays, we determined the GdnHCl concentration required to denature 50% of the PrP^{Sc} molecules. The $[GdnHCl]_{1/2}$ values were 2.9 M and 3.7 M for the shorter- and longer-incubating prion strains, respectively. Intrigued by this finding, we measured the conformational stabilities of many prion isolates from synthetic and naturally occurring sources. When the incubation times were plotted as a function of the $[GdnHCl]_{1/2}$ values, a linear relationship was found. Unexpectedly, our investigation indicates that less stable prions replicate more rapidly than stable prions.

ORAL-16

TWO-RUNG MODEL OF A LEFT-HANDED ß-HELIX FOR PRIONS EXPLAINS SPECIES BARRIER AND STRAIN VARIATION IN TSEs.

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In this study, a new ß-helical model is proposed that explains species barrier and strain variation in transmissible spongiform encephalopathies. The left-handed ß-helix serves as a structural model that can explain the seeded growth characteristics of ß-sheet structure in PrP^{Sc} fibrils. Molecular Dynamics simulations demonstrate that the left-handed ß-helix is structurally more stable than the right-handed ß-helix, with a higher ß-sheet content during the simulation and a better distributed network of inter-strand backbone-backbone hydrogen bonds between parallel ß-strands of different rungs. Multiple sequence alignments and homology modelling of prion sequences with different rungs of left-handed ß-helices illustrate that the PrP region with the highest ß-helical propensity (residues 105-143) can fold in just 2 rungs of a left-handed ß-helix. Even if no other flanking sequences participate in the ß-helix, the two rungs of a ß-helix can give the growing fibril enough elevation to accommodate the rest of the PrP protein in a tight packing at the periphery of a trimeric ß-helix. The folding of ß-helices is driven by backbone-backbone hydrogen bonding and stacking of side chains in adjacent rungs. The sequence and structure of the last rung at the fibril end with unprotected ß-sheet edges selects the sequence of a complementary rung and dictates the folding of the new rung with optimal backbone hydrogen bonding and side chain stacking. An important side chain stack that facilitates the ß-helical folding is between methionine residues 109 and 129, which explains their importance in the species barrier of prions. Because the PrP sequence is not evolutionary optimised to fold in a ß-helix and because the ß-helical fold shows very little sequence preference, alternative alignments are possible that result in a different rung able to select for an alternative complementary rung. A different top rung results in a new strain with different growth characteristics. Hence, in the present model, sequence variation and alternative alignments clarify the basis of the species barrier and strain specificity in PrP-based diseases.

ORAL-17

NOVEL PRION PROTEIN CONFORMATION AND GLYCOTYPE IN ATYPICAL CREUTZFELDT-JAKOB DISEASE

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Here we report a 69-year-old woman who presented with behavioral and personality changes followed by rapidly evolving dementia. Post-mortem examination of the brain showed an atypical Creutzfeldt-Jakob disease (CJD) phenotype characterized by intracellular prion protein deposition and the presence of axonal swellings filled with amyloid fibrils. Biochemical analysis of the pathological prion protein (PrP^{Sc}) disclosed a previously unrecognized PrP^{Sc} tertiary structure lacking diglycosylated PrP^{Sc} species. Genetic analysis revealed a wild-type prion protein gene with methionine/valine heterozygosity at the polymorphic codon 129. The present results define a new prion disorder with disease phenotype and PrP^{Sc} glycotype similar to familial CJD with frontotemporal presentation.

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ORAL-18

BSE AGENT SHOWS AN ENHANCED VIRULENCE AFTER PASSAGE IN SHEEP

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Sheep are fully susceptible to BSE infection and the ensuing disease is un-differentiable from scrapie in terms of pathogenesis and clinical signs. The biological properties of BSE adapted in sheep, and more particularly its capacity to infect other species is an animal and human health concern. We investigated, using transmission in various mice models, biological properties of BSE passaged in sheep by comparison to the original cattle BSE and various sheep scrapie prions. Our results indicate, in all mice models we used (bovine, porcine, ovine or murine), that sheep BSE have an increased attack rate and/or a reduced incubation time when compared to the effects of the original cattle BSE isolate. The reduced incubation duration was conserved in subsequent passage in BoPrP-Tg mice indicating that alterations of biological properties in BSE in sheep are not due to differences in infectious titre. In the meanwhile, pathological and biochemical features in mice inoculated with sheep BSE prions were very similar to those exhibited by cattle BSE prions but differed from those observed with sheep scrapie isolates. The enhanced virulence of BSE after passage in sheep raises new concerns about the infectivity of this new prion to infect other species including human.

ORAL-19

IINTERSPECIES PRION TRANSMISSION IS CONTROLLED BY CONFORMATIONAL COMPATIBILITY BETWEEN PRPSC AND HETEROTYPIC PRPC

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The threats to humans and livestock from interspecies prion transmission are difficult to assess because the factors controlling this process remain uncertain. To address this we have used transgenic mouse models to understand the roles played by PrP primary structure, prion strains and the species specificity of protein X in controlling interspecies prion infection in the context of cervid transmission barriers. Cervid prions are of particular concern because chronic wasting disease (CWD) of North American and South Korean cervids is the only recognized prion disease of wild animals and its increasing geographic range, contagious nature, and environmental persistence have raised concerns about prion dissemination and the potential for further interspecies transmission. We show that conformational compatibility of PrP^{Sc} in a prion strain and PrP primary structure in a new host is the most important determinant of interspecies prion transmission barriers. Although prion strains can acquire totally new host range properties following heterologous conversion of PrP^{C} in a new host, the strain-related biochemical properties of PrP^{Sc} may remain relatively stable. We also show that the cervid PrP polymorphism at residue 132, which is equivalent to the human PrP 129 polymorphism, is a crucial determinant of cervid prion transmission and has a profound controlling effect on PrP^{Sc}-related prion strain properties. Our transgenic approaches modeling trans-species prion susceptibility in cervids also speak to the possible origins of CWD since cervid transgenic mice are also vulnerable, to varying degrees, to sheep scrapie prions, the degree of susceptibility being strain related. One particularly well-characterized sheep scrapie isolate, SSBP/1, caused disease as efficiently as CWD prions from diseased deer or elk. Finally, while transmissions in transgenic mice based on the protein X model of prion propagation produced chimeric prions, passage of which resulted in novel cervid prions with an extended host range compared to CWD-cervid prions, the unexpected susceptibilities of such mice to CWD and mouse prions are inconsistent with the previously hypothesized role of protein X in prion propagation.

ORAL-20

SYNTHETIC PRION STRAINS AND PLASMA LIPOPROTEINS

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How PrP^{Sc} enciphers the information required to direct the replication of one isolate from a large ensemble of different strains is unknown; moreover, the biophysical explanation for the unprecedented plasticity of PrP^{Sc} presents a perplexing conundrum. Using synthetic prion strains formed initially from recombinant MoPrP(89–230) that was polymerized into amyloid, we investigated conformational stability as a function of incubation time in mice. The stability of prion isolates was determined by the GdnHCl concentration required to denature 50% of the PrP^{Sc} molecules, denoted $[GdnHCl]_{1/2}$. When incubation times were plotted as a function of the $[GdnHCl]_{1/2}$ values for 30 prion isolates from synthetic and naturally occurring sources, a linear relationship was found with a correlation coefficient of 0.93. These findings demonstrate that (i) less stable prions replicate more rapidly than do stable prions and (ii) a continuum of PrP^{Sc} structural states enciphers a multitude of incubation-time phenotypes. In other investigations, the similarities between PrP^{Sc} and lipoproteins with respect to hydrophobicity and complex formation with phosphotungstic acid led us to perform binding studies. Prions from patients with sporadic Creutzfeldt-Jakob disease bound to VLDL and LDL but not to HDL or other plasma components. Apolipoprotein B (apoB), which is the major protein component of VLDL and LDL, bound PrP^{Sc} through a highly cooperative process. The apparent binding constants of native human PrP^{Sc} for apoB and LDL ranged from 28 to 212 pM. Whether detection of PrP^{Sc} in VLDL and LDL particles can be adapted into an antemortem diagnostic test for prions in the blood of humans, livestock and free-ranging cervids remains to be determined.

ORAL-21

ROLE OF PrP MEMBRANE ANCHORING IN BRAIN AND EXTRANEURAL PATHOGENESIS OF PRION DISEASES.

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Prion protein (PrP) conversion from the normal protease-sensitive form (PrPsen) to the disease-associated protease-resistant form (PrPres) is an important event in the pathogenesis of prion diseases. Normal PrP exists primarily bound to the cellular plasma membrane by a glycosylphosphatidylinositol (GPI) linkage, and conversion to the disease-associated form is believed to occur mainly as a membrane-associated event. Our previous experiments demonstrated that PrP lacking the GPI anchor could be converted to protease-resistant PrP in tissue culture cells and in cell-free in vitro conditions. To test the in vivo influence of GPI anchoring on prion disease infection and disease, we generated and studied transgenic (tg) mice which express only the anchorless GPI-negative PrP. Following scrapie infection of such tg mice we observed replication of scrapie infectivity, accumulation of PrPres in brain mainly as an amyloid form, and vacuolation primarily in white matter areas. Surprisingly there was no typical fatal clinical disease over an observation period of 600-700 days; however, in the later phases minor clinical defects were detectable by neurobiological testing. The lack of fatal prion disease in these tg mice suggests that either the amyloid form of PrPres has a reduced level of neurotoxicity and/or that membrane anchored PrP is required for the usual neurotoxic effects. Further analysis indicated that PrPres could be detected in the heart and blood of infected tg mice, and cardiac studies showed deficits consistent with restrictive cardiomyopathy typical of the early stages of amyloid heart disease in humans.

ORAL-22

TSE STRAINS AND THE ROLE OF PRP IN HOST SUSCEPTIBILITY

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The full extent of the diversity of TSE strains in animals and humans has not yet been established. Distinct TSE strains have been identified in mice by serially passaging infectious material from sheep, goats, cattle and humans and multiple possible variations in strains are being identified in these species by PrP glycotyping. Extensive surveillance for BSE and scrapie in Europe and elsewhere has revealed the existence of a number of previously unrecognised TSE strains. It has not yet been established whether these represents a newly diversified strains of TSE or whether they have existed for some time and been identified through the increased surveillance. TSE strains have been shown to be associated with differences in conformation, degree of protease resistance and glycoform ratios. Using our glycosylation deficient mice we are addressing the relationship between these characteristics and the TSE strain. The ability of a TSE strain to infect a new host is thought to lie in the sequence or structural similarity between the host PrP and that of the donor of infectivity. Host PrP is clearly a major factor determining susceptibility and we have demonstrated *in vivo* using gene targeted transgenic mice that changes in the species of PrP and also single amino acid changes within a species can have a dramatic but often unpredictable effect on host susceptibility. Moreover we have demonstrated that alterations in host PrP glycosylation can alter susceptibility not only within a species but also between species. Understanding the mechanism underlying host susceptibility, strain determination and strain targeting remains however a major challenge for TSE research.

ORAL-23

TSE STRAIN VARIABILITY IN SHEEP

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Scrapie in sheep and goats has been known for centuries. Following transmission to rodents, at least six different primary scrapie strains have been isolated and characterised historically by analysing the incubation times and profiles of the histopathological lesion in the brains in a standard panel of inbred RIII, C57Bl and VM mice. The PrP^{Sc} signature in most of these classical scrapie isolates is rather similar in terms of its PK resistance and glycoform pattern in immunoblot. However, the intensified epidemiosurveillance of small ruminants in the EU since 2002 lead to the recognition of previously disregarded scrapie cases with atypical histopathological and immunohistochemical features and immunochemical PrP^{Sc} patterns in the brains of the affected animals. Nor98 (the first reported case of this kind) and SCR2 (an isolate dating back to 1989 in the UK and now recognised as such) are prototypes of the large majority of these atypical cases. Similar cases have been described in most EU member states which so far all seem to belong into the same category. Atypical scrapie is characterized by a lower resistance of the accumulated PrP^{Sc} to proteinase K digestion as compared to classical scrapie strains, explaining why the vast majority of these cases are not detected by most of the so far applied BSE rapid tests. The most obvious characteristics are the altered immunoblot profile comprising of at least 5 bands and including a small fragment of approx. 11 kDa that represents the core fragment of PrP^{Sc}. The anatomical distribution of PrP^{Sc} deposition also varies from classical scrapie, as the cerebellum is the most affected localisation, while the brainstem may be free of detectable PrP^{Sc}. The infectivity of such atypical scrapie cases has been demonstrated using two different transgenic mouse lines. However, the origin of this strain is still under discussion and a spontaneous occurrence cannot be ruled out.

ORAL-24

STRAIN VARIABILITY IN BOVINE ATYPICAL TSES

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Since 2003, some cases of prion diseases in cattle have shown unusual features as assessed by molecular characterization of the protease-resistant prion protein (PrPres) and/or histopathology, when compared to the unique features of BSE described previously. Similar cases have now been recognized in a number of countries, and an overview of the current situation will be presented. Such studies have allowed to refine the molecular definition of such cases using Western blotting, referred as H-type (Biacabe et al., 2004) or L-type (BASE)(Casalone et al., 2004). While a single strain of infectious agent had previously been recognized when BSE was transmitted to a panel of genetically defined inbred wild-type mice, the recent unusual findings raised the question of transmission of prion disease from such unusual isolates. We could show transmission of prion disease from unusual BSE isolates in murine experimental models (including wild-type and transgenic mouse lines). Most importantly, data obtained during the characterization of experimentally infected mice showed different features when compared to those previously described in mice infected with typical BSE isolates. The unusual PrPres molecular features initially described in the brain of cattle by Western blot were maintained following transmission of the agent into mice. In this presentation, the potential origin of such cases, including the possible existence of "sporadic" forms of prion diseases in cattle, will be discussed.

ORAL-25

PRION STRAINS IN HUMAN PRION DISEASES

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The prion protein (PrP) genotype, as determined by the polymorphic codon 129, is thought to have an effect on the conformational characteristics of the scrapie PrP (PrP^{Sc}) revealed by the size of the protease-resistant PrP^{Sc} fragment or PrP^{Sc} type. Based on PrP genotype and PrP^{Sc} type we have proposed a classification of sporadic Creutzfeldt-Jakob disease (sCJD) in six subtypes. Each of these subtypes appears to be associated with a distinct PrP^{Sc} or prion strain. The purpose of this presentation is to review the current state of human prion strains, of the disease phenotypes with which the prion strains associate, and of current classifications of human prion diseases. Alternative classifications proposed by others or based on our own experience in examining over thousand cases of prion disease will be discussed. Of special interest is the recent identification of a novel phenotype of human prion disease associated with a distinct PrP^{Sc} strain that is largely protease sensitive. The contribution of transgenic mice expressing human PrP^{C} to the study of human prion strains and disease phenotypes will also be examined. (Supported by NIH AG-14359 and CDC UR8/CCU515004 awards and the Charles S. Britton Fund).

ORAL-26

GERSTMANN-STRÄUSSLER-SCHEINKER DISEASE (GSS) AND PRION PROTEIN CEREBRAL AMYLOID ANGIOPATHY (PRP-CAA)

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GSS and PrP-CAA are degenerative dementias inherited in an autosomal dominant pattern and associated with the deposition of PrP in the cerebral parenchyma and cerebral blood vessels, respectively. GSS has been found to be associated with ten missense mutations in PRNP. PrP-CAA has been found to be associated with three nonsense mutations in PRNP. We report clinical, and genetic data collected from 34 affected and 19 unaffected gene-carriers from 7 families in which the following mutations in the PRNP gene were found: P102L-129M, A117V-129V, H187R-129V, F198S-129V. We report pathologic, and genetic data collected from 52 affected and 3 unaffected gene-carriers from 15 families in which the following mutations in the PRNP gene were found: P102L-129M, P102L-129V, A117V-129V, G131V-129M, Y145STOP-129M, H187R-129V, F198S-129V, D202N-129V, Q212P-129M, Q217R-129V. Clinically, GSS is characterized by a movement disorder and dementia. PrP-CAA is characterized by a dementing illness reminiscent of Alzheimer disease. The age at onset varies between the fourth and eighth decades; however, in GSS associated with the F198S mutation, we have observed a statistically significant difference ($p<0.001$) in the mean age at onset of clinical signs between individuals homozygous for valine at residue 129 (mean: 43.7 years) and individuals heterozygous methionine/valine at residue 129 (mean: 56.5 years). Pathologically, deposition of intraneuronal insoluble tau occurs in association with the Y145STOP, F198S, D202N and Q217R mutations while it is sporadically present in association with the A117V and H187R. Spongiform degeneration is only seen in GSS associated with the P102L mutation, but not in all cases. Transmission of GSS into experimental animals has been shown to occur only following the inoculation of brain tissue from individuals with the P102L mutation.

ORAL-27

CONTROLLING AMYLOID GROWTH IN ALL DIMENSIONS

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The great progress made in defining the structure of protein and peptide amyloid assemblies, particularly the arrangement of peptides in ß-sheets, is counterbalanced by the still poor understanding of the higher organization of ß-sheets within the fibril and overall fibril/fibril associations. The assembly pathway and basis of amyloid toxicity may well depend on these higher-order structural features. For example, significant evidence points to association between sheets as the rate limiting step in fibril assembly, and a critical metal binding site has now been identified that involves residues from different individual sheets. Here we review experiments that are identifying some of the issues associated with sheet-sheet association by investigating simple model peptides derived from the central core of the Aß peptide implicated in Alzheimer's Disease. These peptides transition between fibril/ribbon/nanotube morphologies in response to assembly conditions, laying the foundation for understanding the folding landscape for these higher order assemblies, revealing potential targets for therapeutic intervention, and opening strategies for the design of highly ordered peptide self-assembled microscale morphologies.

ORAL-28

NEONATAL LETHALITY IN TRANSGENIC MICE EXPRESSING PRION PROTEIN WITH A DELETION OF RESIDUES 105-125.

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PrP^C possesses neuroprotective properties, but deletion of specific regions of the molecule is known to unmask neurotoxic activities of the protein. To identify domains of PrP important for its neurotoxic and neuroprotective properties, we have engineered transgenic mice that express a form of murine PrP deleted for a conserved block of 21 amino acids (residues 105-125) in the unstructured, N-terminal tail of the protein. These mice spontaneously developed a severe neurodegenerative illness that was lethal within 1 week of birth on the *Prn-p*$^{0/0}$ (PrP-null) background. This phenotype was reversed in a dose-dependent fashion by co-expression of wild-type PrP, with endogenous levels of wild-type protein (*Prn-p*$^{+/+}$ background) delaying death until 30-60 days, and 5X over-expression of wild-type protein (produced by introduction of a Tga20 transgene) delaying death beyond 1 year. The phenotype of Tg(PrPΔ105-125) mice is reminiscent of, but much more severe than, those described in mice that express PrP harboring larger deletions of the N-terminus (Δ32-121 and Δ32-134) (Shmerling et al., *Cell* 93:203-214, 1998), and in mice that ectopically express Doppel, a PrP paralog, in the CNS (Rossi et al., *EMBO J*. 20:694-702, 2001). The dramatically increased specific toxicity of PrPΔ105-125 is most consistent with a model in which this protein has greatly enhanced affinity for a hypothetical receptor that serves to transduce the toxic signal. Our results define the region encompassing residues 105-125 as a crucial determinant of the neurotoxic and neuroprotective activities of PrP, and they suggest new models for how the physiological function of PrP^C might be subverted to generate neurotoxic signals during prion infection and after exposure to the toxic peptide PrP106-126.

ORAL-29

ROLE OF PRION NEUROTOXICITY IN TSE PATHOGENESIS

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Cerebral accumulation of an altered form of the prion protein (PrPSc) is believed to cause the neuropathological changes observed in transmissible spongiform encephalopathies (TSE). The coincidence of the topological distribution of neuropathological changes and PrPSc deposits and their temporal correlation in experimental scrapie strongly suggest that PrPSc is the primary cause of neurodegeneration. However, the evidence that some prion diseases arise in the absence of detectable PrPSc has led to the hypothesis that other abnormal PrP species could be the actual proximate cause of neurodegeneration. In the past we investigated the direct neurotoxic effect of PrPSc using the synthetic peptide PrP 106-126 that induced neuronal death and glial proliferation, reproducing in vitro the main neuropathological hallmarks of prion diseases. More recently, we synthesized a peptide corresponding to the smallest amyloid subunit found in Gerstmann-Sträussler-Scheinker patient brains spanning the sequence 82-146 (PrP 82-146) that polymerizes into fibrils with the tinctorial properties of amyloid. To investigate the relationship between the structure and the biological activity of the peptide, we used non-amyloidogenic variants of PrP 82-146 with a scrambled amino acid sequence in the region 106-126 and 127-146. The results indicate that both amyloid and non-amyloid forms of PrP 82-146 are toxic to neurons, the effect being mediated by oligomeric aggregates, whereas only the amyloid was able to stimulate astroglial proliferation. Investigations also showed that the biological effects of PrP 82-146 are influenced by the expression of endogenous PrP. These results, together with recent studies using recombinant PrP and transgenic mice support a direct role of oligomeric aggregates and amyloidogenic fragments in TSE pathogenesis.

ORAL-30

TARGETING CELLULAR PRION PROTEIN REVERSES EARLY COGNITIVE DEFICITS IN PRION-INFECTED MICE.

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Currently no treatment can prevent the progressive cognitive and motor decline associated with widespread neurodegeneration in prion disease. However, we previously showed that depleting endogenous neuronal prion protein (PrP^{C}) in prion-infected mice halted progression to clinical symptoms and prevented neuronal loss, as well as reversing early spongiform degeneration[1]. Therefore, we now asked whether the recovery of early pathology reflects a capacity for functional as well as morphological recovery. We asked whether early pathology is associated with neurophysiological, cognitive and behavioural deficits and whether these could recover in parallel with reversal of spongiform degeneration. We therefore examined hippocampal function, testing novel object recognition memory and species-specific behaviours in vivo, and measured synaptic function ex-vivo over several weeks in prion infected animals. We used transgenic mice in which reversal of early spongiform pathology in prion infection occurs due to Cre-recombinase mediated PrP depletion in neurons at ~8 weeks post infection as well as control mice that do not undergo PrP depletion[2]. We found that prion-infected mice of both genotpes developed early cognitive and behavioural impairments associated with impaired hippocampal synaptic function, long before the occurrence of motor symptoms and neuronal loss, and also before synapse loss was apparent. Remarkably, when neuronal PrP^{C} was depleted, learning and behavioural deficits reversed and synaptic function recovered, in parallel with reversal of early pathological change. Further, these occurred before extensive PrP^{Sc} deposits accumulated and recovered rapidly after PrP^{C} depletion, supporting the concept that they are caused by a transient neurotoxic species, distinct from aggregated PrP^{Sc}. Our combined neurophysiological and behavioural analyses provide the first direct evidence for early synaptic failure in prion disease producing functional impairment, before neurodegeneration is established. These data suggest that early therapeutic intervention in human prion disease might also lead to recovery of cognitive and behavioural symptoms.

1. Mallucci, G. *et al.* Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science* 302, 871-874 (2003)
2. Mallucci, G. R. *et al.* Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. *EMBO J.* 21, 202-210 (2002)

ORAL-31

PRION-INDUCED AMYLOID HEART DISEASE WITH HIGH BLOOD INFECTIVITY

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Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of infectious diseases that cause neurodegeneration and death. The majority (>95%) of normal host derived prion protein (PrPsen) exists as a membrane bound, glycophosphatydilinositol (GPI) anchored protein. We recently (Science 2005, vol.308) constructed transgenic (tg) mice where the C-terminal 21 amino acids were not transcribed, resulting in a secreted form of PrPsen in which >98% of that molecule existed in a soluble non-membrane bound form. Intracerebral inoculation of these tg mice with numerous murine scrapie strains (RML, ME7, 22L) resulted in amyloid deposition in the brain and excessive buildup of abnormal folded prion protein (PrPres) in the absence of overt disease manifestations normally associated with scrapie over 700 days post infection (dpi). However, aberrant physiologic events in terms of delayed neural transmission and loss of long term potentiation could be found within 150 days post infection. Novel distribution patterns of PrPres within and around endothelial cells lining blood vessels occurred within the brain. Examination of blood demonstrated that both infectivity and PrPres could be readily detected by 280 dpi in both serum and cellular fractions of RML scrapie infected tg mice. Moreover, multiple extraneural tissues, such as spleen heart lunge, pancreas, and kidney, also showed PrPres deposition. Focus on the heart indicated that similar to brain, deposits of both PrPres and amyloid that was infectious occurred. The deposition of amyloidogenic PrPres within the hearts of scrapie-infected tg mice resulted in disordered cardiac function including dramatic alterations in both systolic (reduced compliance) and diastolic (stiffening of the heart) function.

ORAL-32

INFECTIVITY IN URINE OF HAMSTERS INFECTED WITH SCRAPIE AND IMPLICATIONS ON MECHANISMS OF HORIZONTAL TRANSMISSION

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Horizontal transmission from animal to animal of transmissible spongiform encephalopathies (TSE) has been documented in the wild and in laboratory animals. The mechanism underlining these transmissions is not fully understood although several studies implicated environmental contamination. Urine and feces could be responsible for environmental contamination since they are excreted and due to the resistant nature of the TSE agent their infectivity could persist indefinitely. Previous studies have indicated that urine from TSE affected animals and humans is not infectious. However, those studies were conducted with a small number of animals and in some cases transmissions were attempted across the specie barrier. We investigated urinary excretion as a potential source of secondary exposure in the environmental.

Urine from hamsters infected with the 263K strain of scrapie was collected using metabolic cages and titered by intracerebral inoculation in the same animal specie with the limiting dilution method. Five-ml equivalents of urine (diluted 1:3) were inoculated. After 267 days post inoculation, 4 animals developed scrapie. Although the study is still on-going, the current data indicate that urine from infected hamsters contains low but measurable levels of infectivity (at least 0.8 + 0.4 infectious doses per ml). These results are in contrast with previously reported studies. We argue that similarly to blood infectivity, quantitation of infectivity in urine requires inoculation of a few milliliters of urine into the same animal specie. Ten percent bladder and kidney tissue homogenates from infected hamsters were also titered using the end point titration method. At 253 days, the titers are $10^{4.5}$ and $10^{3.6}$ ID_{50}/ml for bladder and kidney, respectively. Furthermore, in a look-back investigation of previous titrations conducted in our laboratory, we observed a pattern of clustering of positive cases consistent with infectivity been transmitted by secondary exposure in the cage environment.

Our results highlight a new pathway of TSE infection in animals and may be in humans. This pathway involves the organs of the urinary system and directly affects the environment v*ia* urine infectivity excretion. Exposure to chronic low doses of infectivity in the environment may be responsible for TSE horizontal transmission.

ORAL-33

CONFORMATIONAL HETERGONENEITY OF THE NORMAL PRION PROTEIN DEFINED BY MONOCLONAL ANTIBODIES

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The prion protein is unique in that it can adopt several stable conformations *in vivo*. These conformational differences are not limited to the normal vs. the infectious conformational isoforms but have also been shown for the normal prion protein by means of *in vitro* translating PrP mRNA. Definite proof of conformational heterogeneity of the normal prion protein *in vivo* has lacked due to absence of specific ligands.
Here, we present conformation-specific monoclonal antibodies (CS-mABs) specific for several conformational isoforms of the normal prion protein. These CS-mABs were raised by immunizing PrP knockout mice either with recombinant PrP produced in E. coli, or with PrP purified from brain homogenates enriched in particular conformational isoforms.
One antibody, termed 19B10, recognized exclusively an unglycosylated PrP population very similar to what to NTMPrP that has been described by in *vitro* translation. Other CS-mABs recognized the CTM isoform. Serial immunoprecipitation experiments and distinct staining patterns of cells gave evidence for specific recognition of these mABs. Disease-dependent expression of these isoforms and the biological functions of these distinct PrP isoforms were analyzed.
With these tools, we were able to prove the concept of conformational heterogeneity of the normal prion protein. Thus, different stable conformations of one polypeptide sequence can be used to differentially modify biological functions associated with the normal prion protein.

ORAL-34

CONSERVED ROLES OF VERTEBRATE PRION PROTEINS II

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Even though many different roles have been proposed for prion proteins (PrPs), e.g. cell adhesion, copper metabolism, neuroprotection and cell signaling, these putative functions do not converge into a molecular mechanism of physiological relevance to vertebrates. In a separate study, we show that a PrP cellular function is essential for normal development in zebrafish, and that this role might be conserved among vertebrates (see abstract by Málaga-Trillo et al.). To better understand this function, we heterologously expressed fish PrPs in mouse neuroblastoma (N2a) cells, and compared their cellular properties with those of their mouse, xenopus and chicken counterparts. Interestingly, only cells expressing the fluorescently labeled constructs show PrP accumulation at cell contacts. This suggests that a conserved role of vertebrate PrPs might be to establish homotypic *trans*-interactions, which could be involved in cell-cell communication and signaling. To test whether this interaction between PrPs can indeed mediate the formation of cell contacts, we employed the *Drosophila* Schneider-2 (S2) cells, a well-established non-adhesive cell-line used to characterize cell adhesion molecules. Notably, S2 cells expressing various vertebrate PrPs acquired the ability to aggregate upon the accumulation of PrP at cell-cell contacts. Moreover, this accumulation was concomitant with the recruitment of the raft-associated reggie/flotillin proteins and the activation of tyrosine kinases, indicating that PrP-interactions between neighboring cells might elicit signal transduction events. We also showed that the repetitive, hydrophobic and globular domains, as well as the glycosylation states play differential roles in this function. Altogether, our data provide the molecular and cellular basis for this evolutionarily conserved PrP role, which may be lost or deregulated upon PrP conversion.

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ORAL-35

THE NOVEL CNS PROTEIN SHADOO AND PRPC SHARE FUNCTIONAL HOMOLOGY

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The mammalian prion protein family currently consists of two proteins: the cellular prion protein PrP^C which is expressed in the central nervous system, and Doppel (Dpl), a molecule with a similar three-dimensional structure expressed in the testis. Although the function of PrP^C has remained enigmatic, it is known to be protective in a number of experimental paradigms. In particular, PrP^C has a potent neuroprotective effect against the toxicity of CNS-expressed Dpl and mutant forms of PrP (ΔPrP). These studies have facilitated mapping of activity determinants in PrP^C and implicated the action of a cryptic PrP-like protein "□". Here we demonstrate that mammalian *SPRN*, a notional third member of the PrP gene family, encodes a GPI-anchored neuronal glycoprotein termed Shadoo. Shadoo undergoes endoproteolysis and somatodendritic sorting events reminiscent of PrP^C and is expressed in the brain from early postnatal life. While Shadoo and PrP^C have overlaps in expression, within the hippocampus and cerebellum Shadoo protein is prominent in neurons or neuronal processes deficient in PrP, suggesting that Shadoo supplies a PrP-like activity to these neuroanatomic sites. Furthermore, in PrP-deficient neurons Shadoo protects against the toxic effect of Dpl expression, and this effect is lost when Shadoo alleles lacking the main PrP homology region are utilized. We infer that the conjectural □ molecule and Shadoo are synonymous. It is of interest that the ancient activity domain shared by Shadoo and PrP^C coincides with the conformationally plastic region of PrP^C prone to misfolding to PrP^{Sc} in prion infections.

ORAL-36

APPLICATION OF THE SCRAPIE CELL ASSAY (SCA) TO DISCRIMINATE BETWEEN DIFFERENT MOUSE PRION STRAINS

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The scrapie cell assay (SCA; Kloehn et al., PNAS 100, 11666-71 (2003)) is a rapid, cell-based method for the quantification of certain mouse prion strains. With a newly isolated cell line we can now complete the assay within 10 days and detect prions quantitatively down to a 10^{-8} dilution of RML scrapie brain homogenate.
In addition, we have developed cell lines that show different susceptibilities to different murine scrapie prion strains. With a panel of four cell lines we can discriminate between at least three prion strains within three weeks, a task that would take a year or more with the classical mouse assay.
Interestingly, from one parental cell line we have isolated sublines that show very different relative susceptibilities to two prion strains. This raises the question not only as to how cells recognize prion strains but also as to how the variability of this property comes about..

ORAL-37

BETWEEN PROPAGATION AND CLEARANCE: PRIONS IN CULTURED CELLS

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The biogenesis and pathogenesis of prions is ideally studied in cell culture. Unfortunately, the *de novo* prion infection of cultured cells is still an unpredictable and often unsuccessful event. We tried to establish and validate novel cell culture systems which are robust and reproducible, allowing the study of prion propagation as well as the analysis of cellular susceptibility factors. In addition, we are using these systems for devising experimental therapeutic approaches against prion infections. We have recently established a novel cell culture system based on neuronal $PrP^{0/0}$ cells which becomes susceptible to prion infection upon reintroduction of a PrP^{c} of choice, without having endogenous wild-type PrP^{c} in the background. Stable, fast and robust expression of exogenous PrP is accomplished by retroviral transduction. By using epitope tagging *de novo* infection can easily be monitored, already after a few passages. The system is susceptible to selected prion strains and is at the same time a donor system by significantly releasing prions in the supernatant. A second line of studies addresses putative anti-prion strategies, e.g. by eliminating PrP^{c} as substrate for prion conversion or by increasing the cellular clearance capacity for prions. In line of the first, we have established various peptide-based aptamers, embedded in a TrxA folding scaffold, by screening a combinatorial library for PrP interactors. Both the addition of recombinant proteins from outside as well as endogenous expression within cells indicates a potential for reduction of PrP^{Sc} propagation. In addition, we have further studied the impact of cholesterol biosynthesis pathways on prion replication. Interestingly, when searching for cellular susceptibility factors for infection by microarray assays, a significant proportion of differentially expressed genes was implicated in regulation of cholesterol. Taken together, this gives solid evidence that the cellular cholesterol homeostasis has an impact on prion propagation. Interestingly and vice versa, also prion infection has a profound influence on cholesterol pathways and it seems that there is an adaptation of cultured cells to productive prion infection.

ORAL-38

THE ROLE OF MFG-E8 IN PRION PATHOGENESIS

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Aberrations in the removal of apoptotic cells in the brain may influence clearance of prions and degradation of PrP^{Sc}. We have addressed this question with mice deficient for milk fat globule epidermal growth factor 8 (MFG-E8), a secreted glycoprotein mainly expressed by peritoneal macrophages, immature dendritic cells, tingible body macrophages and follicular dendritic cells.
MFG-E8 acts as a bridge linking apoptotic and phagocytic cells, and therefore directly mediates the uptake of the apoptotic cell by the phagocyte.
MFG-E8 is also expressed in the brain, suggesting also a role in removal of apoptotic cells in the CNS. $MFG\text{-}E8^{-/-}$ mice show profound acceleration in prion pathogenesis after intracerebral inoculation and a less pronounced acceleration after intraperitoneal inoculation. $MFG\text{-}E8^{-/-}$ mice have increased levels of PrP^{Sc} in the brain at terminal stage. These results suggest an involvement of MFG-E8 in prion clearance.

ORAL-39

DEPOSITION OF PRP AMYLOID IN THE ABSENCE OF TRANSMISSIBLE DISEASE.

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The Prion hypothesis predicts that the aetiological agent of TSE disease is an abnormally folded isoform of a host glycoprotein, PrP. All current EU approved TSE diagnostic assays are based on the detection of this abnormal proteinase K (PK) resistant protein (PrP^{Sc}) in post mortem brain tissue, and its identification is taken as indicative of the presence of TSE infectivity. However, the relationship between PrP^{Sc} and TSE infectivity is still unclear, and it is unknown which particular isoform of PrP^{Sc} (PK-res, PK-sen, amyloid) is infectious. Gerstmann-Sträussler-Scheinker P102L (GSS P102L) disease is an autosomal dominant human TSE characterised by the accumulation of PrP amyloid in the brain. Two phenotypically distinct forms of GSS P102L exist, both have diffuse PrP and PrP amyloid plaques, but only one has spongiform degeneration. We present results of studies designed to determine whether a pathogenic PrP species that is not infectious exits in GSS P102L. We have inoculated brain extracts from GSS P102L of both phenotypes into gene targeted transgenic (Tg) mice expressing the equivalent mutation (P101L) in murine PrP. Brain extracts from a patient with diffuse and amyloid PrP deposition and spongiform degeneration transmitted 100% to 101LL Tg mice (290d), but extracts from a patient with diffuse and amyloid PrP and no spongiform degeneration transmitted inefficiently (1/22) with animals surviving over 600 days. However several non clinical mice lacking disease associated vacuolation had PrP plaques in the sub-callosal region of the brain. Brain homogenate from such mice failed to transmit disease on subpass in 101LL Tg and wild type mice, and no disease associated vacuolation or diffuse PrP deposition was found on pathological examination. However the 101LL but not wild type mice showed PrP plaques in the same region as previously observed. These data suggest that PrP amyloid is neither neurotoxic nor infectious, and its presence does not cause the development of TSE disease.

ORAL-40

SYSTEMS BIOLOGY APPROACH TO PRION DISEASE PATHOGENESIS

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A systems view of disease attempts to understand disease initiation and progression in terms of specific-perturbations and their dynamic transitions. The unusual nature of prion disease and the variety of strains and pathologies prompted exploration of a systems approach to identify networks perturbed by infection and to determine which perturbations are essential for various aspects of pathogenesis. Using the Affymetrix GeneChip mouse array 430 2.0, we tracked changes in gene expression for two prion strains (RML and 301V) and five lines of mice over their entire incubation periods; PrP null mice also were inoculated. Differentially expressed genes (DEGs) with consistent temporal patterns across multiple mouse-prion groups were considered likely to be associated with fundamental prion disease processes. However, the goal of this study was not to compile a list of DEGs, but rather to integrate multiple types of data to provide a new perspective on disease for hypothesis building and testing. Array data were used in conjunction with gene ontology, protein interaction and gene regulatory databases to construct hypothetical pathways and gene regulatory networks associated with major pathological events, including glial activation, synaptic degeneration, cell death, and protein degradation. These pathways were integrated with temporal changes in regional PrP^{Sc} distribution, pathology, and regional gene expression among the different host-agent combinations. The mismatch between rate of PrP^{Sc} accumulation and disease onset and differences in DEGs in specific combinations were used to formulate and test hypotheses on the involvement of specific pathways in disease. For example, lack of changes in a pathway involved in generation of reactive oxygen species (ROS) in transgenic mice with short incubation times suggested that ROS might not be an essential component of neurological dysfunction. In accord with this prediction, overexpression of SOD1, shown to be effective in our Alzheimer's disease models, had no effect on prion incubation time. Data and analysis tools are available on the internet in our searchable Prion Disease Database.

ORAL-41

RECENT PROGRESS IN PRION BIOLOGY

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Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative diseases of humans and animals. The underlying infectious agent, the prion, accumulates not only in the central nervous system (CNS) but also in secondary lymphoid organs. I will revisit the role of the immune system in peripheral prion pathogenesis, while focusing on the mechanisms by which extraneural and extralymphatic prion infectivity develops. Interestingly, the same pro-inflammatory cytokines and homeostatic chemokines that are involved in lymphoid neogenesis and compartmentalization of immune cells appear to represent the crucial molecular switches responsible for the establishment of extraneural prion reservoirs.

ORAL-42

GENETIC DISSECTION OF THE ETIOLOGIES AND PATHOGENESES OF ALZHEIMER'S AND PARKINSON'S DISEASES AND RELATED DISORDERS

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Genetic analyses have implicated mutations in the APP gene as one cause of early onset, autosomal dominant, Alzheimer's disease. These mutations, like mutations in the presenilin genes, alter the amount of the peptide, Aβ42 produced during APP processing. Genetic analysis of a recent genome screen for late onset Alzheimer's disease, has implicated the APP locus as risk factor locus, for late onset disease although frank mutations have not been found: this suggests that genetic variability in APP expression contributes to the risk of this form of the disorder: not surprising, perhaps, given the longstanding association between trisomy 21 and AD. Similarly, mutations in the tau gene cause autosomal dominant tangle disease (FTDP-17), and genetic variation at the tau locus, but not coding changes, is associated with the sporadic tangle diseases, progressive supranuclear palsy and corticobasal degeneration: this suggests that genetic variability in either tau expression or in tau splicing contributes to the risk of these diseases. Finally, mutations in the α-synuclein gene cause autosomal dominant Lewy body disease, and genetic variability (haplotypic association) at the α-synuclein locus contributes to sporadic disease: our recent demonstration that one cause of autosomal dominant disease is a triplication of the α-synuclein locus, indicates that the most likely explanation of this observation is that this haplotypic association reflects the fact that genetic variability in the control of α-synuclein contributes to disease risk. These observations have in common support for the notion that these common diseases are initiated by overexpression of key pathogenic proteins which are close to their threshold of solubility.

ORAL-43

ON THE SEARCH FOR AN IN-VIVO TEST FOR HUMAN AND ANIMAL TSES

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Transmissible spongiform encephalopathies have received considerable attention because of the huge BSE epidemic which affected more than 185.000 clinically and fatally diseased bovines. It has been estimated that roughly 3 million infected animals that were still in the preclinical state were slaughtered and entered the human food chain in the United Kingdom and elsewhere. Transmission of BSE prions to man has eventually caused a variant form of Creutzfeldt-Jakob disease in more than 170 humans primarily in the UK, but also in France, Italy, Japan and elsewhere. As a preventive measure in the European Union the risk of human BSE exposure is minimized by BSE rapid testing of all cattle >30 months of age and by the removal of specified risk materials from slaughter cattle which are considered to possibly contain BSE infectivity in incubating animals.

Diagnostic tests for TSEs can generally be divided into ante- (or in-vivo) and post-mortem tests. The term in-vivo diagnostic tests implies the ante-mortem diagnosis of preclinical TSE infections in humans and animals. These tests are based on the detection of abnormal prion protein in bodily fluids or easily accessible tissues (e.g. lymphatic tissue biopsies). Other tests rely on the detection of surrogate markers which are indicative for a dysfunctions in the homeostasis of the body or for cellular degenerative processes. However, depending on the species, the genetic background of the individuum and/or on the prion strain involved, there may be considerable differences in the detectability of these markers in preclinical animals. For example in variant CJD in humans, abnormal prion protein can already been found in lymphatic organs during the incubation time, while in other human prion diseases such a strong lymphotropism of the infectious agent has not been reported.

In sheep, the prion spread and distribution has been thoroughly studied in the past using animals carrying the ARQ or VRQ alleles of the prion protein (PrP). In such animals BSE and scrapie PrP^{Sc} are deposited in the lymphoreticular system from early after the infection. However, the TSE pathogenesis largely depends on the PrP genotype, i.e. no such pronounced depositions are observed in sheep carrying the PrP^{ARR} allele.

To elucidate the still unknown pathogenesis of bovine spongiform encephalopathy (BSE), we have carried out an oral BSE challenge and sequential kill study on 56 calves. Our results demonstrate that BSE prions spread from their primary entry site, the Peyer's patches in the distal ileum, via the autonomous nervous system to the central nervous system (CNS) with essentially no further involvement of the lymphoreticular system. Moreover, BSE associated abnormal prion protein (PrP^{Sc}) was already detected in the brainstem of an animal 24 months post infection, which is 8 months earlier than reported before.

For the development of a functional in-vivo test for TSEs in humans and animals it is therefore of crucial importance to take the species specific and the prion strain specific as well as the genetic effects into consideration.

ORAL-44

BIOCHEMICAL DETECTION OF PRIONS IN BLOOD

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The development of highly sensitive assays for biochemical detection of PrP^{Sc} is considered a top priority for minimizing the spread of the disease. We have optimized and automated the protein misfolding cyclic amplification (PMCA) technology for high sensitive detection of PrP^{Sc}. PMCA enables the specific and reproducible amplification of exceptionally minute quantities of PrP^{Sc}. Indeed, after 7 rounds of PMCA we were able to generate large amounts of PrP^{Sc} starting from a 1 $x10^{-12}$ dilution of scrapie hamster brain, which contains the equivalent to approximately 26 molecules of protein monomers. According to recent data this quantity is similar to the minimum number of molecules present in a single unit of infectious PrP^{Sc}, indicating that PMCA may enable detection of as little as 1 oligomeric PrP^{Sc} infectious particle. The unprecedented amplification efficiency of PMCA leads to several billion folds increase of sensitivity for PrP^{Sc} detection as compared to standard tests used to screen prion infected cattle and at least 4000-times more sensitivity than the animal bioassay. The extremely high sensitivity of PMCA enabled detection of PrP^{Sc} in blood samples of hamsters at the symptomatic and pre-symptomatic stages of the disease. These findings represent the first time in which PrP^{Sc} has been detected biochemically in blood, offering a high promise for developing a non-invasive early diagnosis of prion diseases.

ORAL-45

SURFACE-FIDA: A NEW DIAGNOSTIC TOOL FOR PRION DISEASES

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The infectious agents of prion diseases are composed primarily of the pathogenic isoform of the prion protein designated PrP^{Sc}, which is generated by a conformational change of the cellular isoform PrP^{C}. In contrast to its cellular isoform, the pathogenic isoform PrP^{Sc} forms insoluble aggregates. Hitherto accredited prion tests use the PK-resistance of PrP^{Sc} as a marker for the disease. Because of varying portions of disease related aggregated PrP, which is not PK-resistant (1,2), these prion tests offer only a limited sensitivity. Therefore prion detection, which does not rely on PK-digestion, would be favourable for a sensitive diagnosis. It would allow the detection of both, PK-resistant and PK-sensitive PrP^{Sc}. Our new test system is based on Dual-Colour Fluorescence-Intensity-Distribution-Analysis (2D-FIDA). It is able to detect and quantify protein aggregates and to distinguish the aggregated from the monomeric or oligomeric state, irrespective of PK-resistance. To increase the sensitivity, PrP^{Sc} was concentrated by immobilizing the particles at the surface of a slide. Consequently the immobilisation reduces the dispersion of the prions from three dimensions in solution to two dimensions on a surface. The surface can be scanned systematically and single prion particles are counted (Surface-FIDA). With Surface-FIDA we are able to distinguish Scrapie infected hamster as well as BSE infected cattle in the clinical stage from a control group (3). Preliminary data showed good tendencies that surface FIDA is adaptive to cerebrospinal fluid of cattle.

(1) Safar *et al.* (1998) Nature Med. 4, 1157-1160
(2) Safar *et al.* (2002) Nat Biotechnol, 20, 1147–1150
(3) Birkmann *et al.* (2006) Biol. Chem., 387, 95-102

ORAL-46

A NOVEL MONOCLONAL ANTIBODY THAT SELECTIVELY RECOGNISES ABNORMAL PRION PROTEIN

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The aggregated prion protein peptide ($PrP^{106-126}$) has been used extensively as a model of prion disease neurotoxicity. We reasoned that the aggregation process may generate epitopes that are not present on the monomeric peptide, and that such epitopes may be shared with the PrP^{Sc} that accumulates in human and animal prion diseases. In an attempt to generate monoclonal antibodies that recognise such putative PrP^{Sc}-specific epitopes, we immunised PrP null mice with the aggregated $PrP^{106-126}$peptide. We then screened resultant monoclonal antibodies with monomeric and aggregated forms of $PrP^{106-126}$ and identified an antibody (designated P1.1) that binds to the aggregated, but not the monomeric $PrP^{106-126}$ peptide. Next we determined the reactivity of P1.1 for abnormal full-length mouse and human prion protein. P1.1 selectively recognises aggregated, in preference to monomeric, recombinant mouse PrP and selectively immunoprecipitates PrP^{Sc} from CJD brain homogenates. These data indicate that P1.1 is a PrP^{Sc}-selective reagent and imply that structural aspects of the aggregated $PrP^{106-126}$ peptide are also features of the abnormal disease-associated PrP found in vivo.
This work was supported by the UK Department of Health.

ORAL 46B

WHO TSE REFERENCE MATERIALS

(Importance of standardized reference materials)

At several WHO TSE Consultations (1), issues related to the development of TSE Reference Materials in general and TSE Blood Reference Materials in particular have been discussed. WHO CJD brain-derived Reference Materials have been prepared (2). The preparations are maintained and distributed by the National Institute of Biological Standards (NIBSC), a WHO Collaborating Centre for Biological Standards.

Several groups recently claimed to have developed promising and even partially validated blood-based methods to identify animals infected with TSE agents during incubation period and humans with CJD. Some of those tests may be presented for regulatory review, requesting marketing authorization from national regulatory authorities. WHO has been asked to take a leading role in developing TSE Blood Reference Materials suitable to evaluate blood-based TSE diagnostic tests and screening tests for donors of blood and tissues. Materials to evaluate blood-based tests of animal TSEs important for human health would also be useful.

As for other reference materials, a TSE Blood Reference Material need not be offered as a working reagent for test developers but as a calibrant to prepare in-house reference materials. Reference materials can also be suitable to assemble panels of replicate TSE-derived and control materials (in dilutions) coded and randomized. Both the original TSE Blood Reference Materials and the panels would be intended for preliminary characterization in international collaborative studies, just as the TSE Brain Reference Materials were studied previously.

Any suitable biological reference material should be available in sufficient quantity to allow comparison of performance characteristics of candidate tests at a global level. If this effort proceeds, WHO would consider reconvening the WHO Working Group on TSE Reference Materials to assist in coordinating the selection of TSE materials and organizing international collaborative studies.

References:

(1) WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies: www.who.int/bloodproducts/tse/en

(2) Proposal to establish WHO Reference Reagents for *in vitro* assays of CJD specimens. WHO/BS/03.1965 Rev.1: www.who.int/bloodproducts/tse/en

ORAL-47

PENTOSAN POLYSULFATE AND AMYLOIDOPHILIC CHEMICALS FOR PRION DISEASES

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Long-term cerebroventricular administration of pentosan polysulfate (PPS), a clinical approach based on our preclinical study in rodent models of prion diseases (Doh-ura et al. 2004), has been carried out in more than 20 patients with various types of diseases. Although its therapeutic efficacy remains to be confirmed, preliminary clinical experience indicates prolonged survival in some patients receiving long-term PPS. Further prospective investigation of PPS administration is necessary to obtain high-quality evidence for its clinical benefits.
Administration of PPS requires surgical implantation of a continuous infusion pump and an intraventricular catheter. Although these devices and methods are licensed for routine clinical use in other neurological conditions, the requirements for a surgical procedure might present an obstacle for future clinical trials with intraventricular drugs compared to oral treatments with such agents as quinacrine or flupirtine. Furthermore, since Dr. Caughey and his colleagues discovered the anti-prion actions of Congo red, its derivatives and those of another amyloid dye, thioflavine, have been considered as anti-prion candidates.
We previously reported that some amyloid-imaging probes for Alzheimer's disease diagnosis are effective as anti-prion chemicals when administered intravenously (Ishikawa et al. 2004). We have developed and tested new amyloidophilic imaging chemicals that show better penetration of the blood-brain barrier. We have concluded that not only permeability, but also retention in the brain is important for anti-prion effectiveness (Ishikawa et al. 2006). This has been finally demonstrated using an orally administered amyloidophilic chemical, which has satisfactory permeability and relatively longer retention in the brain than the imaging chemicals and which is remarkably effective in prolonging the incubation times of intracerebrally infected mice.
Our findings with these amyloidophilic chemicals are encouraging, but further improvement of their safety and pharmacokinetic profiles is necessary before clinical application can be considered. Additional problems exist with prion strain-dependent effectiveness of these chemicals and with their reduced effectiveness if administered at later stages of the disease. Details of each research area introduced here will be presented in respective posters.

ORAL-48

PRPSC EXPOSURE OF TYR-TYR-ARG: MARKER, PROBE AND TREATMENT TARGET

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The prion diseases are characterized by the template-directed conversion of normal cellular prion protein (PrP^{C}) into an abnormal, protease-resistant isoform (PrP^{Sc}). Conversion of prion protein in disease is associated with the loss of certain molecular surface epitopes, and the acquisition of others, including the tripeptide motif Tyr-Tyr-Arg. Dendritic cells from scrapie-infected sheep display cell surface immunoreactivity for Tyr-Tyr-Arg, consistent with the key role of these peripheral immunocytes in prion neuroinvasion. Exposure of the Tyr-Tyr-Arg epitope in PrP misfolding puts constraints on mechanisms of PrP disease conversion, and suggests that the short beta sheet of PrP^{C} must dissociate as an intermediate to PrP^{Sc} conversion. Misfolding-associated exposure of Tyr-Tyr-Arg provides a unique immunotherapy target. We have demonstrated that: 1) monoclonal antibodies directed against the Tyr-Tyr-Arg epitope deplete scrapie-infected ScN2a cells of PrPres, the protease-resistant core of PrP^{Sc}; 2) the infectious titre of 301V prion inocula treated *ex vivo* with Tyr-Tyr-Arg mAbs is reduced by 5-10 fold in subsequent mouse bioassay; and 3) CD1 wild-type mice immunized with Tyr-Tyr-Arg peptides are partially protected against experimental transmission of RML scrapie by intracranial inoculation. The Tyr-Tyr-Arg research program provides a prototype for neo-epitopes exposed during protein misfolding, which may have applications for other posttranslational disorders of the proteome.

ORAL-49

LENTIVECTOR MEDIATED RNAI KNOCK-DOWN OF PRION PROTEIN EXPRESSION IN NEURONAL CELLS AND MICE AIMED AT A SOMATIC GENE THERAPY OF PRION DISEASES

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RNA interference (RNAi) is a conserved mechanism by which small interfering RNAs (siRNAs) specifically silence target genes. Experimental evidence suggesting that reducing the expression of PrP could be therapeutically beneficial turns RNAi into a promising approach for the treatment of Creztfeldt-Jakob disease. Quite recently, the therapeutic properties of RNAi directed against PrP were verified in scrapie infected cell lines. To further explore the potential of RNAi for the treatment of scrapie infected animals, we generated lentiviral vectors expressing short hairpin RNAs (shRNAs) specifically targeting the mouse PRNP gene. In neuronal cell cultures these lentivectors efficiently silenced PrP^{C} and suppressed the accumulation of PrP^{Sc}. *In vivo* studies using PrP overexpressing tga20 mice revealed reduced PrP^{C} levels after intracerebral stereotactic application of lentiviral shRNA vectors. In addition, mice chimeric for lentivector transduced embryonic stem cells were generated. These animals showed a considerable reduction of PrP^{C} levels in those areas, where a significant proportion of cells was derived from the lentivector transduced embryonic stem cells. Importantly, scrapie-infected chimeric mice displayed a delayed onset of scrapie symptoms and a significant extension of survival times. Taken together, our data suggest that lentivector mediated RNAi could be an approach for the treatment of prion disease.

ORAL-50

SODIUM PENTOSAN POLYSULPHATE USE IN RODENT MODELS OF SCRAPIE AND BSE

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There is no proven treatment for TSEs. Sodium pentosan polysulphate (PPS) is a heparin analogue with anti-coagulant, anti-thrombotic, anti-inflammatory and anti-viral activity. We and others have shown that PPS increases survival time and reduces susceptibility if given parenterally within days to weeks of scrapie inoculation in rodents. The aim of this study is to evaluate, in rodent models of scrapie and BSE, whether PPS has general efficacy in reducing susceptibility to TSEs at doses, and by routes, that are both clinically relevant and without deleterious effects. The effectiveness of PPS is model dependent, with route, dose and timing of delivery also influencing outcome. PPS increases survival time by the greatest amount in those models where the lymphoid phase plays a significant role in pathogenesis. Repeated parenteral delivery of drug can further increase survival time, even to normal lifespan. Proof of concept of oral efficacy is surprising given the low oral bioavailability of PPS. Per oral PPS is licensed in the USA for interstitial cystitis, is well tolerated long-term and avoids the effects on haemostasis seen with parenteral delivery. Oral PPS may therefore have potential as a post-exposure prophylactic where, for example, a blood donor is identified as having vCJD shortly after their blood has been used for transfusion. In addition, we found that where rodents survived PPS entering the CNS compartment hours after inoculation with ME7 scrapie, they lived significantly longer than untreated controls. PPS may therefore have efficacy even after neuroinvasion. In scrapie infected cell lines PPS appears to inhibit or disrupt *de novo* conversion of PrP^{c} to PrP^{sc}, with no significant reversion to PrP^{c} detected in its presence. This may explain the general finding from experimental animal models that the later PPS therapy is initiated the smaller the increase in survival obtained. As a result of this work we have designed a generic TSE model screening platform to test whether novel therapeutics, pre-selected *in vitro,* outperform PPS and to reduce animal use.

This work is funded by the Department of Health, UK

ORAL-51

USE OF TETRACYLINES FOR THERAPEUTIC INTERVENTION OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

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In the last 15 years we have been working for the identification of molecules with anti-prion activity. We found that tetracyclines are potentially very useful drugs since they (i) are already used in human therapy, (ii) interact with and revert the protease resistance of PrP^{Sc} extracted from scrapie-infected animals and patients with all forms of Creutzfeldt-Jakob disease (CJD), (iii) decrease the prion titre, and (iv) prolong survival of peripherally infected animals. In the last five years, a small series of CJD patients received compassionate treatment of doxycycline, and a retrospective analysis showed a significantly longer survival than untreated patients. On this ground a double-blind pilot clinical trial in CJD patients has been recently commenced in Italy. To extend the therapeutic potential of tetracylines, the efficacy of direct infusion of the compound in advanced stages of infection is being investigated. Since intraventricular infusion of doxycycline solutions caused overt acute toxicity to animals, we decided to entrap the drug into multilamellar vesicles (MLV). Syrian hamsters were inoculated intracerebrally with 25 µl of a 10^{-4} dilution of 263K-infected brain homogenate. Then a single intraventricular infusion of 25 µg/animal of doxycycline entrapped into MLV was administered after 30 days. At the time of writing, after one month from drug treatment, no deaths caused by doxycycline toxicity was observed.

ORAL-52

DECONTAMINATING PRIONS – REQUIREMENTS AND SOLUTIONS

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The unique resistance of prions to usual decontamination techniques poses a very difficult infection control challenge to health-care facilities, but also to the pharmaceutical industry and more widely to all the industries exposed to the risk.

The drastic procedures recommended (1M sodium hydroxyde or 2% sodium hypochlorite for one hour, 134°C autoclaving for 18 min) are not applicable in numerous situations. To implement new techniques, a rapid evolution of the recommendations implies a consensus on the validation methods which will be acceptable by health authorities. Different experimental models have been compared to evaluate the efficiency of classical prion decontamination methods versus new original procedures compatible with fragile devices. Reproducible results have been obtained in a 263K hamster model and a BSE mouse model of intracerebral infection by contaminated wires exposed to different treatments. We observed that limited concentrations of alcali or stabilized bleach, when properly formulated with detergents and used at specific temperatures, could exhibit similar efficiency on prions than the classical NaOH or NaOCl treatments. Short time exposures and compatibility with material render these treatments adapted for practical use. Similarly a dry system based on vaporized hydrogen peroxide under vacuum turned out to be more efficient than autoclaving whilst beeing compatible with electronic devices. These experimental models combined with in vitro protocols based on the detection of PrPres allow to investigate the effectiveness of new versus standard treatments and to explore the mechanisms of action involved.

Thus, new efficient procedures of prion decontamination can be evaluated in standardized experimental validation systems. As new protocols arise they should be considered for recommentation by official guidelines.

ORAL-53

VARIANT CREUTZFELDT-JAKOB DISEASE AND BLOOD INFECTIVITY IN ANIMAL MODELS

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Patients with variant Creutzfeldt-Jakob disease (vCJD) accumulate infectivity and disease-associated prion protein (PrP^{TSE}) in lymphoreticular tissues more extensively than patients with classical CJD. These findings suggest that the blood of vCJD patients may also carry higher levels of infectivity. Three instances of secondary transmission of infection associated with blood transfusion have recently been reported in the UK, increasing the concern about blood safety. We have shown that during the clinical phase of the disease, buffy coat from mice inoculated with mouse-adapted human strains of Gerstmann-Sträussler-Scheinker disease (GSS) had a higher level of infectivity than buffy coat from mice inoculated with vCJD. In contrast, during the preclinical stage of disease, both buffy coat and plasma from vCJD-inoculated mice had higher levels of infectivity than mice inoculated with GSS. We also showed that infection is efficiently transmitted by i.c. and i.v. routes for buffy coat and platelet poor plasma. Here, we present data on rates of transmission by i.v. inoculation of whole blood from animals infected with vCJD, and discuss the results in connection with other investigations of TSE transmission through blood or blood components using a variety of animal models.

ORAL-54

PRIONS AND FATS: PROTECTION OR DESTRUCTION?

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The inactivation of prion infectivity and the degradation of the PrP27-30 peptide backbone are influenced profoundly by solution conditions. The qualitative and quantitative comparison of degradation and inactivation of prion infectivity, both determined with the most stable form of the TSE agent, the so-called prion rods, demonstrated differences in the heat-mediated reduction of a factor up to 10^5. The presence of fat, fatty acids, and particularly glycerol has a profound capacity to protect the peptide backbone integrity of PrP27-30 against heat degradation. Whereas the protective effect of fat and fatty acid is lost with increasing temperature as compared to the heat degradation in water (1, 2), the protective effect of glycerol dominates over the degradation activity of water. Under all conditions analysed the inactivation of prion infectivity is achieved several orders of magnitude more efficiently than the degradation of the PrP peptide backbone occurs. Whereas inactivation in presence of fats is increased compared to pure water conditions, it is decreased in presence of glycerol. The inactivation in fat water mixtures exceeds that of the pure components. From our systematic analyses it can be concluded that the industrial conditions of the basic oleochemical process of hydrolytic fat splitting inactivate safely an unforeseen contamination from BSE-infected raw material and guarantee prion-free products (3). The distribution of prions between fat and water phases was analysed quantitatively. The phase distribution experiments indicated that PrP27-30 migrates to the fat water interphase. Only by increasing temperature, detergent concentration, or salt concentration a notable amount of PrP27-30 was obtained in the water phase. From the experimental data, a systematic interpretation of the mechanisms of prion inactivation and degradation and of the interactions contributing could be derived. An analysis of heat-induced structural changes of PrP27-30 as well as of a possible alteration of the PK resistance is underway.

(1) Appel et al., J Gen Virol. 2001, 82, 465-473
(2) Müller & Riesner, Eur J Lipid Sci Technol. 2005, Nov 11, Vol. 107: 833-9
(3) Müller et al., Eur J Lip Sci Technol, submitted

ORAL-55

SOIL CLAY CONCENTRATES PRION PROTEIN AND PRION INFECTIVITY

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Better control of domestic animal prion diseases is currently achieved through sanitary provisions and genetic selection for resistant sheep.The persistence of an environmental reservoir for infectivity would counteract this progress, particularly in the wild. Abiotic mechanisms of prion retention and dissemination in soil were first investigated by studying interaction between a pure clay (Montmorillonite or Mt) and a model oligomeric prion protein mimicking the pathological isoform; these physico-chemical approaches (mainly FTIR-spectrometry, RMN studies, biochemical and immunochemical depletion studies) allowed to reason infectivity studies with infected brain homogenates adsorbed on Mt. Clays are known for displaying a high adsorption capacity for proteins, due to their negative permanent charge and their high specific area (800 square metres for one gramme of sodic montmorillonite). Adsorption of PrPrec to Mt ranged from 1g protein / g Mt at pH 3-5 to more than 2 g protein / g Mt at neutral/alcaline pH. The mechanism relied mainly on the two-domain structure of the prion protein, allowing a strong interaction of the positively charged N-terminal domain and a hydrophobic-like interaction of the full-length protein with the Mt surface (Revault et al., 2005, BBA, 1724,367). Protein desorption from Mt could not be achieved by saline, detergent or chaotropic reagents supporting irreversibility of adsorption in environmental conditions. Moreover when competing with other biological material such as serum proteins, PrPrec was selectively adsorbed by Mt. So Mt behaves as a strong concentrating agent for prion protein (Rigou et al., 2006, Environ.Sci.Technol, 40,1479). In natural conditions Mt might constitute "infectivity hot points" from trace amounts of infected biological material recurrently deposited on soil like in whelping areas. Mt efficiency in capturing infectivity from infected organs was checked in bioassays on transgenic mice. Infectivity experiments emphasized the infectious capacity of contaminated Mt devoid of any biological debris after one night contact with infected brain homogenates. Fate of Mt-adsorbed labelled PrP in brain after inoculation or in gut after ingestion was followed by dynamic imaging techniques. Implications of these results for environmental control of prion diseases will be discussed taking into account recent publications from other research groups.

ORAL-56

CANADIAN MEDIA REPRESENTATIONS OF BSE AND VCJD AND PUBLIC RISK PERSPECTIVES

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Successful risk analysis requires an understanding of public perspectives on the risk. Such knowledge allows both risk managers and other interested and affected parties to better understand the risk problem to be addressed, the options available for risk management and the best way to communicate about the risk. Public views and judgments on science, policy and risk issues are greatly influenced by the media. A media content analysis can thus provide invaluable insights into prevailing public reaction to the risk. A print media analysis of the portrayal of *Bovine Spongiform Encephalopathy* (BSE) and *variant Creutzfeld Jacob* Disease (vCJD) was conducted from the time of the initial discovery of a cow with BSE in Alberta, Canada on May 20, 2003 through to the most recent BSE case in British Columbia, Canada on April 13, 2006. The focus of the analysis was the elucidation of the comparative frequency, importance and message framing of coverage relating to both health risk and to social and economic impacts, and the attendant influences on public risk perspectives. The two leading national newspapers (*The Globe and Mail* and *The National Post*) and three primary Alberta newspapers (*The Edmonton Journal, The Calgary Herald* and *The Lethbridge Herald*) were chosen for the sample. Articles were initially screened based on applicability to the research focus. Relevant articles from all samples were used to determine an overall frequency of different types and themes of media stories. An in-depth content analysis was then conducted of the Alberta newspapers to examine factors relating to the social construction of the risk. The impact of articles appearing in the first ten days after the first BSE discovery were examined to determine if these initial stories served to establish a common heuristic or 'trigger' that the public may have then repeatedly drawn on to reinforce and make sense of subsequent reporting of the same issue over time and multiple occurrences. The results from this study serve to better understand the reactions of the Canadian public to this risk issue, and to explain apparent differences in public reactions previously documented in the United Kingdom and Europe (e.g. Washer 2006).

Poster Session

EPIDEMIOLOGY

EPI-01

THE DISTRIBUTION OF SHEEP SAMPLED FOR SCRAPIE IN GREAT BRITAIN

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Estimates of the prevalence of scrapie in Europe depended until recently on mandatory reports of clinical cases in adult animals. This method of surveillance was suspected of underestimating the true prevalence, because the denominator population was unknown. Therefore the denominator populations of the active surveillance methods established since 2002 in Great Britain should be identified. Sheep in the abattoir, fallen stock and dead in transit surveys are sampled after being transported to a limited number of locations. Therefore geospatial biases can be expected in these surveys. Holdings providing sheep to the fallen stock survey were identified and located from County-Parish-Holding (CPH) numbers. The holdings providing sheep to the abattoir and dead in transit surveys were identified from the flock tags recorded from sampled sheep, by tracing back to source premises recorded in the Animal Movement Licensing Scheme (AMLS) database. Over 90% of the holdings associated with sampled sheep could be traced (c. 2000 out of 2160 holdings in the fallen stock survey 2005, c. 5500 out of 6047 in the abattoir survey). All the surveys sampled Great Britain unevenly, with different regional biases. The abattoir survey sampled western regions most intensely, especially Wales, whereas the fallen stock survey was most intense to the east. Both surveys sampled the Highlands and Islands of Scotland and the south-east of England sparsely. Differences between the geospatial distributions of samples in the surveys were associated with differences in the characteristics of holdings sampled, including numbers of sheep. The impact of these uneven spatial distributions on surveillance will depend on the relationship between the pattern of sampling and the distributions of scrapie infection and PrP genotypes.

EPI-02

IINCIDENCE DATA TO DESCRIBE THE ITALIAN SCRAPIE EPIDEMIC

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While prevalence data of the Scrapie epidemic in Italy are promptly available since the implementation of active surveillance in 2002, incidence data have never been used to depict the descriptive epidemiology of the disease. The aim of this work is to quantify the incidence of Scrapie in outbreaks reported during the period 1995-2005.In order to collect information, farmers of 215 flocks were interviewed (97% outbreaks) using a standardised questionnaire, containing the following subjects: number of sheep and goats (stratified into three age classes: < 2 year old, 2 to 4 year old, >4 year old), number of suspected ill animals (with Scrapie-like symptoms) observed during the 12 months before the confirmation. Incidence was calculated both in the total population of Scrapie-affected flocks and within each flock. By each species and age, within flock specific incidence was also calculated. Within flock incidence density, during the 12 months preceding the confirmation of the positivity of the flock, was 2,1 cases per 100 animal/years in sheep (CI 95% 2,0-2,2) and 8,8 cases per 100 animal/years in goats (CI 95% 8,1-9,6).Remarkable differences in the age adjusted incidence were evident by time, place and characteristics of the affected animals. Incidence differences between the two species and in the geographical areas, could be due to the presence of specific risk factors, e.g. the use of a vaccine against M. agalactiae in the Central and Southern Italian regions in the mid 90's. The different type of surveillance (active vs passive) may explain the difference incidence as well. The study of age-specific incidence within flock indicates as expected, that the 2 to 4 years old small ruminants showed the highest incidence of Scrapie.

EPI-03

AN ASSESSMENT OF THE EFFICIENCY OF THE FIRST TWO BANS TAKEN AGAINST BSE IN FRANCE

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Following the emergence and exponential increase of the BSE epizootic in the UK, two bans have been successively put in force in France in order to control the disease: in 1990 a first ban on meat-and-bone meal in cattle feed; in 1996 a second ban on cadavers and specified risk material in farm animal feed. Despite these measures, BSE have been detected in animals born after these two bans. Out of the 989 cases detected on March 1st 2006, 847 were born between the first and second ban, and 103 after the second one. In order first to estimate the trend of the epizootic and second to understand the risk factors for BSE in France since the first ban is in force, different studies have been carried out by our teams: i) an analysis of the trend of the prevalence in the successive birth cohorts using logistic regression models; ii) a spatial analysis of the BSE prevalence over time and in relation with the density of the populations of pigs and poultry using Bayesian graphical modelling methods and based on a Poisson distribution with spatial smoothing; iii) an attempt to evidence risk factors for cases born between the two bans using a case-control study involving 182 cases and 182 matched controls using conditional logistic regression; iiii) an attempt to raise hypotheses for the contamination of cases born after the second ban using a case study. Altogether, the results of these complementary approaches, in accordance with the results of modelling issued from other team studies, allow to deliver a global frame for the trend of the epizootic since the disease emerged in France and for the risk factors for cattle infection over time. They lead finally to assess the efficiency of the first two bans implemented to control the disease. The presentation would be the occasion to present for the first time an integrated overview of these studies and their results.

EPI-04

ACTIVE SURVEILLANCE ON TSES OF SHEEP AND GOATS IN MARCHE REGION SINCE 2002 TO 2005

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The Regulation (EC) 999/2001 and subsequent modifications, has established the rules for monitoring plan on TSEs of sheep and goats in the Member States. In Italy, the plan allocates an annual sample to the regions for testing healthy slaughtered and dead animals older than 18 months, based on their populations. This work is an evaluation of the active surveillance plan, during the period 2001 – 2005 and of the epidemiological situation about scrapie in Marche region. A total of 161.961 small ruminants was considered as population target (census ISTAT 2001). The annual sample for healthy slaughtered and fallen stock animals and testing percentage were described. 100% of expected samples in healthy slaughtered animals has been done during 2004 and 2005, while in 2002 and 2003 the testing percentage was lower than 100% because of the large sample size. 100% of expected samples in dead animals has been done every year. However the percentage of monitored small ruminants flocks is still low if compared to flocks which are present in the region. The active surveillance program, allowed to detect 8 scrapie outbreaks in sheep flocks of Marche region (prevalence = 0,9% I.C. 95% 0,49 – 1,53). The eradication measures enforced on these outbreaks, according to the Regulation (EC) 2003/1139, detected a prevalence of infection inside flocks, ranging from 1,7% to 16,6%. All the index cases of the outbreaks, were characterized by a sensible genotype to scrapie, with the exception of the sheep in the Gualdo (MC) outbreak, which was confirmed to be the first case of atypical scrapie in the region.

EPI-05

CLINICAL SYMPTOMS REPORTED - AND DECREASE IN MILK PRODUCTION - OF BSE AFFECTED COWS DETECTED BY ACTIVE SURVEILLANCE IN THE NETHERLANDS.

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Active BSE surveillance by testing all slaughtered cows and fallen stock is a strong tool to estimate the BSE prevalence. Introduction of active surveillance mostly results in an increase of detected cases. We present data that underline the assumption that passive surveillance underestimates the number of cases with clinical signs.
Ninety percent of the BSE case farms in The Netherlands participated in a case-control study. A part of the questionnaire used in this study concerned the clinical symptoms observed by the farmers. Also milk production records were asked for BSE cows and within-farm control cows matched by parity.
The number of notified clinically suspected cows in The Netherlands was first low and increased in 1996 and 2001, presumably as a consequence of the increased awareness of farmers and veterinarians in these years. In these specific years BSE was a news media topic and new control measures were implemented. The reports about clinical signs collected during interviews of farmers show that 14 out of 16 fallen stock and 19 out of 36 slaughtered animals had clinical symptoms at hind sight. Milk production records show a significant decrease in the last lactation of BSE cows.
Due to the a-specific nature of many of the BSE symptoms, especially at the beginning of the clinical stage of the disease, clinical detection can be difficult. The effectiveness of a passive surveillance system for BSE is therefore strongly depending on awareness of farmers and veterinarians that BSE could occur, their willingness to report cases even if the diagnosis is not evident, their feeling of urgency to report clinical suspects and their knowledge of clinical symptoms. The present results underline that estimated prevalence or absence of BSE in a certain country could very well be underestimated when based on passive surveillance.

EPI-06

NATURAL SCRAPIE INFECTION IN EXPERIMENTAL SHEEP FLOCK IN LELYSTAD.

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CIDC-Lelystad maintains two sheep flocks of Texel mixed breed. At the start in 1992, in one of these flocks a scrapie infection was introduced by purchase of scrapie affected sheep. This infection is sustained by natural infection within the flock. The other flock is kept scrapie free and can serve as a control flock with minimal infection pressure. The 2 flocks are kept outdoors and count about 65 and 35 heads respectively. Five allele types (ARR, ARH, AHQ, ARQ, VRQ) and most possible genotype combinations are maintained in the scrapie flock. All sheep are yearly tested by tonsil biopsies and immunohistochemistry (IHC) to monitor the scrapie status of the flocks. At the end of their life sheep are necropsied and different organs are collected in duplo and stored at -20 and in formaline. Obex and tonsil are tested by IHC for diagnosis of scrapie.
The main characteristics of the two sheep flock will be described. The attack rate of scrapie in the different genotypes of the sheep born and raised in the scrapie flock are presented.
Epidemiological data suggest that at least two scrapie strains are residing in the scrapie flock. This is supported by preliminary strain typing results in a bioassay. All VRQ/VRQ and ARQ/VRQ sheep and about half of ARQ/ARQ sheep are positive in brain and lymph nodes at the end of life with longer incubation times for the ARQ/VRQ and ARQ/ARQ sheep. Most ARR/VRQ sheep stay negative or have a long incubation time without evident lymphoid involvement. None of the ARR/ARR and ARR/ARQ animals were scrapie positive.
The scrapie flock provides a well characterised living source of materials for development or first validation of (pre)clinical tests and reference materials.

EPI-07

RISK FACTORS FOR SCRAPIE AT THE INDIVIDUAL ANIMAL LEVEL

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Scrapie is a disease that, at a flock level, occurs at a low incidence. Many naturally-affected flocks do not experience a within-flock epidemic, however, for some individual flocks a within-flock epidemic can have a significant impact. There are still many unknowns as to mechanisms and routes of transmission, and risk factors for scrapie at the individual animal level, particularly in naturally-affected field flocks. Over the last ten years, this study has worked with several scrapie-affected flocks in Great Britain, in an attempt to collect enough data at the individual animal level to study such risk factors. Thirty eight flocks have provided sufficient data to be included in the analyses. Descriptive analyses of individual flocks has been followed by survival analysis of cohort population data and conditional logistic regression of case control population data. Some of the results of these analyses will be presented. They include i/ confirmation that prion protein (PrP) genotype influences the occurrence of clinical scrapie, although variation in the effect of some PrP alleles is observed between flocks, ii/ evidence for maternal transmission of scrapie, but not paternal transmission, and iii/ a bimodal age distribution of scrapie cases that may be due to delayed exposure to the infectious agent in some animals. The analyses confirm the strong 'protective' effect of the ARR allele for clinical scrapie in all flocks and highlight areas for attention in the development of disease control strategies for individual flocks. This study was funded by the Department for Food and Rural Affairs.

EPI-08

MORTALITY FROM SPORADIC CREUTZFELDT-JAKOB DISEASE IN PIEMONTE, 1999-2005: CLINICAL AND MOLECULAR ANALYSIS

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The average yearly mortality rate from sporadic Creutzfeltd-Jakob disease (CJD) in Italy, 1993-2000, is 1.04 cases per million inhabitants, while the reported rate in Piemonte, 1999-2000 is 1.41 per million (Puopolo et al 2003). Aim of the present investigation was to assess incidence in 1999-2005 and analyze trend in mortality rate, clinical, pathological, molecular data, and factors associated with survival duration of all incident cases of CJD in the resident population of Piemonte, 1999-2005. Since 2002 all incident cases are referred to the local Regional Human Prion Diseases Center established in Torino. Forty-eight (31 females,17 males) sporadic CJD cases and seven familial cases (E200K and L210 mutations) were identified. All sporadic cases were probable CJD. Autopsy was performed in 41 patients. Mean age at onset of symptoms was 66 year (range 36-82); mean disease duration 5.6 months (range 1-18). Average yearly mortality rate from sporadic CJD, 1999-2005 was 1.68, ranging from 0.91 in 2003 to 3.63 in 2005. Mortality rate by gender resulted higher for females (2.10) than males (1.22). Neuropathology confirmed the diagnosis in all cases; no case had the vCJD phenotype. MM at codon 129 was found in 74,4% of studied patients and was preferentially associated with PrPsc type 1; MV-type 2 was found in 10% of cases. Multivariate analysis of survival duration showed that diagnosis before 2001 and type 2 PrPres were significant predictors of longer survival (p 0,006 and 0,005 respectively). In conclusion, no significant variation in mortality rate was found in Piemonte, 1999-2005. The centralized referral of cases is of crucial importance in the continuing epidemiological surveillance of CJD.

Supported by Regione Piemonte.

EPI-09

EPIDEMIOLOGY OF HUMAN PRION DISEASES IN PIEMONTE AND VALLE D'AOSTA IN 2002-2005 PERIOD: A REPORT OF THE FIRST 4-YEAR ACTIVITY OF THE REGIONAL HUMAN PRION DISEASE CENTER.

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Since 2002, laboratory investigations and autopsies of suspected Creutzfeldt-Jakob disease (CJD) and the other prion diseases cases from Piemonte and Valle d'Aosta (North-West Italy) have been all unified in the Regional Human Prion Disease Center in "Maria Vittoria" and "Amedeo di Savoia" hospitals in Torino. These activities have paralleled the recording of patients' clinical and instrumental data to get a comprehensive view of human prion diseases in our districts. We aimed to analyse the epidemiology of human prion diseases in Piemonte and Valle d'Aosta in 2002-2005 years. In the whole period 2002-2005 a definite diagnosis of prion disease was made in 38 patients (24 females, 14 males). Sporadic CJD was diagnosed in 30 cases whereas sporadic fatal insomnia in 2 ones. PRNP gene sequencing disclosed a mutation in 6 patients (4 E200K, 1 P210L and 1 D178N). No case of accidentally transmitted or variant CJD was reported. The annual mortality rate for all prion diseases per million of inhabitants was 2.12 (CI 95% 0.79-3.45) in 2002, 0.83 (CI 95% 0.01-1.65) in 2003, 1.67 (CI 95% 0.50-2.84) in 2004 and 3.10 (CI 95% 1.51-4.69) in 2005.
Mortality rates of prion diseases in Piemonte and Valle d'Aosta in 2002-2005 have paralleled the rates observed in the whole Italy and also the strikingly high value observed in 2005 is not significantly different from the national value. The unification of autopsies and specific laboratory investigations in one center may represent a good model to improve case ascertainment and active surveillance on human prion diseases.

Regional Human Prion Disease Center is supported by the Regione Piemonte (Ricerca Sanitaria Finalizzata 2002-2003-2004)

EPI-10

ESTIMATION OF PAST AND FUTURE BSE CASES IN ITALY

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BSE in Italy was firstly detected in 2001, and during the period 2001-2005, 125 autochtonous cases were identified by active surveillance and 1 by passive surveillance. During the first two years of surveillance the reported cases were 89, then the infection decreased up to 8 cases in 2005.
Detailed epidemiological models, from the classical mathematical modelling route, were developed and applied to BSE in several European countries. A simplified model was developed in 2004 and applied to the Italian BSE epidemic. First data (updated to 31/12/2004) showed that the main infection occurred in 1993-2000 (the peak in 1996) and 235 cases were expected before 2001, 556 in 2001-2003, 171 after 2003. Our purpose is to update the data to 31/12/2005 and compare the new results with the old ones.
The model is a backward calculation based on some demographic features of the observed cases (as date of birth or age at BSE detection), of the population (as survival function, age distribution) and of disease (as incubation period). First step of the model is the estimate of the age of cases at infection, obtained by combining the distribution of incubation period with the age at detection of BSE. Demographic data about population allow us to know the size of the cohorts of each case. Assuming that all the animals belonging to the same cohort shared the same risk of the cases detected in that cohort, we estimate the distribution of the age at infection for the cohorts, combining the size of the cohorts with the age at infection of cases. Re-introducing the survival function and the incubation period in this last estimation we obtain an estimate of the number of infected animals that reached alive at a certain age in a certain year. The application of this model to data updated to 31/12/2005 shows results not dissimilar from those previously reported.

EPI-11

MONITORING FOR CHRONIC WASTING DISEASE (CWD) IN WILD RUMINANTS IN SLOVENIA

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Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) of wild ruminants, diagnosed up till now only in USA and Canada, both in captured and free living animals. Most of European countries experienced BSE and scrapie so far, including our country and epidemiological situation for these diseases is thoroughly monitored and well known. However situation about CWD is in general unknown in Europe, except of a few data acquired in some of the countries on voluntary basis. The aim of our study was to obtain initial information on CWD status in the population of red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and fellow deer (*Dama dama*) in our country, with the emphasis on examination of animals coming from the regions where TSEs in domestic animals were diagnosed. Animals included in the study were killed or found dead. Altogether heads of 32 red deer, 27 roe deer and 28 fellow deer were collected, and samples of the brain and lymphoid tissues from the head gathered and examined for CWD. Brain stems were tested with rapid post-mortem test and the rest of the brain and lymphoid tissues were fixed and paraffin embedded for whole brain histopathology and immunohistochemistry. All samples tested negative for CWD with rapid post-mortem test, all brain regions including cerebral cortex, brain stem with medulla and obex, and cerebellum were negative for specific spongiform lesions in histopathology, and immunohistochemistry for prion protein was negative, too.
These preliminary results on a small number of cervids indicate that the epidemiological situation for CWD in our country is good at the moment; however more intense and long-term survey is needed, especially in the regions where scrapie became endemic.

EPI-12

BSE: THE UNITED STATES OF AMERICA'S EXPERIENCE, SURVEILLANCE AND STRATEGY

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Throughout Europe and Asia, there seems to be a genuine misunderstanding of how the U.S. commenced BSE surveillance and controls in the late 1980s and continued throughout the 1990s to monitor both CJD in humans and BSE in ruminants, and to prevent their spread. Three agencies of the U.S. government play a key role in our national integrated BSE controls: USDA, FDA, and the CDC. With the aforementioned agencies and guidance from the Harvard Risk Assessment Study, the U.S. feels that all of the firewalls implemented from the late 1980s have safeguarded the U.S. from BSE or CJD problems. The epidemiologic study that has, as of June 15, 2006, tested in excess of 730,000 high-risk cattle and over 21,000 clinically normal adult cattle, as part of the enhanced BSE surveillance program, starting in 2004, bolsters this point. Surveillance in humans, also begun in the 1980s, further confirms the success of the BSE firewalls and will be discussed. To date, two native-born American cattle were diagnosed with atypical BSE and one imported cow from Canada was diagnosed with typical BSE. The lack of understanding of the U.S.'s program has cost upwards of $5 billion in international trade, yet has had zero impact on the consumption habits and public opinion of the U.S. public. In this presentation, we will cover the veterinary, public health, food safety and human epidemiologic implications of BSE and CJD in the U.S., our experience, surveillance and strategy.

EPI-13

FREQUENCY OF SCRAPIE RESISTANCE ASSOCIATED ALELLE LISINE-171 IN SPANISH OVINE BREEDS

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Spanish ovine genotyping programme is coordinated by Ministry of Agriculture, Fisheries and Food and is carried out by one National Reference Laboratory (Laboratorio Central de Veterinaria) (R.D. 1312/2005) and 6 authorised laboratories. Since the beginning, more than 1.000.000 animals has been analysed from 40 ovine pure breeds. At LCV SNaPshot technology is used to detect polymorphisms located at codons 136, 154 and 171 of *prnp* gene. Five most common alleles were VRQ, ARQ, ARR, AHQ and ARH, nevertheless the ARK allele was also found. For that purpose an improved method has been designed for Scrapie genotyping and ARK detection by microsequencing.
More than 2000 animals belonging to 15 pure breeds show at least one allele ARK, all of then are health animals included in the National Genotyping Programme. No ARK alleles have been found in Scrapie affected cases. Most of animals are from breed Ojinegra de Teruel (39.2%), Assaf (12.3%), Rasa Aragonesa (16.1%) and Navarra (12.3%). Frequencies of polymorphism Lysine-171 are less than 1% in the others 13 breeds and near 7% in two of them.
Results of ARK incidence in Spanish ovine breeds and distribution will show and data concerning to resistance associated alleles in ARK carrying animals.

EPI-14

DEVELOPMENT OF METHODS FOR THE ANALYSIS OF THE PRESENCE AND STABILITY OF PRIONS IN WASTEWATER

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Experimental approaches on the presence and behaviour of prion proteins in the wastewater generated in abattoirs, are required in order to provide experimental data for the evaluation of sanitary risks related with the dissemination of the contamination and development of good management practices. Some quantitative risk assessment studies have hypothesized a possible transmission of prion diseases through sewage and sludge from abattoirs where TSE cases have been identified, especially BSE. As only post-mortem tests are available and all slaughtering processes are finished when the TSE case is confirmed, some SRM infectivity could enter the sewer inducing a prion contamination. Furthermore, prions are highly resistant, markedly insoluble and amphipathic. The objective of this work was to develop a methodology to deal with prions in slaughterhouse sewage, providing experimental tools to evaluate their possible presence and their persistence in these matrices. Thus, different slaughterhouse sewage samples were spiked with either scrapie or BSE brain homogenates and brain tissues in suspension were recovered by centrifugation. The corresponding pellet was resuspended, PK-treated and concentrated using the Bio-Rad TSE Purification kit with some modifications, and finally analyzed by Western blot. The designed procedure has been able to detect 2ug of BSE- and 0.5ug Scrapie-affected tissue for 30ml of sewage sample analyzed. This procedure was applied to the analysis of 17 sewage samples collected in slaughterhouses processing cows of ages older than 24 months. Eight of these samples were collected in slaughterhouses one day after the identification of a TSE positive case between the processed animals. None of the samples was found to be positive for PrP^{sc}. The stability of PrP^{sc} in wastewater was experimentally studied at 20ºC. Persistence curves for either scrapie or BSE were obtained in the experiments analyzing raw wastewater samples and in samples collected after a primary treatment. In conclusion, the developed methodology for detection of PrP^{sc} in slaughterhouse sewage may represent a sensitive technique useful for detecting either Scrapie or BSE associated PrP^{sc} wastewater and as a tool for the study and the control of the dissemination of prions in the environment.

EPI-15

BORN TO BE RESISTANT: HISTORY OF A SELECTIVE CULLING.

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In May 2003 an outbreak of scrapie in a Biellese breed flock was confirmed in Piemonte.The flock was entirely genotyped for selective culling according to Regulation 1492/2004/EC. Owing to the low frequency of the ARR allele, the foreseen derogations (Reg. 260/2003/EC) were applied, thus delaying the destruction of susceptible animals for up to five breeding years. The aim of this work was to analyse the cumulative survival probability of each sheep associated to its own genotype. With regard to susceptibility the genotypes were grouped in three different classes according to the "National plan of genetic selection". In order to model the hazard for each individual to come down with scrapie, survival analysis techniques were applied to 1198 sheep who entered the study during a thirty-month follow up time. 53 scrapie positive sheep were detected in the study period. After the complete culling of all the VRQ carriers sheep, the origin and the end of the study were fixed respectively a day after the initial big culling (22 October 2003) and on 30 April 2006. Animals still alive at the end of the study were considered as right censored, whereas animals who left the flock because of a reason other than scrapie, were considered left censored. Follow up time was split into very small time bands (one day each) in order to suppose that only few animals died in each time band. The Kaplan – Meier estimate of the survivor function was obtained. To verify the null hypothesis (the three classes have the same probability to survive) the Logrank test was performed. In order to include in the model as covariates both age and genotype, a semi-parametric analyses (Cox proportional hazards model) was fitted. After adjustment, statistically increased risk was evident only for the susceptible genotype and the age-class 24-48 months.

EPI-16

EVALUATION OF A CLINICAL PROTOCOL TO DETECT SCRAPIE

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This work presents the results of a three-years study carried out in a large outbreak of scrapie affecting sheep of Biellese breed. Aims of the study were to record clinical signs consistent with scrapie, to evaluate the accuracy of a standardised protocol in detecting the disease. 49 animals were submitted to a complete neurological examination. A three-part standardized data collection form was filled in for each animal and an *ad hoc* database was implemented. For clinical history taking, a list of the main clinical signs found in Italian outbreaks was provided to obtain an objective record of the signs the breeder described. The neurological examination followed the standard procedure for assessing mental status, posture, gait, postural reactions and proprioception, cranial nerves, spinal reflexes and sensitivity. A positive clinical history for scrapie included at least two clinical signs as reported by the breeder. A sheep was considered suspicious if it met with at least two of the following criteria: positive clinical history, abnormal fleece, abnormal mental status/behavior, abnormal gait, abnormal postural reaction/proprioception, legs dirty with ruminal material or saliva and positive nibble reflex. The accuracy of the clinical test has been evaluated using the routinary confirmatory tests (histopathology, immunohistochemistry) as the Gold Standard. 18 animals were scrapie affected out of 49 sheep clinically examined. By using the software Stata 9.2, sensitivity, specificity. and predictive values have been calculated. On the whole this clinical test showed good diagnostic performances: the positive predictive value was 93% (CI95% 66-100) whereas the sensitivity was not so good (Se 72%; CI95% 47-90). Moreover the test showed a good ability in avoiding false positive results (Sp 97%; CI95% 83-100) and, although the high prevalence in the study group, a good negative predictive value (NPV 86% CI95% 70-95).

EPI-17

EPIDEMIOLOGICAL PATTERNS OF SCRAPIE OUTBREAKS IN SHEEP FLOCKS IN GREAT BRITAIN

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Scrapie is an infectious disease, but one that is unusual in that the incubation period leading to clinical disease is governed by a strong host genetic component and a strong dose-response relationship. These factors can interact to yield intriguing epidemiological patterns. Here, we describe patterns in the outbreaks of confirmed classical scrapie in 30 sheep flocks. The flocks are part of a large farm-based case-control study, undertaken by the Institute for Animal Health (IAH) since 1998. Data on confirmed cases were derived from the Scrapie Notification Database (SND) held at the Veterinary Laboratories Agency (VLA), while those on flock characteristics were collected by IAH staff using a questionnaire. All flocks were blood sampled for PrP genotyping. Taking the first confirmed case as the beginning of an outbreak, the duration of epidemics varied between flocks from 1 to 9 years. Confirmed cases occurred in 6 (out of 15) PrP genotypes. These were predominantly in VRQ-bearing animals, but some cases also occurred in animals carrying the ARQ allele. The most common genotypes of cases were ARQ/VRQ and VRQ/VRQ. In most flocks, cases were confined to these two genotypes, but some flocks had markedly different affected genotypes. A measure of flock-level susceptibility was defined based on the relative frequency of genotypes in the flock and the risk of scrapie in those genotypes. The total number of cases per 1000 sheep increased significantly with flock-level susceptibility. However, no association was found between flock-level susceptibility and incidence (cases per 1000 sheep per year) or outbreak duration. The age-at-onset varied markedly within and between flocks, but certain trends were observed. In particular, it increased significantly during outbreaks, and the mean age-at-onset within a flock was similar for all genotypes, except for VRQ/VRQ where it was significantly lower. In one flock, animals of the ARQ/ARH genotype developed clinical signs at a significantly earlier age than VRQ/ARQ animals.

EPI-18

SURVEILLANCE OF TSE IN WILD RUMINATS IN NORTH-WESTERN ITALY.

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Chronic Wasting Disease (CWD) is a Transmissible Spongiform Encephalopathy (TSE) which has been identified in captive and free-ranging cervids more than 20 years ago in North America, where is currently in epidemic phase. The evidence of the circulation of TSEs in European population of wildlife ungulates does not exist, but the high prevalence of scrapie infection in Italy and the synchronous/alternate use of common grazing areas by domestic and wild ruminants may suggest the possibility of the spread to the latter. Furthermore, due to the strong similarities between CWD and scrapie, i.e. horizontal transmission, clinical signs and lesions, scrapie has been proposed as possible origin of CWD. Surveillance of TSE in wild ruminants is not mandatory in Europe according to 999/2001 EC Regulation, and data available to date are the results of voluntary plans. Aim of the study is the definition of the maximum prevalence of TSE infection in roe deer (*Capreolus capreolus*), chamois (*Rupicapra rupicapra*), red deer (*Cervus elaphus elaphus*) culled or found dead in North-Western Italy. Brain stem or spleen from animals (older then 1 year) of the above mentioned species are collected and submitted to a USDA approved CWD rapid test (Idexx Herdchek CWD Antigen Kit) and one scrapie rapid test approved according to 260/2006 EC Regulation (Bio-rad TeSeE). Data such as species, sex, age, origin, *causa mortis* are collected in an *ad hoc* database. In case of positivity to one rapid test, the sample is submitted to confirmatory tests (immunohistochemistry and western blotting). Preliminary results on 90 samples don't support the presence of TSE. Collection of a much wider sample will allow to verify the endemic presence of TSE among free-ranging ruminants in our study area or, alternatively (in the case of negative results), to define the expected maximum prevalence of TSE in the studied population.

EPI-19

A MODEL OF EPIDEMIOLOGICAL STUDY OF VARIANT CREUTZFELDT- JAKOB DISEASE IN ITALY

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The prevalence of preclinical or subclinical variant Creutzfeldt-Jakob disease (vCJD) in Italy is currently unknown. In order to assess the feasibility in our Country of previous epidemiological studies performed in the UK, we retrospectively screened 1000 anonymous surgical appendicectomy and tonsillectomy specimens for the presence of disease-associated prion protein (PrP^{Sc}) in lymphoreticular tissue. We retrieved appendix and tonsil samples removed between 2000 and 2005 from patients aged 10-60 from the Histopathology Institute of Università Politecnica delle Marche. Such time range was chosen since the maximum incidence peak of Bovine Spongiform Encephalopathy (BSE) in Italy was estimated to be between 1990 and 1995 and the median vCJD incubation time is postulated to be in the order of ten years from the exposure to BSE prions. We analysed only cases with at least five secondary lymphoid follicles because PrP^{Sc} in vCJD was reported to be present in approximately 20% of follicles. PrP^{Sc} was detected by immunohistochemistry with monoclonal antibody 3F4 after sequential pre-treatments with hydrolytic autoclaving and guanidine isothiocyanate. Lymphoid tissue from the single Italian vCJD case was used as positive control in each experimental session. No positive cases were identified by our analysis. Due to the small sample size and unknown test sensitivity during the prolonged incubation period, this negative result cannot reassure that relevant community infection is unlikely. Nevertheless, we may provide a convenient and inexpensive protocol for vCJD prevalence screening on a national scale. Supported by the Italian Ministry of Health (grant Regione Marche 14, Ricerca Finalizzata 2003).

EPI-20

FEEDSTUFF FACTORIES AND BSE IN FRANCE – SPATIAL ANALYSIS

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A spatial analysis was carried out in order to analyse the reason why the risk of Bovine Spongiform Encephalopathy (BSE) was spatially heterogeneous in France, during the period following the feed ban of Meat and Bone Meal (MBM) to cattle. The hypothesis of cross-contamination between cattle and monogastric feedstuff, as well as the hypothesis of the contamination of animal by products used in animal feed (fat, phosphates) was assessed via a spatial analysis focused on the use of MBM, animal fat and bone derived minerals in factories.
Data concerned the 629 BSE cases born in France after the ban of MBM (July 1990) and detected between July 1st, 2001 and December 31, 2005, when the surveillance system was optimal and not spatially biased. Animal density data were provided by the 2000 census and the data on the 327 feedstuff factories producing compound feed for cattle were collected from administrative inspection. The first step of the analysis was to define areas concerned by the delivery areas of the same factories and the level of each risk factor, as well as the spatial risk of BSE. The second step analysed the crude link between the BSE risk and the risk factors at the area level, based on categorisation and chi square test. Finally the third step was a disease mapping, based on hierarchical Bayesian models (Poisson distribution with spatial smoothing and covariates) elaborated by directed acyclic graphs method. Parameters were estimated by a Markov Chain Monte Carlo simulation method.
The results evidenced the role on the BSE risk of the use of MBM for monogastrics in the factories, pointing out the cross-contamination between cattle and monogastric feedstuffs as the main probable source of infection for the BSE cases born after the ban of MBM to cattle.

EPI-21

PATHOGENIC PRION PROTEIN ADSORPTION TO QUARTZ SAND

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Soil may serve as a reservoir for prion infectivity, and soil-bound prions remain infectious. The mechanisms controlling the environmental transport of prions and their interaction with soil particles remain poorly understood. As an initial step toward understanding prion interaction with soil, we conducted batch sorption experiments to examine PrP^{Sc} adsorption to quartz sand as a function of pH, ionic strength and prion protein concentration. The electrokinetic properties of both PrP^{Sc} and the quartz sand were determined under relevant solution conditions, as was PrP^{Sc} aggregate size. Prion protein interaction with quartz surfaces was strongly pH-dependent with maximal sorption occurring near the apparent isoelectric point of the PrP^{Sc} aggregates. Our findings were in qualitative agreement with predictions based on colloid stability theory. Our results suggest that the composition of the soil solution influences prion mobility in soils and therefore the accessibility of soil-associated prions to grazing animals and other species that ingest soil.

EPI-22

BAYESIAN ESTIMATION OF THE TRUE PREVALENCE OF BSE IN BELGIAN CATTLE

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In 2002 and 2003, the mean apparent prevalence of BSE in Belgium was of 6.29 10-5. Since 2001, the detection system of this zoonosis has been based mainly on the use of a “rapid” test. The only rapid test in use in Belgium at that time was the Platelia® test, commercialized by Bio-Rad. At the development of the test, its sensitivity and specificity were estimated at 100% [95% CI: 99 - 100%] and 100% [95% CI: 99.7% - 100%] as the ability to accurately identify a non-infected animal. However, this test has generally been used in order to screen non-clinical populations. Under these conditions, the sensibility and specificity of the test could differ from the initially calculated values, and the true prevalence of BSE could diverge from the apparent prevalence. The sensibility and specificity of the rapid test, as well as the true prevalence of BSE in Belgium, have thus been estimated by Bayesian methods for the years 2002 and 2003. Prior distributions were obtained from expert opinion, and likelihood was based on data from tested animals. The posterior distribution of the true BSE prevalence used Markov Chain Monte Carlo Gibbs sampling. In 2002 and 2003, the true BSE prevalence in Belgium is situated in a credibility interval (CI) going from approximately 10-7 to 10-5. The specificity and sensitivity of the rapid test were estimated at 99.99% [CI: 99.99 – 100%] and 91.23% [CI: 81.69 - 91.64%] respectively. This study shows the importance of a critical study of the sensitivity and specificity of BSE diagnostic tests, as well as the apparent prevalence of this zoonosis, as their discrepancy with initial values could have important consequences for active and passive epidemiosurveillance of BSE.

EPI-23

CHRONIC WASTING SURVEILLANCE (CWD) IN BELGIUM : PRELIMINARY DATA

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In accordance with the opinion of the European Scientific Steering Committee, a preliminary (active) surveillance scheme was installed, in order to improve the knowledge of the CWD status of wild cervids (roe deer & red deer) in Belgium. For this we used 866 samples of spleen (roe deer and red deer) collected in the South-Eastern part and 206 samples of spleen and 222 brain samples from roe deer of the Northern part of Belgium. We used both the TeSeE capture ELISA (Bio-Rad) as the antigen-capture enzyme-linked immunoassay (EIA) (Idexx) technique combined with immunohistochemistry (R524, 2G11 & 12F10) as a confirmatory test. There were no indications on the occurrence of TSE (CWD) in any of the samples. A Bayesian framework was used for the estimation of the true prevalence of CWD in Belgium. This was estimated to have a median value of zero with a 95th percentile value of 0.00115 for the spleen samples of the South-Eastern part and 0.0049 and 0.0045 for the spleen and brain samples of the Northern part respectively.

EPI-24

TRENDS IN AGE-AT-DETECTION IN BOVINE SPONGIFORM ENCEPHALOPATHY CASES: A USEFUL INDICATOR OF THE EPIDEMIC CURVE IN BELGIUM

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There were 118 BSE cases in Belgium before 1 January 2004. The trends in age at time of detection of the BSE cases were analyzed. This parameter was used as a predictor tool for the current stadium of the BSE epidemic curve in a country. The following indicator variables were considered: date of birth, breed, date of detection, mode of detection, monthly number and age of animals slaughtered and rendered. The trends in age at detection in function of date of birth is a very poor epidemiological indicator. The increasing of the average age of BSE cases at the time of detection is due to the decrease of cases. That appears to be a reliable indicator of the onset of a decrease in the epidemic curve in Belgium. By means of simulations using fictitious data sets compared to real data from Great Britain, a relation between the trends of age distribution at the time of detection and the stage of the epidemic curve was demonstrated. Trends in age at the time of detection of the BSE cases may be of use in those situations where absolute numbers of BSE cases cannot be determined accurately; that constitutes the originality of this epidemiological indicator.

EPI-25

THREE YEARS OF CHRONIC WASTING DISEASE SURVEILLANCE IN WILD RUMINANTS OF THE STELVIO NATURAL PARK, IN NORTHERN ITALY

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Chronic Wasting Disease (CWD) is a transmissible spongiform encephalopathy (TSE) of both captive and wild ranging cervids such as deer and elk in North America. Until now, there is no evidence that CWD exists in wild ruminants in Italy or any other European country. However, the European Commission strongly recommends the surveillance for CWD (Regulation (EC) No 999/01 and further amendments). In the present work 200 hunted or found dead wild ruminants - European red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) - from the Stelvio Natural Park (northern Italy) underwent CWD investigations between 2004 and 2006. Medulla oblongata, palatine tonsils and retropharyngeal lymph nodes were collected from each animal. The obex was sectioned longitudinally and one half was used for the diagnosis by rapid test (Prionics® Check-Western, Bio-Rad TeSeE® ELISA), while the other was immediately fixed in 10% neutral buffered formalin for histological and IHC examination. Tonsils and retropharyngeal lymph nodes were also fixed for IHC. The Mab F99/97.6.1 was used as primary antibody. This antibody recognises a conserved epitope (residues QYQRES) of the prion protein of sheep, cattle, mule deer and Rocky Mountain elk. All the animals investigated resulted negative by rapid test on the obex. By histological examination, no vacuolisation was observed in the obex. By IHC no positive immunolabelling was observed in the obex, and in lymphoid follicles of the tonsil and retropharyngeal lymph node. In our survey no evidence of prion infection in wild ruminants was observed. Our results are in accordance with those obtained in previous investigations carried out in other Italian alpine regions. In conclusion, the existence of TSE in wild ruminants in Italy and a risk of TSE transmission to humans resulting from the consumption of their venison are rather unlikely.

EPI-26

SECONDARY TRANSMISSION OF SPORADIC CREUTZFELDT-JAKOB DISEASE VIA SURGERY IN THE UK?

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Sporadic Creutzfeldt-Jakob disease (sCJD) is the most common form of the human TSEs. The disease-associated form of the prion protein, PrPsc, is the principle component of the sCJD infectious agent, but how or even whether it is acquired remains uncertain. Until recently, the sCJD agent was thought to be primarily confined to the central and peripheral nervous systems. However, the use of increasingly sensitive techniques has enabled abnormal prion protein to be detected in some peripheral tissues of sCJD cases, including skeletal muscle and spleen. These data, together with strong evidence of transmission of variant CJD through blood, and the relative resistance of PrPsc to routine decontamination processes, have led to increased concern that sCJD might be transmitted through routine surgery.
This study describes the first analysis of reported surgical histories from a large series of sCJD cases (n=370) and general population controls (n=922) from Great Britain. The results of this analysis will be compared with those from two large case-control studies carried out previously in Australia and Europe. Temporal and geographical links will be described between sCJD cases (n=697), who underwent neurological and gynaecological surgical procedures in the UK, to determine whether it is plausible that transmission could have occurred through contaminated instruments during these procedures.

Poster Session

RISK ASSESSMENT

RA-01

ESTIMATING THE TEMPORAL RELATIONSHIP BETWEEN PRPSC DETECTION AND INCUBATION PERIOD IN EXPERIMENTAL BOVINE SPONGIFORM ENCEPHALOPATHY (BSE) OF CATTLE

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The age of cattle at which central nervous system (CNS) and associated structures should be removed at slaughter to protect human and animal health from exposure to BSE infection cannot be accurately determined based on current data. This study addresses the detection of disease specific PrP (PrP^{Sc}) relative to time after exposure in CNS and certain peripheral nervous system ganglia tissues, from cattle dosed with 100g or 1g of BSE infected brain and then killed sequentially throughout the disease course, using immunohistochemical labelling, a Western blot incorporating a sodium phosphotungstic acid precipitation step (WBNaPTA) and BioRadTeSeE, diagnostic methods. Data from experimental studies of attack rate and dose/incubation period response for the doses were obtained from experimental attack rate studies. The development of a statistical model accounted for the differences in incubation period and probability of infection between the different dose groups. There was little overall difference in the timing of detection of PrP^{Sc} among different CNS tissues, although immunohistochemistry detected PrP^{Sc} in the medulla oblongata (the current statutory testing target site) earlier than the other two testing modalities. There was a significant difference in the estimated timing of detection relative to incubation period, according to dose; the point at which 50 percent of the animals would be detected by immunohistochemistry applied to rostral medulla was estimated to occur at 1.7 months and 9.6 months before onset of clinical signs for the 1g and 100g dosed cattle respectively. There was a very low probability of detection in any of the tissues, by any of the tests, at more than 12 months before clinical onset. PrP^{Sc} was inconsistently detected in dorsal root ganglia and not at all in certain sympathetic nervous system ganglia associated with the vertebral column. When dorsal root ganglia were positive, detection was always after the CNS was positive.

RA-02

RISKS FROM THE USE OF MAMMALIAN MEAT AND BONE MEAL AS A SOIL IMPROVER

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Landspreading is now being considered a viable utilisation route of mammalian meat and bone meal (mMBM) by many European Countries and which is allowed under new European legislation (European Commission regulation No 1774/2002). These changes have meant that Category 3 waste (including mMBM derived from animals slaughtered for human consumption) which has been appropriately heat treated and ground to a specified particle size, can be spread on non-pasture agricultural land. Two separate case studies (study 1 in Great Britain and study 2 in Ireland) on the potential exposure to Transmissible Spongiform Encephalopathy (TSE) infectivity following the spreading of Category 3 abattoir waste on non-pasture agricultural land was carried out. Both models use Monte Carlo simulation techniques to account for parameter uncertainty and variability. For Case Study 1, the average TSE infectivity on non-pasture agricultural land per year from sheep with scrapie was found to be higher (5 orders of magnitude) than that estimated for BSE in cattle (1.5 Ovine Oral ID_{50} per tonne of mMBM compared to 1.5×10^{-5} Bovine Oral ID_{50} per tonne of mMBM). The mean estimate for BSE in sheep was 8.1×10^{-6} Ovine Oral ID_{50} per tonne. For Case Study 2 the mean level of infectivity in Category 3 produced mMBM was assessed to be 2.36×10^{-5} ID_{50}/tonne of MBM. The spreading of this MBM resulted in infectivity on non-pasture land of 1.62×10^{-8} Bovine Oral ID_{50}/m^3. The mean simulated probability of infection per year per bovine animal was 1.11×10^{-9}. The two studies indicate the low risk associated with the re-use of mMBM as a fertiliser and signal the option to change national legislation in line with EU TSE legislation without comprising animal or human health.

RA-03

ASSESSMENT OF HAZARDS FROM ENVIRONMENTAL PERSISTENCE OF TSE INFECTIVITY

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TSE infectivity may enter the environment by various routes and persist in the ground, spread from the original source to contaminate an extended area and ground water. We are addressing whether infectivity survives within a carcass over time; survives without containment, whether it is disseminated; and whether environmental conditions affect the survival and/or transport of infectivity through soil. We are performing two field experiments using a fast draining sandy loam and a slow draining clay loam. A series of 10 bovine heads have been spiked with TSE infectivity and buried in the two soils, contained within lysimeters, for sequential annual exhumation and analysis. Boluses of TSE infected brain are also buried in the centre of two lysimeters and soil samples taken from them regularly. Rainwater flow-through is also analysed. These experiments are complemented by laboratory and soil column studies of the interaction between soil and its components and TSE infectivity, using recPrP and PrP^{Sc} as a surrogate marker. PrP binds strongly to both the sandy soil and clay soil, and to pure sand (quartz). Elution was only achieved with Sarkosyl. Elution from clay soil elution also required proteinase K digestion. Hence a different mechanism of binding may occur to components of the clay soil which are largely absent from the sandy soil. This binding occurs via the N-terminal domain of PrP and is not disrupted by Sarkosyl. These results form the basis of a method for eluting PrP^{Sc} and TSE infectivity from soil. Results so far suggest that TSE infectivity may bind strongly to soil components and has very limited mobility in soils with controlled rates of water percolation. TSE infectivity could therefore persist in one location for long periods of time.

RA-04

BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF PRP IN EWE MILK.

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Because of the possible presence of the BSE strain in sheep and goat flocks some concerns have long been raised about the possibility that small ruminant milk could carry some infectivity. These concerns have been reinforced recently by the demonstration that infectivity is currently reported in blood from animal or human infected by the BSE strain associated with the presence of blood related cells in milk. In addition, recent works have shown that PrPsc accumulates in mammary glands from sheep affected by scrapie and mastitis. In this context it is critical to analyse more precisely the relationship between PrP and milk components.

In this study, we have analysed the PrP content of the main milk fractions (casein whey, fat and cells) using biochemical (ultracentrifugation, western-blot) and immunological (western-blot, ELISA) methods.

By using a panel of monoclonal antibodies directed against different epitopes located along the PrP sequence and by combining immunoprecipitation techniques with western-blot measurements, we could demonstrate that PrP is essentially present in milk fractions (casein whey, fat and cells) as an N-terminally truncated protein. Ultracentrifugation analysis showed that PrP exists in casein whey and fat fractions as an amphiphilic protein bearing a GPI anchor.

Measurements made on the different ewe milk fractions using an adapted sandwich ELISA showed that important concentration of PrP are present in casein whey (75-600 ng/ml) and fat (150-600 ng/ml) while much lower concentration are measured in cells extracts. Taking into account the proportion of each milk fraction it clearly appeared that casein whey accounts for more than 95% of the total PrP content of milk with approximately 5% for fat and less than 0.1% for cells. Measurements performed on colostrums showed that it contains about twice more PrP than milk.

Finally, we have optimized immunoprecipitation methods in order to bind on magnetic beads more than 95% of the total PrP content of the different milk fractions. This was obtained by using a mixture of monoclonal antibodies including an antibody having demonstrated its capacity to immunoprecipitate aggregated PrPsc in TSE infected brain extracts. These beads will be inoculated in ovine transgenic mice in order to detect infectivity possibly associated with PrP in the three milk fractions.

RA- 05

CURRENT ANALYTICAL DEVELOPMENTS FOR THE DETECTION OF CNS-BASED SPECIFIED RISK MATERIAL (SRM)

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In the wake of the BSE-crisis, particularly after the emergence of a new variant of Creutzfeldt-Jakob disease, preventive measures where established for the protection against a potential human TSE exposure risk. Bovine BSE infectivity is concentrated in tissues of the central nervous tissue (CNS) and closely related to tissues of the peripheral nervous tissue. These CNS-based tissues compose the majority of the so called specified risk material (SRM), at least 95%. Until now the removal of SRM from the human and animal food chain and their innocuous disposal was given top priority. In view of infectivity titres, the legal definition of SRM includes the specification of the animal species (domestic ruminants) and age of the animal (older than e.g. 12 months) from which these tissues were derived.

In addition to the SRM ban, the individual testing of bovines for the presence of PrP^{Sc} guarantees that potential contamination of meat with CNS-based SRM during slaughter would not cause any additional human exposure risk. As a reaction to the steadily decreasing incidence of BSE the European Commission proposed in its TSE-Roadmap, as a strategic goal, the reduction of BSE-tests in bovines and the increase of age limits for SRM. As both preventive measures have to be performed systematically and individually, they are extremely expensive. Thus, such a strategic goal would be welcomed widely. In prion disease research, however, many questions remain unanswered and it appears from the point of risk assessment to be unwise to reduce the existing measures without compensation.

Since 1996 immunochemical methods for the detection of CNS in meat products have been developed and validated. In some countries these methods have been successfully applied to control and to ensure the SRM ban for the human food-chain. In future, such spot-check methods could compensate the reduction of primary systematic preventive measures. Here we report on the current developments in methods for the detection of CNS-based SRM. In particular we identify drawbacks of immunochemical and molecular biological methods as pertaining to stability of markers and species- / age-specificity. The development of a new analytical approach using GC/MS and certain CNS-correlated fatty acids is demonstrated. GC/MS facilitates the detection of CNS in accordance to the legal definition of specified risk material (SRM) and shows the potential to become a future reference method for CNS-based SRM detection.

RA- 06

EVALUATION OF RISK FACTOR OF BSE AND VCJD TRANSMISSION REGARDING HUMAN CODON 129 POLYMORPHISM

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Prion diseases comprise a group of neurodegenerative transmissible spongiform encephalopathies (TSE). The epidemic of mad cow disease in UK in 1990s and its recent spread to humans have received broad attention all over the world. Human prion gene presents a common polymorphism at codon 129, resulting in either methionine or valine.

Recent studies showed that the polymorphism at codon 129 of the human prion gene has a profound effect on the susceptibility in human prion disease. In order to quantify the species barrier of prion transmission, we developed a seed titration method to evaluate it. Prion peptides corresponding to human PrP sequence 108 to 144 and bovine PrP sequence 111 to 147 were used as a model system. The seeding efficiency among bovine PrP (111-147) and human PrP (108-144) with different genotypes at codon 129 were evaluated by time-resolved Circular Dichroism (CD) spectroscopy. Our finding suggested that the individuals with methionine homozygote at codon 129 (huPrP129M) has the highest susceptibility to bovine PrP fibrils, followed by the heterozygote (huPrP129M/V), and those with valine homozygote (huPrP129V) has the lowest susceptibility. Moreover, the risk of acquiring vCJD from human was also assessed by the same peptide system. Interestingly, the risk factor of intra-species prion transmission among different human genotypes was higher than the bovine → human transmission, especially for the heterozygote (huPrP129M/V) and the valine homozygote (huPrP129V).

RA- 07

CLUSTERING OF PRP^{RES} IN CENTRAL BRAIN REGIONS OF BSE-INFECTED MACAQUES (M. FASCICULARIS)

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According to biochemical and epidemiological findings bovine spongiform encephalopathy (BSE) was transmitted to humans causing variant Creutzfeldt Jakob disease (vCJD). Previous studies have shown intracerebral (i.c.) transmission of BSE affected brain from cattle can cause TSEs in cynomolgus macaques (*M. fascicularis*). The lesion profile resembles that of vCJD. Recently, oral infection of *M. fascicularis* with macaque-adapted BSE material was reported.
In cooperation with five European partners a quantitative study for the transmission of the BSE agent to *M. fascicularis* was initiated to assess the risk of vCJD infection in humans through contaminated food products. Titration was performed orally and intracerebrally to determine the minimal infectious dose for cynomolgus monkeys.
Here we report the outcome of the intracerebral infection with 50 mg BSE brain homogenate in six non-human primates. All animals showed clinical symptoms of TSE after an average of 1100 days. Using immunohistological and biochemical methods prion protein (PrP) deposits were confirmed in the brains of all animals. Using Western blot analysis the glycosylation pattern was compared to the inoculum and to the pattern of different CJD subtypes. The glycopattern TSE infected cynomolgus macaques resembles human CJD type 2. Simian PrP^{res} was detected with the monoclonal anti prion antibody 11C6, which revealed a higher sensitivity in comparison to 12F10 and 3F4. We further analysed the distribution of PrP^{res} by microdissection of seven different brain regions of all infected macaques. High concentrations of PrP^{res} were found in central brain regions, as *gyrus cinguli, nucleus caudatus, vermis cerebelli* and *basis pontis*. In contrast, in the peripheral regions *gyrus frontalis, gyrus parietalis* and *gyrus occipitalis* PrP^{res} was hardly detectable.
Thus, the incubation period related to the life expectancy, the PrP^{res} glycosylation pattern as well as the distribution in certain brain regions resemble those in vCJD patients. The relative abundance of PrP^{res} in macaques will be compared to that of orally infected animals.

RA- 08

ENVIRONMENTAL BEHAVIOUR OF PRION PROTEINS

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Before the risks connected to the past BSE epidemics were fully recognized, prions were discharged into the environment from rendering plants, abattoirs and landfills (Gale et al. 2001). Prions can still be occasionally dispersed through blood and even probably feces by infected animals and cause contamination of pastures and surface water (Belay et al. 2004). In this work we investigated the interactions among recumbinant murine (23-231) and human (90-231) prion proteins and environmental colloids.
Recumbinant murine prion protein 23-231 was expressed in *Escherichia Coli* cells cultures and purified according to the method described by Negro et al. (2000). Human 90-231 recumbinant prion protein was prepared as described by Corsaro et al. (2002). Interactions of prion proteins with humic substances in solution were investigated by differential UV spectroscopy. The first and second derivative were used to measure bathochromic effects and calculate exposure indexes. The relative contribution of the α helixes, β-sheets and random coil structures to the tertiary structure of the protein in solution was evaluated by circular dichroism before and after addition of humic an fulvic acids. Interaction between recombinant hamster or human prion proteins and humic acids in solution results in structural rearrangements of the proteins which affects side chains. Circular dichroism spectra confirmed the existence of interaction between the protein and show an increase in β-sheet stucture for both proteins examined. Elaborations by the Autodock program coupled with experimental results suggest that the most probable sites of interaction are represented by the positively charged exposed groups of the histidine 115, the lysine 6 and arginine 79. Humic substances of both aquatic and terrestrial origin strongly interact with prion proteins and can therefore affect their behaviour in the environment.. These interactions together with those observed with clays explain the irreversible adsorption observed on soils rich in organic matter and clay and the lower affinity displayed by a sandy soil (Leita et al. in press).

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RA- 09

HOW MUCH SHEEP MEAT WE EAT IN EUROPE: DATA FOR RISK ASSESSMENT

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The confirmation of the first case of BSE in a goat, in 2005, raised concern on the human risk of exposure to BSE via sheep and goat meat consumption. Unfortunately few data are available on this kind of consumption and most of nutrition surveys do not specifically account for items like sheep or lamb meat. Our purpose was to estimate the order of magnitude of the sheep meat consumption in the European population. This exercise is part of an exposure assessment aiming at characterizing the intake of a potential BSE infective dose through small ruminants' meat. Two nationwide surveys, providing data on the weekly or daily intake of sheep meat in the United Kingdom *(National Diet and Nutrition Survey)* and in Italy (*INN-CA study)* were used to validate Eurostat data as a reliable source. Using the Eurostat data on human population sizes, production of sheep carcasses and the import/export of sheep meat in the EU15 countries from 1995 to 2005, we estimated the overall EU15-wide consumptions and the daily individual meat intake over the last 10-year period. The overall annual consumption of sheep meat in both Italy and UK obtained through survey data was lower than that from Eurostat but of the same order of magnitude. The EU15-wide figures for the overall annual consumption (in 1000-ton) and daily individual intake (in grams) were respectively 1186.4 and 8.5 for the year 2004. We provide helpful and previously unavailable data on the sheep meat consumption. Moreover we show that Eurostat data may represent a valuable source when carrying out food exposure assessment. The difference observed between national surveys and official statistics may come from different food classification used. Finally our findings are population averages with skewed distributions: further information is needed on the "actual consumers". It is further recommended to initiate collection of such data from more Member States using a harmonized approach in order to be able to compare results.

RA-10

SEASONALITY OF EXPOSURE OF CATTLE TO BSE IN FRANCE

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In order to detect a potential birth seasonal pattern of exposure for the risk of Bovine Spongiform Encephalopathy (BSE) in France, we fitted a logitic model adjusted for age at detection, birth cohort and month of birth. We used exhaustive BSE data provided by the screening plans in force in France since July 1st 2001. 640 BSE positive cases and 7,981 009 negative animals over 48 months, detected from July 1st 2001 through December 31st 2005, were included in the study. Age and birth cohort were categorized into 16 groups using 12 month intervals, while season of birth was taken into account on a monthly basis. Two logistic models were run, in which parameters were estimated by the maximum likelihood method, respectively for dairy cattle and beef cattle, because calving seasons and feeding patterns are different between these production types in France. Categories at risk of BSE for the age at detection and birth cohort factors were identical for dairy and beef cattle. BSE prevalence was maximal for animals aged from 60 to 83 months at detection and for cohorts born from July 1st 1993 to June 30th 1995 (birth cohorts 93/94 and 94/95) and decreased for latest birth cohorts. Dairy cattle born from July to September were significantly more at risk for BSE than those born in other months while BSE prevalence in beef cattle was significantly higher for August and November months of birth. Our results agreed with previous studies in the UK which evidenced a seasonal pattern of exposure, probably due to specific herd and feeding schemes. More detailed data about specific seasonal feeding practices and particularly consumption of concentrates could help understanding the epidemiology of the disease.

RA-11

INFLUENCE OF BOVINE GASTROINTESTINAL MICROBIOTA ON SCRAPIE ASSOCIATED PRION PROTEIN (PRP^{SC})

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The influence of complex microflora residing in the gastrointestinal tract of cattle on prion protein plays a crucial role with respect to early pathogenesis and the potential infectivity of faeces resulting in environmental contamination. However, it is unknown whether infectious prion proteins, considered to be very stable, are inactivated by microbial processes in the gastrointestinal tract of animals. Feedstuffs consumed by ruminants are initially exposed to microbial fermentation in the rumen prior to gastric and intestinal digestion. Especially the polygastric digestion of ruminants represents an efficient system to degrade food proteins by microbial fermentation processes in rumen and colon. In this study, rumen and colon contents from healthy cattle, taken immediately after slaughter, were used to assess the ability of these microbial consortia to inactivate PrP^{Sc}. Therefore, the consortia were incubated with brain homogenates of scrapie (strain 263K) infected hamsters under physiological anaerobic conditions. Recently, studies were published indicating the ability of complex ruminal and colonic microbiota of cattle to decrease scrapie associated prion protein up to immunochemically undetectable levels in Western blot under physiological conditions. Subsequently, comparatively analysing the concomitance of the loss of anti-prion antibody 3F4 immunoreactivity and the inactivation of PrP^{Sc} *in vivo* hamster bioassays were performed. However, the results demonstrated significant prion infectivity after degradation of infected hamster brain through the gastrointestinal microflora of cattle. Thus, infectivity is still present and may enter the host, irrespectively of the mechanism, by PrP^{Sc} at levels below the threshold of immunochemical detection, or by a sub-fraction of infectious prion protein not detectable by immunochemical methods. Finally, the possibility of present infectious molecules or structures other than PrP^{Sc} must be seriously considered. Conclusively, these data highlight the deficiency of using Western blot or immunoassay formats in TSE inactivation assessment studies, and raise the possibility that the environment might be contaminated through cattle shedding infected faeces.

RA-12

EXOGENOUS RISK FACTOR OF CJD IN REGIONS WITH ITS INCREASED OCCURRENCE IN SLOVAKIA.

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Slovakia is characterized by unique proportion of genetic CJD (gCJD) with E200K mutation. Since the penetrance of the mutation is incomplete (59%) probably also other factors may influence the disease course.The precursor of the prion (PrP^{sc}) is a normal cellular prion protein (PrP^{c}), which under physiological conditions binds copper (Cu) and this linkage regulates the resistance of cells to the oxidation stress and apoptosis. Experimentaly was demonstrated that in a case of Cu deficiency other metals (mainly manganese) can replace Cu, forming misfolded metallo-glycoprotein with a similar physical-chemical properties as PrP^{sc}. The role of described changes in the pathogenesis of prion diseases is not clear. The majority of Slovak gCJD originate from nothern and southern parts of Central Slovakia. In both regions are factories (ferromanganese and glass) - potentionall sources of atmospheric Mn contamination. Previously we demonstrated significant Mn/Cu disbalance in the soil and food chain in the Slovak CJD clusters area. Our objective was to determine Mn, Cu levels in the CNS of CJD patients as a pilot study for further investigation of Mn/Cu disbalance as a possible co-factor in the development clinical manifestation of CJD. Mn and Cu concentrations in 24 CNS samples (8 gCJD, 8 sporadic (sCJD), 8 controls without neurol.signs) were determined by flame AAS. Analyses demonstrate i) mean Mn level 0,6088mg/kg for gCJD, 0,3675mg/kg for sCJD, 0,3000mg/kg for controls, the difference between genetic and control groups was significant (p=0,003) ii), mean Cu status in gCJD 5,475mg/kg, sCJD 4,738mg/kg, controls 4,700mg/kg; iii) mean Mn/Cu ratios in gCJD 11,4338, sCJD 7,9738 and controls 6,4888. Mn/Cu ratios in our genetic group show strong disbalance of metal ions in CNS in comparison to those of control group. Results warrant further studies, whether the observed coincidence of endogenous (genetic) risk and a possible environment. risk factor may be involved in the ethiopathogenesis of gCJD and to contribute to the focal CJD accumulation in Slovakia. Work was supported by Science and Technology Assistance Agency, contract No. APVT-21-019004.

RA-13

CONSUMER CONFIDENCE AND BSE IN CANADA

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BSE is a medical and animal health problem with wide ranging social and economic consequences. Using Canada as a case study, this paper tracks the surprisingly resilient consumer confidence in beef and beef products in the wake of the BSE case in Alberta on May 20, 2003. This strong and unwavering consumer confidence in Canada was not seen in other countries with BSE. Using qualitative data collected in Alberta in the summer of 2006, quantitative data on beef consumption compiled by Statistics Canada and Alberta Beef Producers, and archival data of media coverage on BSE and related issues since May 2003, this paper will show that the resilience of Canadian consumer confidence in beef and beef products in 2003 was driven by a multitude of factors that are outside the scientific-based framework of associating risk (to human) with prion/BSE (in the beef products). Some of these non-BSE factors are nationalism and regional identity, the concurrent outbreak of SARS, the regional and provincial politics of rural economy, and the lessons learned from past economic fallouts due to BSE in the U.K. The findings suggest that risk assessment in food safety and its subsequent translation into food consumption behaviour are not necessarily straightforward or easily predicted. The scientific information about BSE and prion and their links to human health is only one of many factors that consumers take into consideration in their own formulation of risk and consumption behaviour. This case study provides a concrete example of what happened in Canada in 2003 and it highlights how the specificity of local events, economic history, and political legacies can contributed to unexpected outcomes in the event of a crisis. The primary data for this paper are collected in multiple sites within the province of Alberta using the following methods: interviews, focus group discussions, document analysis. The major informants are institution personnel (government, beef industry, private businesses, universities, unions), beef producers, rural and urban consumers, retailers and other private citizens.

RA-14

DESIGNING A RISK MANAGEMENT FRAMEWORK FOR HUMAN AND ANIMAL PRION DISEASES IN CANADA

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In Canada the occurrence of five bovine spongiform encephalopathy (BSE) cattle within three years has led to an estimated six billion dollar economic loss in direct and indirect costs. Much concern has been focused on the economic impacts to the Canadian agricultural sector while the outcomes to farm families remain largely uncalculated. Our analysis attempts to design an over-arching risk management framework for prion diseases with the first step of identifying the psychological and social outcomes that have occurred at the individual (farm worker), family, farm business, community, regional, national and international levels. The occurrence of BSE in cattle has resulted in a ripple effect with concern about human health, increased stress to farm families, contaminated blood transmission, and iatrogenic infectivity. Unique Canadian Aboriginal Peoples (First Nations, Métis and Inuit) may be at higher risk for negative impacts as wild cervid populations of white-tailed deer and elk are affected by chronic wasting disease, a related prion disease. The transmission risk from cervid to human populations is not yet known. Cataloging the outcomes allows for appropriate prioritization and allocation of resources to effectively respond to and manage ongoing prion disease risks.

RA-15

RISK ASSESSMENT OF SELECTION FOR RESISTANT PRP ALLELES ON MILK PRODUCTION IN VALLE DEL BELICE

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To avoid any risk of scrapie or BSE in sheep to humans the EU decided that selection against susceptible *PrP* alleles should be implemented. This means that all susceptible *PrP* alleles should be eradicated from the EU sheep population. Such a selection can adversely affect any traits which are directly or by linkage associated with *PrP* genotypes. A study has been undertaken to determine if *PrP* genotypes or individual *PrP* codons have an effect on milk production traits. A total of 501 Valle del Belice sheep with both production as well as *PrP* genotype information was available. In total these ewes had information on 5472 test-days from 796 lactations. The analysed traits were: Milk yield, Fat yield, Protein yield, Fat+Protein yield, % Fat, % Protein and Somatic Cell Score (SCS). A variety of mixed models were used for the analyses accounting for additive, dominance and epistasis effects of up-to three *PrP* codons. An effect of the *PrP* gene in Valle del Belice dairy ewes on milk, fat and protein yields appears significant. No significant effect on fat and protein percentages and on somatic cell score was found. Given the strong influence of a single animal, the only one with the AHQ/AHQ genotype, the significances are doubtful; without this animal only milk yield remained significant. Selection to obtain homozygous ARR/ARR animals is expected not to result in any production changes, because the ARR/ARR animals were producing on the population average.

RA-16

CANADIAN FOOD AND ENVIRONMENTAL RISK PERCEPTIONS FOLLOWING THREE BSE INCIDENTS: A NATURAL EXPERIMENT

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Our objective is to measure whether and how Canadians' food and environmental risk perceptions have changed following a series of BSE incidents. We report analysis of data from a natural experiment based on responses by a representative group of Canadians to surveys conducted in January 2003 (prior to the first BSE incident in a Canadian-origin cow) and October 2005 (subsequent to three BSE incidents), following extensive media reporting. Each survey queried respondent's risk perceptions, based on a four point rating scale ("high risk" to "almost no risk") and "don't know" for 8 food issues, including BSE, presented in random order. A separate set of randomized questions related to perceptions of environmental risks of 6 issues associated with agriculture, including BSE, used similar rating scales. For the 2003 sample, n=646; for the 2005 survey n=1567. Each is reasonably representative of the adult Canadian population. The level of concern expressed in the choice of "high risk" ratings for BSE as a food issue fell from 31% to 24% between January 2003 and October 2005 while "moderate risk" and "slight risk" ratings rose from 10% to 27% and 18 % to 28%, respectively. Ordered probit models are tested in which socio-economic, demographic, and trust variables are postulated to explain the probabilities of the categorical risk rankings for each food and environmental issue. These are tested separately on the data for each period and on the data set pooled for the two periods, including time dummy variables. Chow-type tests show significant differences in parameters that determine respondents' BSE risk perceptions between the two time periods. Respondents with higher education were more likely to consider BSE to be highly risky in 2005 than in 2003. Older respondents and those living in Quebec were less likely to rate BSE as a high food risk in 2005 than in 2003. Risk communication issues that may have influenced these changes are discussed

Poster Session

GENETICS

GEN-01

POLYMORPHISMS IN THE PROTEIN CODING REGION OF THE PRION GENE (PRNP) OF SOME EUROPEAN AND ASIATIC CERVID SPECIES

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Chronic wasting disease (CWD) is a natural Prion disease which currently affects a number of cervid species in North America; these include Mule deer, White-Tailed deer, Rocky Mountain elk and Moose. Association between variation in the primary sequence of the PrP gene and disease modulation has been shown for CWD and a number of studies have analysed the genetic variability of the PrP gene in North American cervid species. European cervid species are currently considered free of this disease and the knowledge of how the PrP gene varies between and within these species is still limited.
We report here the results obtained in two studies of the PRNP gene in various European deer species. Study one analysed 82 red deer (*Cervus elaphus)* from mainland Scotland while the second study analysed 50 red deer from a Scottish island (Isle of Rhum). Additionally, archival samples from one *Axis axis,* two *Cervus eldii, one Dama dama and* four *Elaphurus davidianus* were analysed. The study focussed on the open reading frame of the PrP gene. DNA was extracted from blood and analysed through direct sequencing of PCR products.
We have seen amino acid polymorphisms at codons 138, 168, 208 and 226. Several silent mutations were also detected at codons 15, 21, 77, 136, 168, 172 and 217. All the animals studied were methionine homozygous at codon 132. Variation at this position has been associated with CWD modulation in Rocky Mountain elk.

GEN-02

A CASE-CONTROL STUDY ON THE ASSOCIATION OF CODON 222 POLYMORPHISM WITH RESISTANCE TO SCRAPIE IN ITALIAN GOATS USING THE PYROSEQUENCING TECHNIQUE.

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Recently an association with resistance to scrapie of the glutamine to lysine mutation at codon 222 was found in Italian goats by Acutis *et al.* (2006), in a case-control study on 177 goats from six scrapie outbreaks. Aim of the present work was to extend that previous case-control study, analysing a higher number of goats and outbreaks, to better assess the possible protective role of the mutation K222. To achieve this objective we set up a protocol to determine the genotype at codon 222 using the high throughput Pyrosequencing technique. We designed a pair of primers amplifying a 237pb fragment including codon 222. The sequencing primer for the Pyrosequencing assay was designed using the SNP Primer Design Software (Pyrosequencing, AB). The assay was validated analysing 253 goat samples previously sequenced with an automated sequencer (194 samples Q/Q at codon 222; 50 Q/K and 9 K/K): all the samples were correctly identified. The new technique was then applied to analyse 582 goats (34 positive and 548 negative) from 15 scrapie outbreaks. In this sample also the 177 animals from the previous study were included. We found 484 goats Q/Q at codon 222 (83,2%), 91 Q/K (15,6%) and 7 K/K (1,2%). All the positive animals were Q/Q. A χ^2 test was performed comparing the frequencies of genotypes between cases and controls: Q/K and K/K were combined in a single group. A significant association of K222 with the scrapie negative status was found (χ^2=6.09 p-value=0.0136). The possible protective role of K222 in Italian goats has then been confirmed, with a statistical association stronger than that found in the previous case-control study.

GEN-03

GENETIC SUSCEPTIBILITY OF ITALIAN SHEEP AND GOATS TO NOR98: A CASE-CONTROL STUDY

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Prion protein (PrP) gene alleles AHQ and $AF_{141}RQ$ are associated with the highest risk of acquiring the atypical scrapie strain Nor98. Furthermore, Nor98 is also found in animals carrying the ARR allele, associated with resistance to classical scrapie. In Italy, until May 2006, 15 Nor98, have been detected, four of which involving goats. A case-control study has been carried out to investigate the genetic susceptibility to Nor98 in Italian sheep and goats, considering the alleles formed by polymorphisms at codons 136, 141, 154 and 171. In sheep, allele frequencies were compared, with a χ^2 test, between 3 cases and 1301 Nor98 negative flockmates. In addition, a χ^2 comparison has been done between allele frequencies of all the 11 ovine cases found in Italy so far and allele frequencies of the Italian ovine population, coming from the genotype analysis of the sample foreseen by Reg. 999/2001 (EC), Annex III, point 7.2. In goats, 3 cases and 139 negative herdmates have been examined. In sheep a significant association of AHQ and $AF_{141}RQ$ with Nor98 was found (χ^2=6.84, p-value=0.009, OR=4.96; χ^2=31.21, p-value=0.000, OR=47.69, respectively). The ARR allele had no significant association, showing that neither it presents an increased risk of acquiring the disease nor it has a protective role as in classical scrapie. In goats the AHQ allele was significantly associated with Nor98 (χ^2=4,35, p-value=0.013, OR=9.12), suggesting that genetic susceptibility in sheep and goats shares common features.

GEN-04

CHARACTERIZATION OF THE PRP POLYMORPHISM IN POSITION 171 BY AN ELISA TEST, APPLICATION FOR THE SELECTION OF SHEEP RESISTANT TO TSE.

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Scrapie is a transmissible spongiform encephalopathy (TSE) which naturally affects sheep and goat. In the last years, concerns have been raised concerning the possible presence of BSE in small ruminant flocks. Following Commission Decision 2003/100/EC many countries have to introduce a breeding program to select for resistance to TSEs in sheep herds based on the PrP polymorphism at codons 136 (A/V), 154 (R/H) and 171 (R/H/Q).The resistance to prion infection is essentially associated with the presence of an arginine residue in position 171 of PrP and is particularly high in homozygous ARR/ARR animals. We described a new ELISA test (sandwich immunoassay) allowing a rapid, simple and cost effective characterization of the PrP polymorphism in position 171 on a plasma or serum sample. This test is based on the use of monoclonal antibody 2A11 which has the peculiar property of not recognizing PrP bearing a R residue in position 171 while it binds very efficiently PrP with an histidine (H) or a glutamine (Q) at the same position. The capture of PrP contained in the serum or plasma sample is achieved by another monoclonal antibody recognizing all PrP molecules independently of the polymorphism in position 171.
This test unambiguously identifies animal bearing a ARR/ARR genotype. In addition it allows classifying with a excellent sensibility and specificity animal bearing a Q/Q or a H/Q genotype with regards to heterozygote R/Q or R/H. It is adapted for selection on a high throughput basis of animals resistant to TSEs.

GEN-05

IDENTIFICATION OF EARLY NATURAL SCRAPIE-SPECIFIC GENE EXPRESSION CHANGES IN TONSIL AND PEYER'S PATCHES OF SHEEP USING A SHEEP CDNA MICROARRAY.

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All currently used routine diagnostics for TSEs are thus far solely based on the detection of pathogically folded accumulated prion protein (PrP). The process of TSE agent-uptake, which most likely takes place in the gut via the ileal Peyer's patches, is still poorly understood, as is the host response to the TSE agent itself. This research focuses on the identification of new biomarkers (other than PrP) that might be useful to extend current TSE diagnostics and/or allowing us to understand TSE pathogenesis better. Therefore, we studied the host response / gene-expression-profiles in the very early phase of a natural scrapie infection in sheep using custom sheep cDNA arrays.
We have generated a cDNA microarray of about 32.500 spots per slide containing 7.369 unique features from a large normalised EST library of Tonsil and Peyer's patches of sheep (non-infected and infected of different PrP genotypes). The unique features were determined by sequencing, clustering and assembling over 13.000 sequenced ESTs into 1.850 contigs and 2.594 singletons. Only about 40% for these sequences could be functionally 'annotated' by BLAST to publicly databases thus far.
To probe the sheep arrays we collected and mRNA preserved many different tissues that include tonsil, Peyer's patches of ileum and jejunum, brain regions, blood, muscle regions and RLNs. These materials were collected at different time points (0, 3, 8 and 32 weeks) of sheep having different PrP genotypes after natural exposure to scrapie or from scrapie-free sheep. Tissues used for array hybridisation were also checked by IHC for scrapie to be able to link the found gene expression differences to the kinetics of PrP^{Sc} deposition. The current status of the gene expression profiling will be presented in relation to PrP genotype, length of incubation, tissue, and PrP^{Sc} accumulation.

GEN-06

CLINICAL AND SUB-CLINICAL SCRAPIE OCCURRENCE IN A NATURAL INFECTED SARDA BREED SHEEP FLOCK SUBMITTED TO DIVERGENT SELECTION FOR RESISTANCE.

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The occurrence of Scrapie in sheep is modulated by the polymorphisms at codons 136, 154 and 171 of the gene coding the prion protein (PrP). Combinations of the polymorphisms in these three codons, create different alleles, of which ARQ, AHQ and ARR are the most frequently found in Sarda breed sheep. So far, Scrapie in this breed affects animals with ARQ/ARQ, ARQ/AHQ, and AHQ/AHQ genotypes. In order to demonstrate that genetic control of population at the PrP locus can be a useful strategy in field conditions, a study on the effect of the introduction of resistant rams was planned, starting in 2001, in a natural-scrapie affected flock. A susceptible blood line (GS) and a resistant blood line (GR) of sheep were created in this flock, mating respectively, one group of sheep with an ARQ/ARQ rams and an other with an ARR/ARR rams. This procedure was also applied in the mating period of the three following years. The female born from each blood line in the period 2002 - 2004, were raised and also included, at 7 months of age, in the mentioned breeding program along with the dams. The two groups were kept in contact together in a barn, except during the mating period. Male offspring of each year was sacrificed at about 24 months of age. Appropriated western blotting and immunohistochemical protocols for PrP^{Sc} detection in the Central Nervous System (CNS) and in the Lymphoreticular System (LRS) were carried out on the male offspring, sheep found with neurological signs, and sheep that died of non-scrapie-related conditions. In this period we found 29 sheep, of which 18 from the progenitor group and 11 from the offspring of the GS group, harbouring PrP^{Sc} in the CSN and/or the LRS. All infected animals carried the ARQ/ARQ or the ARQ/AHQ genotype. In our monitored flock of scrapie-affected Sarda breed sheep the genetic control of population at the PrP locus proved to be a useful strategy to avoid spreading the disease among the offspring. Indeed, the ARR allele is strongly associated with resistance to scrapie, in spite of the high infective pressure in the flock, since susceptible and resistant animals were kept together, even in the lambing period.

GEN-07

SPORADIC FATAL INSOMNIA IN A FATAL FAMILIAL INSOMNIA PEDIGREE

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We describe a case of sporadic fatal insomnia (sFI) occurring in a family in which several members carried the D178N mutation in the *PRNP* gene and died of fatal familial insomnia (FFI). A 43-year-old woman presented with an 11-month history of diplopia, withdrawal, confusion, memory loss, unsteady gait and inability to sleep with episodes of agitation and dream enactment. After a progressive course characterized by cognitive impairment, marked gait ataxia, signs of autonomic hyperactivity, and myoclonus the patient died 24 months after the onset of symptoms. The patient did not have any personal contact with FFI affected relatives and her closest one was a paternal uncle, the son of her grand-grand mother. Analyses of DNA from various tissues of endo- ecto- and meso-dermal origin, including 5 different regions of the CNS revealed no pathogenic mutations and methionine homozygosity at codon 129 of *PRNP*. Brain histopathology and PrP^{Sc} typing showed typical features of FI such as thalamic and olivary atrophy, focal spongiform degeneration limited to the cerebral cortex, relative sparing of basal ganglia and cerebellum, and relatively low amount of PrP^{Sc} type 2A accumulation.
sFI represents the rarest among the sporadic human TSE subtypes described to date with less than twenty cases described worldwide and only three cases diagnosed in Italy since the establishment of TSE surveillance. Similarly, only six unrelated FFI families have been observed in Italy to date, making the probability of a chance association between sFI and FFI in the same family extremely low. Thus, we believe that our observation emphasizes the importance of undiscovered factors modulating the susceptibility to human prion diseases.
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GEN-08

PRP GENE POLYMORPHISMS IN BARB BSE CATTLE IN GREAT BRITAIN

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The aim of this study was to establish whether polymorphisms in the open reading frame (ORF) or promoter regions of the PrP gene from cattle born-after-the-reinforced ban (BARB) are associated with increased susceptibility to BSE disease. These cattle, born after August 1996, are diverse in breed, age and geographical location and because of regulations on feed ingredients should not have been orally exposed to the BSE agent in contaminated feed. The ORF (1.1 kb) of the PrP gene from 101 BARB BSE cases and the promoter region (5 kb including exon 1 and 2) from 49 BARB BSE cases were sequenced. In addition, when suitable animals could be located matched control animals were also sequenced. Within the ORF, four silent single nucleotide polymorphisms (SNPs) were detected corresponding to codon positions L23 (leucine), Q78 (glutamine), P113 (proline) and N192 (asparagine). One non-conservative polymorphism was found in the which affected the number of octapeptide repeats in the PrP N-terminal region. Genotypes with 6:6, 6:5, 5:5 and 6:7 repeats were detected. For the PrP promoter region, 51 common polymorphisms were identified including two major indels (insertions/deletions) of 23bp, located upstream of exon 1, and 12 bp, located in intron 1. Five main promoter haplotypes were determined from the promoter sequence data generated. No polymorphisms identified in the PrP gene coding region or promoter region were found to be associated with increased susceptibility to BSE in the BARB cases when compared to controls. However, within the BSE BARB group, a homozygous SNP in the ORF was found to be putatively associated with an absence of clinical symptoms ($p < 0.05$), but this may be explained by breed and is being investigated further. This project was funded by Defra, UK.

GEN-09

GENETIC VARIABILITY OF THE PRP GENE IN GOAT BREEDS FROM NORTHERN AND SOUTHERN ITALY

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Prion protein (PrP) gene polymorphism in Italian sheep breeds have been well investigated, whereas little is known about goats. Aim of this work is to determine the variability of PrP gene in goat breeds from Piedmont and Basilicata, a Northern and a Southern Italian region respectively. Blood samples were collected from 300 goats of the four most common breeds in Piedmont region and from 216 goats of 10 breeds from Basilicata. PrP gene polymorphisms were detected by direct DNA sequencing on both strands of the PCR products. Thirteen *PrP* polymorphisms were identified: G37V, T110P, G127S, M137I, I142M, I142T, H143R, R154H, P168Q, T194P, R211Q, Q222K, S240P (the dimorphisms I142T and T194P are novel). This gave rise to 14 protein variants (nine in the Northern breeds and 12 in the Southern breeds). The allele with the mutation S240P only has been found at the highest frequency in both groups (38.8% and 54.2% in Northern and Southern goats respectively). Mutations G37V, M137I, M142T, H143R and T194P have not been detected in Northern goats while G127S and R211Q have not been found in Southern breeds. For G37V, H143R, G127S and R211Q the associations are statistically significant (χ^2=23.95, p-value=<0.05; χ^2=20.48, p-value=<0.05; χ^2=10.27, p-value=<0.05; χ^2=45.51, p-value=<0.05 respectively). I142M is present at higher frequency in Northern goats (12.3% *vs* 0.8%) (χ^2=39.83, p-value=<0.05). Q222K (2.7% *vs* 14.6%) and R154H (4% *vs* 7.6%) are significantly associated with breeds from the south (χ^2=48.21, p-value=<0.05; χ^2=5.68, p-value=<0.05, respectively). The present study revealed a high variation in the caprine PrP gene and clear differences between Northern and Southern breeds, with mutations exclusively or significantly associated with one group or the other.

GEN-10

PHENOTYPIC DETECTION OF THE PRION-LIKE ALTERATIONS OF MAMMALIAN PRP PROTEIN IN THE YEAST MODEL

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Mammalian prion diseases associated with formation of the abnormal aggregated isoform of Prion Protein (PrP^{Sc}) are incurable and fatal. Factors regulating prion propagation remain unknown to date. We have generated a yeast-based system for screening the agents involved in regulation of PrP conformational switches. Our system is based on the properties of yeast prion protein Sup35, which is a translation termination factor. Yeast cells containing a prion form of Sup35 exhibit nonsense suppression, detectable in the strains with reporter nonsense mutations as a growth on selective media. Prion properties of Sup35 are exclusively determined by the N-terminal prion domain. We have substituted the prion domain of Sup35 with a mouse sequence coding for the 90-231aa fragment of PrP. Yeast strain bearing a plasmid with *PrnP-SUP35MC* gene under the control of copper-inducible promoter, and containing a deletion of the chromosomal copy of *SUP35* has been generated. Chimeric PrP-Sup35MC protein compensates for the termination function of Sup35 in our system. However, derivatives exhibiting nonsense suppression have been selected. We have shown that nonsense suppression is a result of partial inactivation of the PrP-Sup35MC protein. The heritable, functionally defective state of PrP-Sup35MC designated as [PrP^{S+}] possesses all characteristics of a yeast prion, such as reversible curability by GuHCl, non-Mendelian inheritance and cytoplasmic infectivity. PrP-Sup35MCp in prion isoform is more resistant to proteinase K digestion, compared to the same protein in a non-prion form. This provides the first example of the PrP-based prion formation in the organism other than mammals. Our phenotypic assay could be employed to screen for the proteins and agents affecting PrP prionization. Supported by grants: Fogarty TW006965-01A1, NIH R01GM58763, RFBR 49002, CRDF ST-012.

GEN-11

DETERMINING THE STRUCTURE AND SEQUENCE OF OVINE GENES WHICH ENCODE POTENTIAL PRP BINDING PROTEINS.

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The PrP protein gene is a major controlling factor of scrapie. Polymorphisms in the PrP gene lead to modulation of disease phenotypes. The mechanisms by which these PrP polymorphisms achieve their effect are still unknown but it is likely that interactions with other host proteins are of importance. We have begun to analyse genes of proteins that have been shown by *in vitro* studies to bind PrP. We have selected genes such as 140kDa NCAM, 37kDa laminin receptor precursor (LRP) and Pint1 for a detailed analysis of their gene structure and genetics in sheep, a natural host of classical and atypical scrapie. Here we report our data on ovine Pint1.
Spielhaupter & Schätzl, (J Biol Chem. 276:44604-44612; 2001) described a prion interactor (Pint1) gene based on a partial mouse brain cDNA expressing a peptide domain that bound to PrP protein in a yeast two-hybrid system. A homologous cDNA is also present in human brain. To be able to analyse this protein interaction in sheep, we have cloned and sequenced Pint1-coding cDNAs from ovine brain and testis. The exon-intron structure of sheep Pint1 was inferred from comparison with the published mouse and human Pint1 gene structures and confirmed in part on sheep genomic clones. Our data reveal novel Pint1 mRNA variants in brain and testis derived through alternative splicing. Some of these alternative mRNAs encode variants in the Pint1 coding region, in one case this change is close to the putative PrP binding domain of Pint1. The PrP-binding domain was sequenced for brain cDNA from sheep, goat, cattle and mouse to analyse species specificities in this protein domain. The expression of Pint1 mRNA from sheep brain was quantified and compared with mouse. We conclude that the roles of Pint1 in ruminants and rodents in scrapie may differ

GEN-12

GENETIC DIFFERENCES IN SUSCEPTIBILITY OF NEUROSPHERE CULTURES TO PRION INFECTION

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Prion protein genotype (Prnp in mice) is the most important determinant of susceptibility and disease phenotype in every known prion disorder. Background genes other than Prnp also significantly influence incubation time in mice and chromosomal locations have been determined for several of these modifier loci. Due to the limited genetic diversity and aneuploidy of prion infectible cell lines and the consequent reliance on expensive and time-consuming bioassays in mice, there is only limited understanding of the cellular mechanisms underlying genetic differences in susceptibility. CNS-stem cell-containing neurosphere cultures were produced to test their suitability for studying the genetics of susceptibility to prions. Efficiency of infection, spread from cell to cell, and rate of prion replication can be discriminated in neurosphere cultures using filter-based immmunostaining for PK-resistant PrPSc or conformation-dependent immunocytochemistry (Giri et al., Proc Natl Acad Sci USA 2006,103:3875). The RML strain of prions produces short incubation times in mice expressing the a allele of Prnp and long incubation times in Prnpb mice. Neurosphere lines were isolated from C57Bl/6J (B6), B6.I-Prnpb, Tg(MoPrP-A)B4053, and Tg(MoPrP-B)C2091 mice; the transgenic lines express comparable levels of the alternative PrP allotypes. Infection could be established at higher dilutions of RML prions and spread of infection through the cultures was more rapid in neurosphere lines expressing PrP-A than those expressing PrP-B, reflecting the short and long incubation times of the mice from which they were derived. For example, by passage 3, most neurosphere colonies were PrPSc-positive in TgB4053 cells incubated with isolate diluted 10-8. The PrP-B producing neurospheres were far less sensitive to infection, with de novo PrPSc production achieved only by dilutions of 10-4 to 10-5. To our knowledge, this is the first demonstration of allelic differences in prion susceptibility in mice modeled in tissue culture.

GEN-13

PRION PROTEIN GENES AFFECT SUSCEPTIBILITY OF CERVIDS TO CHRONIC WASTING DISEASE

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The primary sequence of the prion protein affects susceptibility to transmissible spongiform encephalopathies (TSE; prion disease) in mice, sheep and humans. The *Prnp* sequence of free-ranging, Wisconsin white-tailed deer was determined and the *Prnp* genotypes of CWD-positive and -negative deer compared. Six amino acid (AA) changes were identified; two of which were located in pseudogenes. Two alleles, a glutamine to lysine polymorphism at codon 226 and a single octapeptide repeat insertion into the pseudogene, have not been previously reported. The predominant alleles, wild-type (glutamine at AA95, glycine at AA96 and glutamine at AA226) and a glycine to serine polymorphism at AA96 (G96S), comprise almost 98% of the *Prnp* alleles in the Wisconsin white-tailed deer population. Comparison of the allelic frequencies in the CWD-positive and -negative deer suggests that G96S and a glutamine to histidine polymorphism at AA 95 (Q95H) are linked to a reduced susceptibility to CWD. The G96S allele does not, however, provide complete resistance, as a CWD-positive G96S/G96S deer was identified. The G96S allele is also linked to slower progression of disease in CWD-positive deer based on the deposition of PrP^{CWD} in the obex region of the medulla oblongata. To further determine the effect of variations of the cervid *Prnp* alleles on susceptibility, deer with known *Prnp* genotypes were orally dosed with CWD inocula prepared from wild-type/wild-type homozygous animals. The experimentally infected wild-type/wild-type animals have succumbed to disease, animals heterozygous for *Prnp* alleles have not.

GEN-14

PRION PROTEIN GENE EXPRESSION IS MODULATED BY DNA POLYMORPHISMS IN THE PROMOTER REGION

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The phenomenon that host genetic factors modulate the susceptibility to prion infection was initially reported in sheep and human, where polymorphisms within the coding sequence of the prion protein gene (*PRNP*) have been shown to lead to an increase or decrease in susceptibility. However, no polymorphism within the bovine PRNP coding region has been shown to be associated with bovine spongiform encephalopathy (BSE). To determine whether polymorphisms within the promoter region are involved in susceptibility or resistance to BSE, DNA polymorphisms of the promoter region of the bovine *PRNP* gene were identified and genotyped in breeding bulls as control and animals tested positive for BSE. Furthermore, the functional relevance of identified polymorphic sites was assessed using a luciferase reporter gene assay. The binding of different transcription factors to DNA fragments in which polymorphisms were detected, was examined. Comparative sequencing and MALDI-TOF based SNP analysis between control and BSE affected animals from 4 different bovine breeds (namely Schwarzbunt, Rotbunt, Braunvieh and Fleckvieh) showed that Braunvieh animals are significantly different from other breeds, exhibiting different allele frequencies for the 12 bp indel polymorphism in control and BSE animals. Genotyping and haplotype calculation of nine polymorphisms in the promoter region resulted in significantly different haplotype distribution in control and BSE animals. Promoter constructs of main haplotypes containing different combinations of polymorphisms revealed that the influence of SNP combinations (haplotypes) is more important for PRNP expression in neuronal cells than the 12 bp indel polymorphism alone. The haplotype resulting in the lowest expression level is underrepresented in BSE animals compared to controls and therefore supports the hypothesis that expression level is correlated to susceptibility to TSE.

GEN-15

A NOVEL RESISTANCE-LINKED OVINE PRP VARIANT AND ITS EQUIVALENT MOUSE VARIANT MODULATE THE IN VITRO CELL-FREE CONVERSION OF RPRP TO PRPRES

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Prion diseases are associated with the conversion of the normal cellular prion protein, PrP^{c}, to the abnormal disease associated PrP^{Sc}. This conversion can be mimicked *in vitro* using the cell-free conversion assay. We have recently shown that this assay can be modified to use bacterial recombinant PrP as a substrate and mimic the *in vivo* transmission characteristics of rodent scrapie. Here we demonstrate that the assay replicates the ovine polymorphism barriers of scrapie transmission. In addition, the recently identified ovine PrP variant $ARL^{168}Q$, which is associated with survival of sheep to experimental BSE, modulates the cell-free conversion of ovine recombinant PrP to PrP^{res} by 3 different types of PrP^{Sc}, reducing conversion efficiencies to levels similar to the ovine resistance-associated ARR variant. Also, the equivalent variant in mice (L^{164}) is resistant to conversion by 87V scrapie. Together these results suggest a significant role for this position and/or amino acid in conversion.

GEN-16

GENETIC ANTICIPATION IN E200K ITALIAN PATIENTS OF THE CALABRIAN CLUSTER

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The genetic forms of Transmissible Spongiform Encephalopathies (TSEs) or prion diseases represent about 10-15% of human TSEs. Genetic TSEs are always associated with a mutation in the prion protein gene (*PRNP*), and occur in families with an autosomal dominant pattern of inheritance. However, the observations that they occasionally occur in families with no previous history of disease and that some *PRNP* mutant carriers do not develop disease during their lifetime raise the issue of factors influencing the penetrance and the age at onset of these diseases. Genetic anticipation has been shown in several neurodegenerative diseases where the gene linked to the disease is affected by trinucleotide repeat instability, with expansion of repeats clearly correlated with an earlier age at onset

Preliminary evidence has shown anticipation in genetic CJD linked to the E200K mutation among Lybian Jews (Rosenmann H. et al 1999). The term anticipation in genetic disease refers to earlier age at onset and /or increased severity in successive generations.

We investigated whether genetic anticipation may occur in E200K Italian patients of the Calabrian cluster. We recorded the age at onset of 28 parent-offspring pairs from 22 pedigrees. When the parent at risk was alive and neurologically healthy we recorded the time at observation as the age at CJD onset. The paired t-test was performed to test the statistical significance of paired differences.

The age at onset for the carrier generation was 70.55 ± 11.5 , while the age at onset of CJD offsprings was 58.8 ±10.7. The differences between the age at onset in the two generations was statistically significant ($p< 0.0001$). This result suggests that anticipation is also present in the Calabrian cluster of E200K cases. The basis for the anticipation in genetic E200K CJD is unknown. A number of genetic and environmental factors might play a role in determining the anticipation phenomenon.

GEN-17

CONSERVED ROLES OF VERTEBRATE PRION PROTEINS I

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Contrary to the wealth of information available on prion pathogenesis, the natural role of PrP remains a largely unsolved issue. For the last 14 years, it has been difficult to approach this question due to the lack of visible phenotypes in PrP knockout mice. To overcome this problem, we performed loss- and -gain-of-function analyses in the zebrafish using a combination of developmental techniques including morpholino knockdown and RNA overexpression of various full-length and deletion PrP fluorescent constructs in wild type and transgenic zebrafish. We have shown that knockdown of zebrafish PrPs produces strong developmental loss-of-function phenotypes, which can be rescued by fish and mammalian PrPs. In order to uncover the molecular and cellular mechanisms behind this conserved function, we carried out a closer analysis of our PrP phenotypes. We observe that lack of PrP-1 results in early and lethal disruption of embryonic cell adhesion, causing developmental arrest at the onset of gastrulation; PrP-2 depletion impairs proliferation and differentiation of cranial neurons, along with a general effect on brain morphogenesis. Strong localization of fluorescently labeled PrPs to embryonic cell-cell contacts is in line with our data showing that PrP homotypic *trans*-interactions are sufficient to induce cell adhesion and signaling in mammalian and insect cells (see abstract by Solis et al.). We also assessed the relative contributions of PrP domains to its function during zebrafish embryogenesis. Proper PrP localization and embryonic cell adhesion (seen as the ability to rescue the PrP-1 phenotype) are affected to a larger degree by deletion of the globular domain than by deletion of the less conserved repetitive domain, indicating a stronger requirement of the former. This is in contrast to previous data postulating an essential role of the repetitive domain PrP in copper metabolism. Altogether, our experiments indicate that PrPs are required at embryonic cell contacts in order to mediate proper cell adhesion during gastrulation, as well as neuronal proliferation and differentiation. Thus, the evolutionarily conserved interaction between PrPs constitutes a positive and important signal for the cell, which may be lost or deregulated upon PrP conversion.

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GEN-18

CHARACTERIZATION OF CREUTZFELDT-JAKOB DISEASE WITH MUTATION E200K OF CENTRAL EUROPEAN TYPE.

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Creutzfeldt -Jakob disease (CJD) with the prion gene (*PRNP*) mutation E200K (CJD^{E200K}) is the most common human genetic prion disease (gPD). It is randomly distributed worldwide and accumulated in geographic (Slovakia) and ethnic (Israel) clusters. Analysis in CJD^{E200K} of different origin revealed Mediterranean, Japanese, Western European and Central European (CE) haplotypes. These types may differ in penetrance, transmissibility etc. CE haplotype shows the highest occurrence in Slovak CJD^{E200K} clusters, causing in Slovakia the highest incidence (65%) of gPDs in Europe. Studied were: 183 definite CJD, 357 relatives of genetic CJD (gCJD) and 950 healthy corneal donors, histopathology, immunochemistry, genetic testing, epidemiology, genealogy. The mean CJD annual incidence in 1975-2005 was 1,45/mill (CJD^{E200K} 0.88/mill, sporadic CJD (sCJD) 0.57/mill).CJD^{E200K} represents 65% of all cases, 50.42% of which are familial.The mean age at onset in two generations differed (14.9 yrs) significantly. E200K mutation was found in 34.73 % of „healthy“ relatives. Genetic testing of corneal donors revealed 2 asymptomatic carriers (AC). Penetrance of the E200K mutation is 59%. CJD^{E200K} experimental transmission results resembled sCJD.The mutated allele co-segregates with methionine at codon 129. Distribution of M129V polymorphism in CJD^{E200K} patients is MM 78.6%, MV 21.40%, in carriers MM 64%, MV 36%.Methionine homozygosity significantly reduced the disease duration.Gene- alogical studies showed a spread of CJD^{E200K} to Hungary, Bohemia, Poland, also to Belgium, France, USA, Canada. Farmers are most represented in gCJD and even their percentage is decreasing, it is significantly higher than in employed population.CE type of E200K characterize: incomplete penetrance, transmissibility comparable to sCJD, 50% of familial pattern, generation gap at onset indicating anticipation, short clinical course in methionine homozygots. Data may serve for comparison to other subtypes of CJD^{E200K} and as basis for future studies of genetic and exogenous (Cu/Mn, infection) fac-tors, co-influencing manifestation of CJD in AC - the gCJD-risk group, which is at risk to develop and to transmitt the disorder and at present the only one available for specific prevention of prion disease.

GEN-19

RESISTANCE TO ATYPICAL SCRAPIE LINKED TO CODONS 136,141,154,171 OF PRNP GENE IN FRANCE.

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In sheep, scrapie disease is genetically controlled by alleles of the PRNP gene at codons 136, 154 and 171. After the discovery in 1998, in Norway of a specific strain (nor98), new atypical cases were found all over Europe. Amongst the specificity of the Norwegian cases, a large proportion of $A_{136}H_{154}Q_{171}$ and $A_{136}F_{141}R_{154}Q_{171}$ allele carriers was demonstrated.
In France, 54 atypical cases were detected by active surveillance so far. The aim of this study is to classify PrP genotypes at codons 136, 141, 154, 171 for their relative susceptibility to atypical scrapie.
We proposed to explore PrP polymorphisms in atypical cases and in a 'control' population. For atypical scrapie cases, PrP genotypes at codons, 136, 141, 154 and 171 have been identified after direct sequencing of genomic DNA. The control population is composed by two data sets. Data set 1 was obtained in 2002 from 100 rams per breed sampled in French breeding centres. A RFLP-PCR technique has been performed to genotype codons 136, 154 and 171. Four alleles were detected ARR, AHQ, ARQ/H and VRQ. The allele ARH was confused with the allele ARQ. Data set 2 was established in 2006 using stocked genomic DNA of ARQ/H-ARQ/H rams born in 2002 and 2003. Animals have been sampled from breeding centres and nearly 100 rams per breed have been studied. PrP polymorphisms at codons, 136, 141, 154 and 171 of these rams have been analysed after direct sequencing. Using both data sets, frequencies of ARR, AHQ, VRQ, AFRQ, ALRQ and ARH alleles have been calculated within breed. The within breed frequencies were weighted by the breed size to give a representation of the global allele frequencies at the control population level.
Our data confirmed a strong association of the AHQ and AFRQ alleles with the susceptibility to atypical scrapie. In accordance with the Norwegians, the AHQ appears less at risk than the AFRQ. The ARR, ARH and VRQ seem to confer some slight, susceptibility relative to the ALRQ.

GEN-20

THE ROLE OF PRENATAL AND POSTNATAL OVINE CHIMERISM IN SCRAPIE CONTROL PROGRAMS

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Naturally occurring chimerism in mammalian species includes prenatal exchange of stem cells between fetuses due to anastomoses of placental vasculature, transfer of fetal cells to the maternal bloodstream during pregnancy, and transfer of maternal cells to progeny via milk or colostrum. We have demonstrated that exchange of cells between lambs sharing a uterine environment results in accumulation of PrP-d in placental tissues with *Prnp* genotypes generally considered resistant to classical scrapie. The objective of this study was to examine the frequency of placental chimerism in multiple births and the effect of prenatal chimerism on genetic analysis of lambs. Further, we examined the role of fetal cells in the maternal circulation of pluripotent ewes as a potential source of *Prnp* genotyping error. Methods included allele-specific polymerase chain reaction assay of *Prnp*, MHC II DRB1 typing, and microsatellite analysis. Relative sensitivity of the methods, age of the animals, and number of pregnancies were considered as contributing factors when evaluating the methods for accuracy and cost efficiency. Sensitive and specific methods for identifying the true *Prnp* genotype of sheep is an important component of the US scrapie control program.

GEN-21

PRNP GENOTYPE DISTRIBUTION IN ATYPICAL SCRAPIE CASES IN PORTUGAL, INCLUDING THE ASSOCIATION WITH THE RECENTLY REPORTED LEUCINE HAPLOTYPE (ALQ)

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Until March 2006, all the cases of portuguese sheep with PrP^{res} deposition in the central nervous system have shown a different pattern of distribution in the brainstem as well as a distinct electrophoretic profile from that observed for scrapie. The PrP^{res} electrophoretic profile has been of the same range and pattern as that described for Nor 98.
This work reports the *Prnp* genotype distribution in sheep confirmed with atypical scrapie (n=126) in Portugal comparing with the *Prnp* genotype profile in Portuguese sheep population.
For *Prnp* genotyping at codons 136, 141, 154 and 171, genomic DNA extracted from frozen brainstem and ear tissues was subjected to PCR amplification. Following purification, PCR products were then submitted to automated cycle sequencing using an ABI Prism 377 DNA sequencer (*Perkin Elmer*).
The majority of the cases were homozygous for ARQ followed by genotypes rarely associated with scrapie that are included in the *National Scrapie Plan* (*NSP*) types 2 and 1. Those genotypes were also the predominant genotypes determined in both Portuguese pure sheep breeds and random sampled ovine animals. The VRQ/XXX genotypes (*NSP* types 4 and 5) highly susceptible to classical scrapie have not been identified, as yet, in any of the atypical scrapie cases, despite their presence in portuguese sheep, albeit at low frequency. Notably, the recent described haplotype ALQ was also detected in an atypical scrapie positive case combined with the ARQ allele. This new haplotype was never reported in Portuguese sheep.
The apparent absence of classical scrapie in Portuguese sheep population and the *Prnp* haplotypes associated with atypical scrapie, suggest a phenomenon of natural selection in this region of Europe. These data should be taken into account in the current EU sheep-breeding programme for selection of non-susceptible haplotypes.

GEN-22

NOVEL PRION DISEASE MUTATION R136S HAS AN INCOMPLETE PENETRANCE DEPENDENT UPON CODON 129 TRANS ALLELE

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A female patient diagnosed with familial early onset dementia, with two previously deceased (age 46 and 47 years) male siblings with the same neurodegenerative disease, started at age 52 with psychiatric symptoms, generalised myoclonus, Parkinsonic symptoms and progressive dementia. The EEG was lentified, MRI scan results were normal and CSF 14-3-3 was negative. The disease had 4 years of evolution. Genetic analysis of the *PRNP* gene revealed that the patient was homozygous for a novel mutation R136S linked to methionine at codon 129. R136S consists in a drastic aminoacid substitution in a conserved region of the gene. We didn't found this mutation in 340 unrelated *PRNP* alleles, thus concluding that it is not a polymorphism. Brain pathology studies on cerebral cortex and cerebellum, revealed little spongiform changes, but a very extensive formation of multicentric large amyloid plaques like in Gerstmann-Sträussler-Sheinker disease. The plaques labeled strongly with prion protein (PrP) antibodies 12F10 and 3F4, revealing massive PrP aggregation and deposition. The plaques showed the predominant involvement of cortical layers 3, 4 and 5 in the cerebral cortex, and the predominant involvement of the molecular layer in the cerebellar cortex. PrP proteinase K resistant western-blot analysis, revealed a distinct pattern with two low molecular weight bands of 5kDa and 8kDa. The patient's mother (age 93), half sister (age 87), daughter and son, were genetically analysed for the *PRNP* gene: both the mother and half sister were heterozygous for R136S and heterozygous at codon 129, but didn't develop this disease; the father's genotype was inferred as a carrier of the R136S (died age 80 without dementia). So, heterozygous for the R136S and for CD129 polymorphism, don't develop the disease. The disease caused by R136S in the *PRNP* gene is not transmitted as an autossomic dominant trait as expected. R136S has an incomplete penetrance dependent upon CD129 *trans* allele. The presence of a normal prion protein with Valine at position 129 prevents the expression of the disease. The induction of the structural conversion of normal PrP^{C} into abnormal PrP^{SC} seems to depend on the "proteic compatibility" between the abnormal and the normal prion coexisting in the cell. The "compatibility" depends on the aminoacid at position 129 that can be either Metionine or Valine, and only molecules with the same aminoacid at position 129 are compatible.

GEN-23

PRION PROTEIN (PRP) GENE ANALYSIS OF ITALIAN ATYPICAL BSE CASES (BASE)

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In Italy a new atypical BSE phenotype, named BASE, has been identified in two animals. Since BASE displays biochemical and pathological similarities with sporadic CJD (sCJD) cases linked to type-2 PrP^{Sc} and methionine/valine (M/V) polymorphism at codon 129 in the *PrP* gene, it is possible that this disorder represents a sporadic form of cattle TSE. This would also explain the fact that BASE cases were older than other affected bovines. We have previously sequenced the open reading frame (ORF) of BASE cases: one animal carried a wild-type PrP genotype and was homozygous for octapeptide repeat number with six copies while the other one carried a silent mutation at codon 78 (CAG/CAA) and was heterozygous for six/seven repeats. No mutation exclusive to BASE have been then identified in the coding region. Aim of this study was the identification of informative *PrP* gene polymorphisms by extending the analysis outside the ORF. A 2 kb prion gene fragment including part of intron II, the PrP coding region and part of the 3' untranslated region (3'UTR) was amplified and sequenced in the two BASE cases, a healthy age-matched control (> 10 years) and three animals carrying different number of octapeptide repeat units, ranging from five to seven repeats. Four single nucleotide polymorphisms (SNP) were identified (reference sequence AJ298878): an intronic base substitution (65165 T>C) and three point mutations in the 3'UTR (66877 C>T, 66932 A>G, 66948 T>C). Alleles were assessed by haplotype cloning in a plasmid vector and sequencing of recombinant clones. Both BASE and healthy animals included in our study were polymorphic at the detected mutation sites. Only one mutation in the 3'UTR (66948 T>C) was found exclusively in the BASE case homozygous for six repeats.

GEN-24

GENETIC VARIABILITY AND SELECTION OF THE CERVUS ELAPHUS PRION (PRP) GENE

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Variation in the sequence of the PrP prion gene has been shown to modulate the susceptibility of Chronic Wasting Disease (CWD), a neurodegenerative disorder affecting cervid populations in North America. Despite their close phylogenetic relationship, European cervid populations are currently considered CWD free. We will present data on the selective forces acting on the PrP gene of *Cervus elaphus*, based on the analysis of the patterns of nucleotide variation in the PrP gene of two target *C. elaphus* populations: European red deer from the Scottish Isle of Rhum and North American elk from Colorado. The investigation of CWD-affected and CWD-free elk aimed at determining the presence or absence of disease specific selection and the role of PrP variants which appear to be associated with disease modulation. A number of novel nonsynonymous cervid polymorphisms were identified: codon 168 in deer, and codon 25 and 191 in elk. Polymorphism M132L which had been previously associated with CWD-susceptibility in elk was found in the Colorado elk population, but not in the red deer. The relationship between genotypic frequencies and disease will be discussed. Other codons which have been associated with CWD modulation in other cervid species were not found to be polymorphic in the two studied populations.

GEN-25

STUDY OF THE D178N MUTATION IN SPAIN: A UNIQUE COMMON ANCESTOR?

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Fatal Familial Insomnia belongs to the group of inherited prion diseases. This neurological disorder is caused by a missense substitution at codon 178 of the PRNP gene and its incidence is very low. However, to date, approximately 50% of the cases registered in Spain, are located in a Northern region (Basque Country). The aim of this work is to study the phylogenetic relationship among D178N mutation carriers in Spain in order to determine whether its distribution is caused by a unique common ancestor or independent mutational events.
The subjects studied were 31 carriers of the D178N mutation (mutated allele associated to methionine allele at codon 129). The variables of the study were six microsatellite markers adjacent to the PRNP gene, comprising a 150kbp length fragment. They were analysed by PCR and fragment analysis. The phylogenetic relationships among the resulting haplotypes were analysed using Network software (v.4.1.1.2.).
Seven haplotypes (H1-H7) associated to the D178N mutation were obtained. Among them, the most frequent haplotype was H5, which was found in 11 individuals, all of them from the Basque Country. The second most frequent haplotype was H3 followed by H1.
The phylogenetic analyses showed, on one side, the agroupation of H6, H7 and H4 haplotypes, and on the other side, the agroupation of H3, H2 and H1 haplotypes. The haplotype H5 was observed to be located far from these two groups. These results suggest that H7 and H4 derive from one haplotype, possibly H6, and that H2 and H1 derive from haplotype H3.
The results obtained by the analysis of the microsatellite markers show the existente of 7 haplotypes associated to the D178N mutation in Spain. Nevertheless, the analysis of phylogenetic relationship reduces to three, the differenciated haplotypes. This suggests that the actual distribution of the D178N mutation in Spain may be due to three mutational events, possibly originated in independent moments, or to an ancestral and unique foundational event.

GEN-26

PRP GENE POLYMORPHISMS IN ATYPICAL SCRAPIE CASES IN GREAT BRITAIN

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As part of the scrapie surveillance programme in Great Britain (GB), during 2002 and 2003, rapid tests were used to screen apparently healthy sheep passing through abattoirs. This survey detected atypical scrapie infection in sheep with PrP genotypes thought to be genetically resistant to the classical form of scrapie. DNA sequencing of the PrP gene promoter, ORF and UTR from the British atypical scrapie cases (n = 69), classical scrapie cases (n = 59) and scrapie free controls (n = 138), was undertaken to identify whether PrP variants, other than the three well characterised polymorphic codons, influenced susceptibility to atypical scrapie infection. Four non-synonymous changes M112T, M137T, L141F and P241S were detected which are most probably associated with the $A^{136}R^{154}Q^{171}$ haplotype. Only the PrP variant containing a phenylalanine residue at amino acid position 141 was found to be more commonly associated with the atypical scrapie cases. In addition to the single nucleotide polymorphisms associated with the ARQ allele, two out of nine atypical scrapie cases with the ARR/ARR genotype were found to contain a 24bp insertion leading to an additional octapeptide repeat. The 3 kb 3' UTR sequence, revealed three main haplotypes. Atypical scrapie cases were found to be associated with the most common haplotype, with all 69 samples found to carry at least 1 copy of this 3' UTR haplotype. This haplotype was however also associated with the ARR allele. Unravelling whether the UTR has an effect, independent of the ORF, in conferring susceptibility to atypical has not been possible to ascertain with the limited number of samples examined. The 5 kb promoter sequence, which included exons 1 and 2 and intron 1 revealed five main haplotypes, with no clear association with atypical scrapie cases. This study was funded by Defra, UK.

GEN-27

GENE EXPRESSION PROFILIES OF SCRAPIE INFECTED MOUSE BRAIN CELLS

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The aims of this study were to understand the overall effect of prion pathogenesis on gene expression and to identify differentially regulated genes in scrapie infected mouse brain (SMB) cells. These cell lines are commonly employed in prion research and can be maintained as infected and uninfected forms. We analyzed the expression profiles of SMB cells using Affymetrix GeneChip Mouse Genome 430 2.0 Arrays, which contain 45,101 probe sets. The data were normalized and analyzed using the GeneSpring software. Clustering analysis showed that the results were highly reproducible between biological repeats. 8,618 genes passed the filter showing 2 fold or greater change in expression, while only 313 genes passed the filter of 8 fold change. Among these 313 genes several genes were found to have a link with prion diseases in previous studies, for example: complement component 2, complement component factor h, histocompatibility 2, matrix metalloproteinase 2 and neuropeptide Y. Many of these 313 genes encode proteins involved in immune response, apoptosis, cell adhesion, stress response and transcription. To validate the micoarray data, TaqMan quantitative PCR of six genes were carried out and the results showed even greater differences between the scrapie infected samples and the controls in gene expression. These data provide insights into the cellular responses during scrapie infection and also offer a model for expression profiling experiments in prion diseased animals.

GEN-28

PROTECTIVE EFFECT OF ARQK176, AT137RQ, AK142RQ AND ARR PRP ALLELES IN SARDA SHEEP EXPERIMENTALLY INFECTED WITH SCRAPIE

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The aim of this study is to investigate the susceptibility to scrapie infection of Sarda sheep carrying the most diffuse PrP alleles (ARQ, AHQ, ARH and ARR) and ARQ variants ($AT_{137}RQ$, $AK_{142}RQ$ and $ARQK_{176}$). Experimental infections were carried out using a well characterised isolate of natural scrapie from Italy. Preliminary results at 1300 and 1390 dpi are reported for intracerebral (i.c.) and oral challenge, respectively.
Survival times after i.c. inoculation according to PrP genotype were: ARQ/ARQ (n=5/5) 462±25 dpi, ARQ/AHQ (5/5) 703±26 dpi, AHQ/AHQ (1/1) 790 dpi, ARQ/ARH (1/1) 1083 dpi and ARH/AHQ (3/3) 1252±88 dpi. None of the sheep carrying the ARQ/ARR (15), ARR/ARR (5), ARR/AHQ (1), ARR/ARH (1) genotypes showed clinical signs consistent with scrapie at time of writing. Interestingly, also sheep carrying additional mutations of the ARQ allele (one $AT_{137}RQ/ARQK_{176}$ and one $ARQK_{176}$/AHQ) failed to show clinical signs despite their putative susceptibility according to 136, 154 and 171 codons.
After oral infection, survival times were observed as follows: ARQ/ARQ (5/5) 832±54 dpi, ARQ/AHQ (5/5) 1115±59 dpi. None of the sheep with the ARQ/ARR (20), ARR/ARR (5), ARQ/ARH (3), ARR/ARH (1) genotypes showed clinical signs of scrapie, to date. Similarly to what observed in i.c. inoculation, sheep with additional mutations - ARQ/$AT_{137}RQ$ (3), ARQ/$ARQK_{176}$ (1) and ARQ/$AK_{142}RQ$ (1) - are so far healthy.
In a parallel study investigating the kinetics of PrPSc distribution in Sarda sheep after oral scrapie infection, individual animals with the ARQ/$ARQK_{176}$, ARQ/$AK_{142}RQ$ and ARQ/$AT_{137}RQ$ genotypes, culled at 9, 12 and 20 months post infection (p.i.), respectively, were negative for PrPSc deposition, whereas ARQ/ARQ animals culled at the same intervals p.i. were positive in nervous and/or lymphoid tissues.
The present study shows that susceptibility to scrapie in Sarda sheep is clearly governed by the PrP genotype and that the most susceptible alleles are in decreasing order, ARQ, AHQ and ARH. Preliminary results at 1300 d.p.i. indicate that the presence of at least one ARR allele could confers resistance to clinical scrapie. Unexpectedly, also single mutations on the susceptible ARQ allele would seem to confer protection to experimental scrapie infection.

GEN-29

PRNP POLYMORPHISMS IN SHEEP WITH DIFFERENTIAL PRNP MRNA EXPRESSION IN BLOOD

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As there is substantial evidence that substrate PrP^{C} is required in specific tissues and cells for PrP^{Sc} accumulation to occur and because of the probable haematogenous spread of PrP^{Sc} throughout the lymphoreticular system during TSE pathogenesis in sheep, PRNP mRNA expression levels in sheep blood was investigated in 234 sheep. PRNP mRNA expression could be detected in only 11.5% of the samples, using a real-time quantitative PCR assay based on SYBR Green I detection, according to standard operating procedure guidelines. To correct for possible experimental variation, proper normalization of the cDNA input of all samples was achieved using 3 stably expressed reference genes (viz. RPL13A, RPS18 and UBC), chosen from a panel of 9 tested candidate reference genes using the geNorm algorithm. Our gene expression results show true biological variation of PRNP mRNA in sheep blood cells. To look for possible polymorphisms related to the observed variable expression, sequencing of 25,000 bp of the PRNP gene was carried out for 20 pairs of strongly related sheep with differential PrP^{C} expression, constituting 7 different races (Ardense Voskop, Bleu du Maine, Hampshire Down, Rouge de l'Ouest, Suffolk, Texel and Vlaams Kuddeschaap) and the 5 predominant alleles (ARR, ARH, AHQ, ARQ and VRQ). In total, 283 polymorphisms were identified, of which 231 were not previously described. All polymorphisms are the subject of a genetic association study currently carried out. Some of these PRNP polymorphisms may not only elucidate the mechanisms controlling PrP^{C} expression at the level of PRNP gene transcription and translation, but may also have an impact on TSE resistance.

GEN-30

SEQUENCE ANALYSIS OF PRION GENE IN SICILIAN AUTOCHTHON BOVINE BREEDS.

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A project for a characterization and biodiversity conservation on Sicilian autochthon breeds of different animal species had been started which includes two bovine breeds of Sicily: *Modicana and Cinisara.* The two bovine breeds are particularly adapted to climatic and geographic environment of the island and are particularly good milk producers. *Cinisara* is a breed confined essentially in the province of Palermo and produces a milk with high percentage of fat and protein; *Modicana* cows are present not only in all Sicilian territory but also In Abruzzo region. With the aim of polymorphism analysis at nucleotide and aminoacids levels a sequencing studies on prion gene *(Prnp)* had been started on group of animals of this two breeds coming from different herds in comparison with bovine of different not pure breeds present also in Sicilian territory, but coming from different geographical area such as *Italian Red Spotted*. A *Charolais* cow and an *African* cow had also been included in the studies.
Blood samples in EDTA had been taken from the animals. DNA had been extracted by magnetic particle purification (King Fisher system). Genomic region corresponding to different part of the gene and to the coding sequence had been amplified and purified by agarose gel with the DNA gel extraction Kit (Genomics Millipore) following manufacture's instructions. Sequencing had been performed by the Kit BigDye Terminator v1.1 Cycle Sequencing and by ABI prism 310 genetic analyzer (Applied Biosystem).
Comparison analysis on coding sequence showed that *Modicana* breed as all other cows have few conservative aminoacids replacements. Interestingly *Cinisara* breed is the only one that, on the contrary, showed a replacement involving the substitution of a Serine (aromates) in Asparagin (large polar) residue. The asparagin residues in the Prion protein can be glycosylated and so this can be a potential new site for glycosilation.
Considering the importance that glycosilation pattern has in distinguishing different prion strains and its role in the susceptibility of cellular PrP (PrPC) to conversion to the disease-associated conformation, PrP^{Sc} further studies will be performed by western blot analysis to evaluate if glycosylation pattern in *Cinisara* cows are different compare to the other bovine breeds.

Poster Session

STRUCTURAL DETERMINANTS OF INFECTIVITY AND STRAINS

S-01

STRUCTURAL STUDIES OF THE PRION REPLICATIVE INTERFACE

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Neurodegenerative prion diseases such as Creutzfeldt-Jakob disease and bovine spongiform encephalopathy have been closely linked to the conversion of normal cellular prion protein (PrP^{C}) to an alternate, misfolded conformation called PrP^{Sc}. A high-resolution structure of PrP^{Sc} would provide invaluable insight into the mechanism by which the conversion process occurs. However, the natural tendency for PrP^{Sc} to self-assemble into aggregates hinders structural studies. Therefore, only low-resolution structure models of PrP^{Sc} are available so far.
Historically, antibody Fab-protein complexes have been used successfully to facilitate the crystallization and structural analysis of molecules that do not crystallize alone. Here Fab can serve to create protein-protein contacts different from those made by the antigen itself, or may effectively bury certain antigenic determinants, thereby reducing the propensity for unwanted aggregation.
Antibody containing a heavy-chain complementary-determining region 3 (HCDR3) sequence graft of PrP 89-112 polypeptide binds specifically to PrP^{Sc} with tight affinity. Our aim is to co-crystallize 89-112 grafted Fab fragments with misfolded PrP and resolve the structure of this complex. Fab fragments of this PrP^{Sc}-specific antibody have been prepared for crystallographic analysis. Microfluidic free-interface-diffusion screens yielded crystals of Fab 89-112 that diffract to 2.7Å with an R sym of 8.6% belonging to space group $P2_12_12_1$. Data refinement is currently underway, and will yield the first X-ray crystal structure of PrP sequence containing an N-terminal PrP^{Sc} binding motif.

S-02

PRION PROTEIN INDUCES SEQUENCE EPENDENT CONDENSATION OF NUCLEIC ACIDS

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We found earlier that prion protein accelerates hybridization of complementary DNA strands by biochemical methods and induces a time-dependent changes in the nucleic acid morphology leading to the ordered aggregation of condensed DNA molecules which appeared as globules by ultra structural studies. The prion protein-induced ordered nucleic acid globules are different from other known morphologically altered nucleic acid structures induced by other DNA-binding cellular proteins. We report here the initial step of DNA condensation in the presence of prion protein by biophysical methods by using the fluorescence properties of an oxazole yellow dye, YOYO, intercalated in DNA. The protein induces fluorescence quenching of the dye which is greater with DNA containing only GC bases compared to the DNA containing equimolar concentrations of four bases. Interestingly, the prion protein is without any significant effect on the fluorescence properties of the dye intercalated in DNA containing only AT bases. Biological polyamines which condenses DNA are less effective than prion protein in quenching of YOYO fluorescence bound to DNA. Prion protein induces only marginal quenching of fluorescence of the dye bound to oligonucleotides which are generally resistant to condensation. Studies also shows that interaction of the protein with DNA induces greater exposure of the bases to the bulk solvent with DNA containing GC bases compared to the one containing four nucleic acid bases. This probably can explain the increased association and condensation of the GC DNA in the presence of the prion protein. The increased base exposure and condensation of GC-rich DNA by prion protein may suggest a biological function of the prion protein and a role in its pathogenesis.

S-03

TSE STRAIN VARIABILITY IN SHEEP

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Scrapie in sheep and goats has been known for centuries. Following transmission to rodents, at least six different primary scrapie strains have been isolated and characterised historically by analysing the incubation times and profiles of the histopathological lesion in the brains in a standard panel of inbred RIII, C57Bl and VM mice. The PrP^{Sc} signature in most of these classical scrapie isolates is rather similar in terms of its PK resistance and glycoform pattern in immunoblot. However, the intensified epidemiosurveillance of small ruminants in the EU since 2002 lead to the recognition of previously disregarded scrapie cases with atypical histopathological and immunohistochemical features and immunochemical PrP^{Sc} patterns in the brains of the affected animals. Nor98 (the first reported case of this kind) and SCR2 (an isolate dating back to 1989 in the UK and now recognised as such) are prototypes of the large majority of these atypical cases. Similar cases have been described in most EU member states which so far all seem to belong into the same category. Atypical scrapie is characterized by a lower resistance of the accumulated PrP^{Sc} to proteinase K digestion as compared to classical scrapie strains, explaining why the vast majority of these cases are not detected by most of the so far applied BSE rapid tests. The most obvious characteristics are the altered immunoblot profile comprising of at least 5 bands and including a small fragment of approx. 11 kDa that represents the core fragment of PrP^{Sc}. The anatomical distribution of PrP^{Sc} deposition also varies from classical scrapie, as the cerebellum is the most affected localisation, while the brainstem may be free of detectable PrP^{Sc}. The infectivity of such atypical scrapie cases has been demonstrated using two different transgenic mouse lines. However, the origin of this strain is still under discussion and a spontaneous occurrence cannot be ruled out.

S-04

DETERMINATION OF AN EPITOPE ON THE RECOMBINANT BOVINE PRP THAT PROVOKES THE IMMUNE RESPONSE IN WILD-TYPE MICE

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High degree of conservation of the prion protein (PrP) amino acid sequence across mammalian species is supposed to be the reason for the absence of the immune response in prion diseases.
However, monoclonal antibody (mAb) E12/2 was obtained from wild-type mouse, that developed prominent humoral immune response against chemically unaltered recombinant bovine PrP (recBoPrP). This mAb specifically recognizes bovine and human PrP, but not PrP from several other mammals and also reacts with truncated forms of PrP (recHuPrP 90-230 and recHuPrP 123-230). From the primary structure comparison of PrPs from all tested species we anticipated that residue 155 (according to human PrP sequence numbering) might be crucial for binding. That was proven by site-directed mutagenesis, since the substitution of His155 to Tyr abolished the reactivity to mutated recHuPrP 23-230. Structural comparison of human, bovine, mouse PrP and H155Y recHuPrP showed that the failure of mAb E12/2 to recognize mouse PrP and H155Y recHuPrP can be explained by this amino acid substitution, as in addition to known differences between these two amino acids the orientation of Tyr compared to His is significantly different. This structural difference strongly suggests increased immunogenicity of this part of recBoPrP. Besides, C-terminal end of helix-1 has already been reported to be important for the species barrier between hamster and mouse that also differ in residue 155.

S-05

STUDYING THE SEQUENCE DEPENDENT AMYLOID FIBRIL FORMATION

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The common characteristic of Prion disease and Alzheimer's disease is the formation of amyloid plaque in brain. Amyloid is a special structural form composed of cross □-sheet structure. Compared with the prion protein, the major components of the amyloid in Alzheimer's disease, Aβ40 and Aβ42 peptides, is much more prone to aggregate into the amyloid. Here, we use Aβ40 peptide as our studying system and try to explore how sequence determines the amyloid formation and why the polypeptide chain tends to associate into amyloid fibril rather than other □-sheet structures. The D-form proline (^{D}P) has been widely used in designed peptide as the ^{D}P-G sequence tends to form a type II' □-turn which is the favorite turn type in the formation of □-hairpin. We substituted each amino acid residue in front of the Gly residue of the Aβ40 peptide to D-form proline individually to create five peptides containing the ^{D}P-G sequence at different positions. The □-structure formation of these peptides was monitored by the Circular Dichroism (CD) spectroscopy. The amyloidogenesis was affected by these mutations differently. Interestingly, we found the V24P mutation could reversibly form a β–sheet-rich species, depending on the peptide concentration. And the structure also shows Thioflavin-T and Congo-Red binding ability.

S-06

A NEW ASSAY IN E. COLI TO QUANTIFY THE AGGREGATION STATE OF PRION PROTEIN MUTANTS

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Transmissible spongiform encephalopathies are mainly characterized by the accumulation of the prion protein (PrP) as an abnormal aggregated form. However only few models help to evaluate and quantify this aggregation state *in vivo*. We present a general method to assess PrP solubility and aggregation. This model is based on the structural complementation between the α- and the ω-fragments of the β-galactosidase enzyme in *E. Coli*. Different constructions of the PrP sequence (full-length protein, or protein lacking the N-terminus, or with extra octa-repeats) are designed, merged in open frame with the α-fragment sequence and cloned in $\alpha^{-}\omega^{+}$ bacteria. These fusions are functional, as they have been sequenced and validated by Western Blot using anti-PrP antibodies. Solubility of the fusions correlates with a high β-galactosidase activity, and aggregation with a low activity: aggregation or solubility of the fusion-protein is thus directly assayed by the enzyme activity. We have therefore developed three bacterial strains with a clear and quantitative phenotype of the aggregation or solubility of the prion protein: one « fully aggregated » model using the full-length prion protein with 11 octa-repeats, one «partially aggregated » model with the full-length protein and one « soluble » model with an N-terminus deleted PrP sequence. The aggregation can be tested in high-throughput assays, since 96 samples can be tested simultaneously: our method can constitute a new screening test for drugs that could disrupt or prevent the aggregation of the prion protein. Furthermore, we will generate a library of PrP mutants from different species, by random mutagenesis, to investigate PrP aggregation. Thus, our model might provide a new insight in the mechanisms underlying prion formation and in the selection of original drugs.

S-07

CONFORMATIONAL ENGINEERING OF SYNTHETIC PRIONS

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The advent of synthetic prions, which are formed from recombinant prion protein (recPrP) folded into β-rich conformations, is enabling new insights into the structural basis of prion infectivity (Legname, et al., *Science*, 2004). Recent evidence suggests that the conformational stability of prions isolated from infected mice correlates directly with the incubation period for the onset of CNS dysfunction (Legname, et al., manuscript in preparation). We sought to establish whether it is possible to generate misfolded PrP conformers with varying conformational stabilities *in vitro*, and if so, whether these conformers result in different incubation periods upon intracerebral injection into mice. recPrP of mouse residues 89–231 was incubated in solution, and the formation of PrP^{Sc}-like conformers was monitored by Thioflavin T fluorescence. In hopes of generating distinct conformations, we varied the composition of the solution with respect to denaturant and salt concentration, pH, and the presence of protein-stabilizing additives; the temperature of incubation was also varied. We have identified conditions for the formation of recPrP conformers that are less stable, or less resistant to guanidine and temperature denaturation, as assessed by loss of Thioflavin T fluorescence and by the fraction of soluble recPrP. Differences in fiber morphology were observed by electron microscopy. Whether these differences will correspond to changes in the incubation period remains to be determined. The ability to create distinct conformations of PrP *in vitro* and to correlate their structural features to prion disease pathology will be of enormous value to understanding the structural basis of prion infectivity.

S-08

ACCELERATED AGGREGATION AND FIBRILLIZATION OF THE PRION PROTEIN IN PRESENCE OF GLYCOGEN

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Natural prions are mainly composed of aggregated and misfolded prion protein (PrP). They are highly infective and normally display high stability against degradation. Although the prion theory was proven by *in vitro* generation of synthetic prions from recombinant PrP only (1, 2), the molecular details of prion formation are not yet fully understood, especially since synthetic prions led only to small titers of infectivity. Therefore it is essential to understand PrP misfolding and aggregation in the context of known non-PrP components of natural occuring prions. Such a common secondary component of natural prions is the polysaccharide scaffold which in prion rods amounts up to 15 % (w/w). We showed that its structure consists of predominantly alpha-1,4-linked glucose with few 1,4,6-branches, indicating a close relationship with glycogen (3, 4). Alpha-1,4-linked glucose polysaccharides have unique structural properties as they can form complexes with hydrophobic molecules. Therefore we studied the influence of such a polysaccharide on the conformational transition of PrP, applying an *in vitro* conversion system, in which PrP is kept soluble at low concentrations of sodiumdodecylsulfate (SDS) and undergoes conformational transition with aggregation after dilution of SDS (5) or fribrillization in the presence of sodium chloride (6). Conformational transition, aggregation and fibrillization of recombinant PrP in the presence of glycogen was examined *in vitro* using circular dichroism spectroscopy, fluorescence correlation spectroscopy, confocal laser scanning microscopy, Thioflavin-T-fluorescence and electron microscopy. Here we report that glycogen supports and accelerates PrP amorphous aggregation comparable to seeded aggregation leading to coaggregates. Also formation of PrP fibrils was highly accelerated in the presence of glycogen.

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S-09

THE CELLULAR PRION PROTEIN: BINDING TO VESICLES AND MEMBRANES OF RAFT-LIKE LIPID COMPOSITION

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The conversion of the cellular isoform of the prion protein (PrP^C) into the disease associated isoform (PrP^{Sc}) plays a key role in development of prion diseases. Within its cellular pathway PrP^C undergoes several posttranslational modifications, i.e. the attachment of two N-linked glycans and a glycosyl phosphatidyl inositole (GPI-) anchor, by which it is linked to the plasma membrane on the exterior cell surface. In order to study the influence of the membrane environment on the conversion process we purified posttranslationally modified PrP^C from transgenic CHO-cells (1, 2). We first analyzed the interaction of PrP^C with model membranes *in vitro* before we will go on to conversion studies.

Binding of PrP^C to model membranes was studied both with lipid vesicles in solution and with lipid bilayers bound on a chip surface. The equilibrium and mechanism of PrP^C-membrane-interaction was analyzed quantitatively by surface plasmon resonance. We could observe high affinity of PrP^C to lipid bilayers, but only in the presence of the GPI-anchor, i.e. binding of recombinant PrP without GPI-anchor was negligible. Additionally binding of PrP^C to raft-like lipid membranes was higher as compared to lipid composition of the inner plasma membrane and PrP^C in the aggregated, β-sheet rich structure exhibited only little interaction with the membranes. Depending on the degree of saturation of binding sites the concentration of PrP^C released from the membrane into the aqueous solution was estimated to be between 10^{-9} to 10^{-7} M. This corresponds to a free energy of the insertion reaction of about -48 kJ/mol.

In future we will study the interaction of infectious PrP^{Sc}-particles with PrP^C presented on the membrane surface *in vitro*.

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S-10

STUDIES ON THE POLYMERIZATION REACTIONS OF GERSTMANN-STRÄUSSLER-SCHEINKER DISEASE PRION PEPTIDE USING SURFACE PLASMON RESONANCE

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The polymerization of amyloidogenic peptides resembles a nucleation-growth process, with an initial lag phase followed by a rapid assembly. The lag phase is the rate-limiting step, possibly associated to changes in the protein folding and leading to soluble oligomers with b-sheet structure. These structures form the nuclei that catalyze a templated assembly, in which a thermodynamically favorable association of peptide onto the ends of the growing (proto)fibrils leads to a fast elongation.
Different approaches permit to follow the time-course of these polymerization phases on a scale of hours-days. However, the characterization of the underlying binding events requires information on both association and dissociation rates, with collection of data on a much shorter time-scale. Recent studies showed that surface plasmon resonance (SPR)-based technology, which monitor molecular interactions in real time (secs), might well serve to this purpose. We have used SPR to study the polymerization of PrP82-146, an amylodogenic prion peptide found in the brains of patients afflicted with Gerstmann-Sträussler-Scheinker disease (GSS), a familial prion disease. Short-term flow of low-order oligomers onto prefibrillar PrP82-146 aggregates (immobilized on the sensor chip) resulted in the significant growth of the latter, with the characteristics of an elongation process. Analysis of association-dissociation curves allowed to suggest a "dock-and-lock" model, in which the "locking" step is due to sequential isomerization steps, each increasing the affinity until a condition of irreversible binding is reached. The corresponding kinetic constants were also determined.
Ongoing SPR studies aim to characterize the binding events which occur: 1) in the different phases of the polymerization process or between species of different size; 2) in the presence of interfering compounds; 3) with PrP82-146 peptides from different animal species ("Heteroprion" project).

S-11

GENERATION OF MAB THAT DISTINGUISHES PRPSC FROM PRPC AND NEUTRALIZES PRION INFECTIVITY

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PrP^{Sc}-specific molecular probes, such as monoclonal antibody (mAb), are indispensable tool for elucidating the entity of prion. To stablish PrP^{Sc}-specific mAbs, we immunized $PrP^{-/-}$ mice with PrP^{Sc} purified from prion-infected mice, and screened mAbs with purified PrP^{Sc}. Finally we obtained mAb 6H10 that appeared to be a candidate for the PrP^{Sc}-specific mAb. The 6H10 reacted with PrP^{Sc} treated with proteinase K, whereas the reactivity of pan-PrP mAbs to PrP^{Sc} was disappeared under the same condition. The 6H10 did not react with PrP^{Sc} pretreated with more than 3M GdnHCl, while the reactivity of pan-PrP mAbs increased with the increase of GdnHCl concentration used for pretreatment of PrP^{Sc}. In histoblot analysis, the 6H10 showed positive reaction to non-denatured histoblot but the reactivity decreased after the denaturation of histoblot by autoclaving. In contrast, the reactivity of pan-PrP mAb was enhanced after autoclaving. These results suggested that 6H10 recognizes a conformational epitope on PrP^{Sc}. Peptide phage display analysis suggested that the extreme C-terminus of PrP may be involved in constituting the epitope for 6H10. The 6H10 immunoprecipitated PrP^{Sc} from prion-infected brains of mouse, sheep, and cattle. Furthermore, pretreatment of purified PrP^{Sc} with 6H10 reduced the infectious titer by $1Log_{10}$ in mouse bioassay. However, 6H10 showed weak reaction to brains of uninfected *Prnp*$^{+/+}$ and *Prnp*$^{-/-}$ mice in histoblot analysis, suggesting that 6H10 has cross-reactivity to other host factor(s) than PrP. Nevertheless, the mAb 6H10 will be a useful tool for analyzing the relationship between biochemical and biological properties of prion.

S-12

IIMMUNISATION WITH A HUMAN PRION PROTEIN PEPTIDE STIMULATES GENERATION OF IDENTICAL PRPSC-SPECIFIC ANTIBODIES IN DISTINCT MICE.

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A crucial feature of all transmissible spongiform encephalopathies (TSE) is the conversion of cellular prion protein (PrP^{C}) to the disease-associated misfolded isoform (PrP^{Sc}). All current TSE diagnostic assays are based on the detection of PrP^{Sc}. Our group previously reported on the successful attempt to produce PrP^{Sc}-specific monoclonal antibody (mAb) by immunisation of wild type Balb/c mice with a 13 amino acid peptide from the C-terminal region of PrP, bound to keyhole limpet hemocyanin (KLH). Further experiments involving immunization with the same antigen showed that generation of conformationally sensitive, PrP^{Sc}-specific antibodies was reproducible. In fact, most of the obtained mAbs specifically recognized the PrP^{Sc} isoform. In this study we sequenced and compared light and heavy chain variable regions of several PrP^{Sc}-specific mAbs. Molecular modelling was used to predict the structure of antigen binding sites. As all investigated antibodies were obtained by the same strategy and most of them showed very similar specificity, we expected to find common binding site features. Remarkably, in two separate cases, antibodies with absolutely identical variable regions were found. In both cases matching mAbs originated from distinct cell fusion experiments, i.e. they were originally produced by distinct mice. Sequences of other mAbs involved in the study showed considerably more differences, although the degree of sequence similarity agreed with the observed similarities in antigen-binding properties. Generation of identical antibodies might be explained by the well-known immune tolerance to PrP. We suggest that the repertoire of conformationally sensitive antibodies that are able to recognize the peptide, but at the same time do not cross-react with self-PrP, is very limited. Selection for high-affinity antibodies, first by the immune system itself, and later by hybridoma screening, further reduces their number.

S-13

TOWARDS AN UNDERSTANDING OF THE MECHANISM OF RECOMBINANT HUMAN PRION PROTEIN AMYLOID FORMATION IN VITRO

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Prion diseases are fatal neurodegenerative disorders of humans and animals. These diseases can be genetic, infectious or sporadic disorders but all of these involve misfolding and aggregation of ubiquitous Prion protein (PrP^{C}). The sequences of PrP^{C} and PrP^{Sc} (from amyloidal plaques) are identical and a purely structural change is thought to cause aggregation and fibrillogenesis. Though NMR and X-ray structures for PrP^{C} are determined still high resolution structure for PrP^{Sc} is unavailable. The pathways of conformational change of prion protein are poorly understood.
It is believed that misfolding and aggregation of PrP^{C} to form PrP^{Sc} have a path through partially or completely unfolded prion protein. In our lab, we have established the non-native conditions and were able to get fibril from recombinant human prion protein and synthetic peptides. The fibrils were characterized by electron microscopy and congo red absorption. NMR spectroscopy is used as primary tool to investigate the aggregation behavior of recombinant human prion protein, which is further supported by solid-state NMR spectroscopy and cryo electron microscopy.
Structural preferences for recombinant human prion protein in non-native conditions are identified by H/D exchange and relaxation experiments. The behavior of neurotoxic palindrome sequence AGAAAAGA (113-120), helix 1, helix 2 and helix 3 will be discussed. They will compare with theoretical predicated models of PrP^{Sc} to describe the folding mechanism.

S-14

THE INFLUENCE OF AMINO ACID SUBSTITUTIONS AND DIFFERENT TSE AGENTS WITH REGARD TO THE CONVERTIBILITY OF THE PRION PROTEIN IN A CELL-FREE ASSAY

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The crucial event in the pathogenesis of prion diseases or transmissible spongiform encephalopathies (TSEs) is an induced conformational change of cellular PrP^{C} into an abnormal form which is designated PrP^{Sc}. This process is also called conversion and leads to modified biochemical properties of abnormal prion protein such as a partial resistance to proteinase K, aggregation and subsequent fibril formation.
However, the molecular mechanism underlying this structural transition remains unknown.
In a cell-free assay the conversion reaction can be mimicked by incubating highly purified recombinant PrP^{C} molecules together with PrP^{Sc} seeds in an appropriate conversion buffer. Under these conditions we can show, that PrP^{Sc} itself can induce the conversion of PrP^{C} into a partially proteinase K resistant form designated PrP^{res}. This newly converted PrP^{res} can be selectively detected by an antibody that reacts to an epitope tag which is absent in the seed derived PrP^{Sc}. In our assay we use procaryotically expressed PrP^{C} as a substrate and mouse passaged scrapie strains/isolates as seeds.
Experiments were carried out using chimerical constructs, which consisted either of murine and ovine PrP^{C} or of murine and bovine PrP^{C}. The chimerical proteins were incubated with three different mouse scrapie strains and with mouse BSE.
The experimental data show that even single or few amino acid substitutions within the prion protein have a major effect on its conversion. Additionally, the reaction also depends on the PrP^{Sc} seed, which is used to induce the misfolding.
Interestingly, these results are generally in line with in-vivo data which were obtained in transgenic mice carrying the chimerical PrP^{C}s.

S-15

RETROVIRUS INFECTION STRONGLY ENHANCES SCRAPIE INFECTIVITY RELEASE IN CELL CULTURE

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Prion diseases are neurodegenerative disorders associated in most cases with the accumulation in the central nervous system of PrP^{Sc}, a partially protease-resistant isoform of the cellular prion protein PrP^{C}. PrP^{Sc} is thought to be the causative agent of Transmissible Spongiform Encephalopathies (TSEs). The prion proteins are GPI anchored proteins localized in Detergent Resistant Microdomains (DRMs) at the plasma membrane and in intracellular compartments such as late endosomes. The mechanisms involved in the intercellular transfer of PrP^{Sc} are still enigmatic. Recently, small cellular vesicles of endosomal origin called exosomes have been proposed to contribute to the spread of prions in cell culture models. Retroviruses such as Murine Leukemia Virus (MuLV) or Human Immunodeficiency Virus type 1 (HIV-1) have been shown to assemble and bud into DRMs at the plasma membrane and into intracellular compartments such as late endosomes/multivesicular bodies. Data indicated that host proteins from these compartments can be recruited into nascent viral particles where they retain their biological function and are able to influence virus replication and cell physiology. These data suggest that prion proteins could be recruited by the viral particles during their formation. Here we present data indicating that prion proteins cofractionate with MuLV Gag and Env proteins in DRMs and in late endosomes. Furthermore we report that MuLV infection strongly enhances the release of PrP^{C}, PrP^{Sc} and Scrapie infectivity in the supernatant of coinfected cells. Under these conditions we found by Immunogold Electronic Microscopy and immunoprecipitation experiments that PrP^{C}, PrP^{Sc} and Scrapie infectivity are recruited by both MuLV virions and exosomes. We propose that retroviruses can be important cofactors involved in the spread of the pathological prion agent.

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S-16

MOUSE SYNTHETIC PRIONS FROM FULL-LENGTH PRION PROTEIN

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We recently described the *in vitro* production of infectious prions from purified recombinant prion protein of mouse sequence 89 to 230, designated MoPrP(89–230). MoPrP(89–230) was refolded into a β-sheet–rich amyloid preparation and inoculated into transgenic (Tg) mice expressing PrP of the same sequence. Multiple novel mouse synthetic prion strains were identified, as judged by: (i) incubation times, (ii) a conformational stability assay, and (iii) neuropathologic changes. In another set of experiments, amyloid fibrils were produced using either truncated MoPrP(89–230) or full-length MoPrP(23–230). These preparations were injected into Tg mice expressing full-length MoPrP(23–231). Mice inoculated with either amyloid preparation developed prion disease in ≈600 days. That prions could be generated *in vitro* supports the hypothesis that PrP^{Sc} is the sole component of the infectious agent responsible for the mammalian prion diseases.

S-17

STRUCTURE OF PRP82-146 AGGREGATES AND CROSS-LINKED OLIGOMERS

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The major component of the Gerstmann-Straussler-Scheinker amyloid aggregates is a prion protein (PrP) peptide fragment containing residues from 81-82 to 144-153 (Salmona et al. 2003, J. Biol. Chem. 278, 48146). In the earlier stages of PrP aggregation, small oligomers with neurotoxic properties were identified (Kazlauskaite et al. 2005, Biochem. Biophys. Res. Commun. 328, 292).We stabilized PrP oligomers by photo-induced cross-linking (PICUP) and characterized their polymerisation process by surface plasmon resonance (Gobbi et al. 2006, J. Biol. Chem. 281, 843).
Here, we present a structural study of PrP82-146 and of its aggregation process, as well as that of PICUP oligomers by Fourier transform infrared spectroscopy (FT-IR) and Circular Dichroism. The monomer is mainly unstructured and starts to form low order aggregates after a lag-phase of 7 days. A this stage, the FT-IR bands around 1623.2 cm^{-1} and at 1689.8 cm^{-1} suggest that an antiparallel β-sheet intermolecular interaction characterized the early aggregates. At later stages, the band at 1623.2 cm^{-1} shifts to 1626.1 cm^{-1} and its intensity grows abruptly as fibrils formation takes place. At 10 days, in the FT-IR spectrum only the band at 1626.1 cm^{-1}, diagnostic of a parallel β-sheets intermolecular interaction, is observed in mature fibrils, indicating that a structural reorganization of early aggregates is at work during fibril assembly. Interestingly, the FT-IR spectrum of photo-induced cross-linked oligomers displays two antiparallel β-sheet bands similar to those of the early aggregates, therefore indicating a similar structure of these intermediates.
These results suggest that oligomers obtained by PICUP-technique can be used as a model to study the structural properties and the biological activity of early, short living intermediate of fibril formation.

S-18

INSIGHTS OF PRPSC STRUCTURE OBTAINED BY LIMITED PROTEOLYSIS AND MASS SPECTROMETRY

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Elucidation of the structure of PrP^{Sc}, essential to understand the molecular mechanism of prion transmission, continues to be one of the major challenges in prion research, and is hampered by the insolubility and polymeric character of PrP^{Sc}. Limited proteolysis is a useful tool to obtain insight on structural features of proteins: proteolytic enzymes cleave proteins more readily at exposed sites, preferentially if contained in loops, and do not cleave β-sheet stretches. We treated PrP^{Sc} isolated from brains of hamsters infected with 263K and Drowsy prions with different concentrations of proteinase K (PK). After PK deactivation, PrP^{Sc} was denatured, reduced, and cleaved at Cys179 with 2-nitro-5-thiocyanatonitrobenzoic acid (NTCB), and fragments analyzed by nanoHPLC-MS and MALDI. For 263K, the known cleavages at positions preceding Gly90, Gly86 and Gly92 were observed. For Drowsy, cleavages at positions preceding Gly92, Gln98 and Lys101 were seen. But also, additional discrete cleavage points were detected at more internal positions, including those preceding Ala117, Gly119 and Ser135. In parallel, a subfraction of PrP^{Sc} corresponding to smaller oligomers that exhibit increased sensitivity to proteases were treated with trypsin, and fragments analyzed by mass spectrometry. These experiments showed preferential cleavage after Arg136 (confirming the relative protease sensitivity of the region around Ser135), and at Arg151. Our results indicate that besides the “classic” amino terminal PK cleavage points, PrP^{Sc} contains, in its middle core, regions that show some degree of susceptibility to proteases and must therefore correspond to sub-domains with some degree of structural flexibility, interspersed with domains of high resistance to proteases. These results are compatible with a structure consisting of short β-sheet stretches connected by short loops and turns.

S-19

AGGREGATION INITIATION IN PRION PROTEIN BY RARE SUBOMAIN MOTIONS

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The prion protein is thought to be the causative agens in transmissible spongiform encephalopathies, such as Creutzfeldt-Jakob disease (CJD) in man or bovine spongiform encephalopathy (BSE) in cattle. It is assumed that the disease is induced by a change of the prion protein conformation from the cellular form, PrP^{C}, into the infectious Scrapie-form, PrP^{Sc}. Little is known about the structural and dynamical features of this pathogenic conformational change. Here we present a novel concept involving rare large scale motions between the subdomains β1-α1-β2 and α2-α3 in the carboxy-terminal, globular part of PrP^{C}. The interface between these two subdomains carries a significant number of destabilizing, pathogenic mutations known to be associated with prion diseases. Based on computational simulations and experimental results from our own as well as from published research we propose that such a large scale motion subsequently destabilizes large parts of the cellular conformer PrP^{C} thus rendering it susceptible to structural rearrangements, including aggregation of now partially unfolded parts of the PrP sequence. We propose that such large scale subdomain-motions occur as a rare event even under equilibrium conditions and that the interaction of such partially destabilized PrP^{C}-conformers, which we name PrP^{C*}, contributes to the formation of infectious oligomeric species of the prion protein.

S-20

DEFINING INTERACTIONS BETWEEN PRONUCLEONTM CONFORMATIONAL LIGANDS AND THE OPTIMAL AGGREGATION STATE OF PRPTSE SUBSTRATES FOR THE MISFOLDED PROTEIN DIAGNOSTIC (MPD) ASSAY

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The Misfolded Protein Diagnostic (MPD) Assay utilizes conformationally sensitive Pronucleon™ peptide ligands for both the capture of the PrP^{RES} substrate and for amplification and detection. One recent focus has been to define the optimal conformational and aggregate state of our ligand and the PrP^{RES} target since the most infective form of the target protein may be a smaller, more soluble aggregate rather than the mature fibrils formed in late stage disease in the brain. This is in line with our observations in both tissue and blood that sample preparation can greatly influence the outcome of the MPD Assay. We have also examined synthetic and recombinant PrP^{C} and fibril PrP materials. To investigate this further, we have created known aggregate states of PrP targets and evaluated their response in the MPD assay. Our fluorescent measurements of ligand folding for synthetic and recombinant PrP^{RES} demonstrate less reactivity than biologically derived PrP^{RES} aggregates. However, we can use aggregates of smaller PrP peptide sequences (eg. $PrP_{106-126}$) to seed the unique MPD amplification reaction. We have also used field flow field flow-fractionation (FIFFF) fractionated PrP^{RES} material from scrapie-infected hamster brain and normal hamster brain equivalent aliquots and evaluated these materials in the MPD Assay. Fractions corresponding to the most infectious prion protein particles (fractions 10-12), having the highest specific infectivity, gave distinctly positive MPD assay results. We also observed reactivity with additional fractionated PrP^{RES} material (fractions 3, 16, and 23 and 29). The periodicity of positively-reacting fractions from the PrP^{RES} brain, corresponding from <30 kD up to 1,000 kD, suggests a unique hierarchical structural assembly of PrP^{RES} oligomeric units acting as unique substrates for the MPD specific ligand, while the normal brain equivalents generated no detectable response in the MPD Assay. Further investigations of FIFFF fractions are focused on the expanded utility of the technique to generate positive control PrP^{RES} material for the MPD Assay as well as for potential identification of inhibiting component fractions in human plasma.

S-21

ELK-LIKE PRION PROTEIN TRIGGERS SPONTANEOUS SPONGIFORM ENCEPHALOPATHY WITH FLORID PLAQUES IN MICE

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Chronic wasting disease (CWD) is a geographically widespread, naturally occurring prion disease of wild and captive North American cervids. The origin of CWD is unknown. Here we investigated an unusual structural feature of the elk PrP^C, a well-defined loop connecting the second strand of the beta sheet with alpha helix 2 (amino acids 165-175) which is rigidified by two local hydrogen bond networks. These hydrogen bond networks are absent from mouse PrP^C [1]. We have expressed a PrP^C mutant in transgenic mice that mimics the elk "rigid loop" (RL). These transgenic mice develop a spontaneous neurologic disease characterized by vacuolar change, gliosis, microglial activation, and PrP plaques in the brain, similar to deer with CWD or patients with variant CJD and typical of a transmissible spongiform encephalopathy. Based on this unusual rigid structural feature of the elk PrP, it is tempting to speculate that the isolated geographic foci of CWD outbreaks across the U.S. and Canada may be due to increased risk for a sporadic disease that can then spread horizontally.

S-22

MISFOLDING AND AGGREGATION OF CAPRION - A PRION PROTEIN & CARBONIC ANHYDRASE CHIMAERA

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There are numerous conformational diseases, such as Alzheimer's disease and the prion diseases, which are caused by aberrant protein folding. The misfolding process often originates from partly folded/unfolded protein. This state resembles the molten-globule state (MG) that is observed for many proteins, among them human carbonic anhydrase II, (HCA II). These intermediates are extensively populated at physiological conditions for heritable mutants of HCA II associated with marble brain disease. The MG of HCA II is known to aggregate by specific interactions of the central part of the β-sheet. This central part, β-strands 3-7, of HCA II contains a vast hydrophobic core. In this study we show that a peptide corresponding to the sequence β-strand 6 (DGLAVLGI) forms microcrystalline flake-like assemblies resembling reported crystals of a seven residue peptide of the Sup35 yeast prion. Identical crystals were formed from an alanine-glycine rich palindrome sequence of the human prion protein, $HuPrP^{113-120}$ (AGAAAAGA) despite that this sequence is predicted to adopt a helical structure. Hence this portion of HuPrP is a polymorphic stretch possibly involved in helix-sheet conversion during prion amyloid formation. We created a chimera protein, designated CAPRION, of HCA II by replacing β-strand 6 with the $HuPrP^{113-120}$ sequence. The CAPRION protein was found to exist in a MG-like state under physiological conditions as assessed by ANS binding and tryptophan fluorescence. Unfolding by the chemical denaturants GdmCl and urea reveals a highly destabilized structure. The aggregation proprieties of CAPRION compared to HCA II with and without presence of seeds of the $HuPrP^{113-120}$-peptide, was assessed by measuring the light scattering of formed aggregates in a pH-dependent aggregation assay. The results show that seeding with the peptide microcrystals induced different conformations of the aggregates. Thus, it is likely that the seeds of microcrystals of the $HuPrP^{113-120}$-peptide can template aggregation by sequence independent interactions during aggregation. The aggregates were characterized with epi-fluorescence microscopy after staining with the dyes Nile Red, Congo Red, PTAA and POMT. The morphology of the aggregates has been classified into two major classes, asymmetrical aggregates and meta-crystalline lamellar aggregates. The two types of aggregates are present at the same time; the latter being more abundant when seeding.

S-23

MOLECULAR DISSECTION OF PRPC-PRPSC INTERACTIONS

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Direct interaction between endogenous cellular prion protein (PrP^{C}) and misfolded disease-associated (PrP^{Sc}) conformers is a key event in prion propagation that precedes templated conversion of PrP^{C} into nascent PrP^{Sc} and prion infectivity. Although almost none of the molecular details of this process are understood, the persistence of individual prion strains suggests assembly of the prion replicative complex is mechanistically precise.
The aim of our study was to systematically map defined regions of PrP sequence that bind tightly to PrP^{Sc}. For this purpose we have generated a panel of 45 motif-grafted antibodies containing overlapping peptide grafts collectively spanning PrP residues 19 to 231. These grafted-antibodies were applied in immunoprecipitation experiments to test their reactivity against PrP^{Sc} and PrP27-30. The binding experiments clearly identify only three distinct and independent high-affinity PrP^{Sc} recognition motifs. The first of these binding motifs lies at the very N-terminal region of the PrP molecule, within residues PrP 23-33; the second motif lies within PrP residues 89-112; and the third is contained within PrP residues 136-158. Additional binding studies performed with PrP-grafted antibodies containing mutations and truncations of the three PrP^{Sc} binding motifs have further defined the core components of these binding regions. The two N-terminal PrP^{Sc}
recognition peptide motifs 19-33 and 89-112 bear a net positive charge, and elimination or reduction of this charge by exchanging lysine and arginine residues for alanine residues severely reduced or abolished reactivity with misassembled PrP conformers. Further, the reactivity of IgGs containing truncations and mutations of the original 136-158 PrP graft suggest that PrP^{Sc} recognition via this motif is chiefly facilitated through two segments of sequence composed of residues 136-140 and 149-158.
This study identifies three distinct regions of PrP^{C} interacting with PrP^{Sc} and yield new insight into critical peptidic components composing one face of the prion replicative interface.

S-24

CHARACTERISATION OF THE EFFECT OF HEAT ON TSE AGENT-STRAINS.

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We are developing models of TSE inactivation processes, focusing particularly on heat inactivation. The degree of diversity in thermostability of TSE agent strains has been investigated in 9 TSE strains, seven of which were passaged in two PrP genotypes to give a total 16 TSE models. They show a wide diversity of properties, with differences in heat inactivation of more than 20 °C. In addition, the rate of inactivation from different TSE models with increasing temperature varied between TSE models. In some cases passage in the alternate PrP genotype had little effect on the resulting inactivation properties but in three cases inactivation occurred at lower temperatures. Mixing two TSE strains showed that both strains behaved as expected from the behaviour of the unmixed control, and that the strain causing TSE disease could be identified. There was no evidence of a direct effect of heating on intrinsic strain properties. No strain with higher thermostability properties was selected, either directly after heating or after passage in an alternate PrP genotype. However TSE models with lower thermostability were observed after passage in alternate PrP genotypes. Physical or chemical treatments of TSE agents prior to heating could in some cases alter thermostability properties. Thermostability did not correlate with pH stability. However biochemical analysis of PrP^{Sc} in the TSE models under investigation showed an apparent correlation between the degree to which PrP^{Sc} was glycosylated and the resistance to heat inactivation of the TSE model. The results illustrate in a novel way the diversity of TSE strains. They require molecular properties of TSE agents to accommodate high resistance to inactivation; and a mechanism, independent of the host, which encodes these differences. It is hard to imagine how a host protein might accommodate these requirements by itself.

S-25

SPONTANEOUS, SEEDED AND CYCLIC FIBRIL FORMATION OF PURIFIED RECOMBINANT AND POSTTRANSLATIONALLY MODIFIED PRP

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An in vitro conversion system has been described earlier (1) in which recombinant SHaPrP was shifted from alpha-helical, monomeric to alpha-helical dimeric, to beta-sheet rich oligomeric and to polymorphic insoluble aggregates by dilution of submicellar concentrations of SDS. The system was modified to produce regular fibrils (2). Here we show for the first time the conversion into fibrils with full length PrP^{C} with glycosylations and the GPI-anchor obtained from transgenic CHO-cells (3, 4).
These structures were characterized by electron microscopy (EM) including immunogold labelling. Similar fibrils were obtained from recombinant and native full length PrP^{C} (2)
The soluble initial state from which the fibrils were generated was characterized by Mr-determination, CD-spectroscopy and mass spectrometry.
From these studies a system for seeded fibril formation was developed. Small amounts of purified full length PrP^{Sc} are able to convert recombinant SHaPrP into a fibrillar state as analyzed by Thioflavin T fluorescence, fluorescence correlation spectroscopy and EM.
A cyclic amplification system could be developed which is similar to that of the Soto group (5) but clearly different in that only purified components were applied, i.e. no cellular extract.

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S-26

RAPID AGGREGATION OF RECOMBINANT OVINE PRION PROTEIN INDUCED BY SUBMICELLAR CONCENTRATIONS OF PHOSPHOLIPIDS.

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Transformation of prion protein (PrP) in its insoluble amyloid form provokes neurodegenerative disorders - transmissible spongiform encephalopathies. PrP is connected to neuron membrane by covalently linked glycosylphosphatidylinositol (GPI) anchor. Previous studies described structural alterations and formation of aggregated structures of PrP bound to lipid membranes. Current study demonstrates that PrPrec may interact with individual phosphatidylinositol and phosphatidylethanolamine molecules non-included into membrane structures, and these interactions induce rapid aggregation of PrP protein, which takes place even in presence of very low concentrations of lipid. Lipid micelles prevent formation of large PrP aggregates and provoke increase in □-sheet structure of protein. Thus, liberation of PrP from membrane and direct interaction with its own GPI moiety, as well as with membrane lipids, can increase risk of the formation of amyloid structures.

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S-27

MONITORING OF CONFORMATIONAL TRANSITION OF PRPSC DURING INTERSPECIES TRANSMISSION BY USING NOVEL PRPSC-SPECIFIC ANTIBODIES

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A panel of PrPSc conformation-specific antibodies can be valuable tools in the structural analysis of PrPSc. In order to develop novel PrPSc-specific monoclonal antibodies (mAbs), we immunized PrP-deficient mice with PrPSc that was purified from scrapie-infected mouse brains. The brain homogenate of a scrapie-infected mouse was coated onto an ELISA plate and incubated with (denatured) or without (native) guanidine prior to hybridoma screening. In this screening, we selected candidate mAbs that could act as PrPSc-specific mAbs; these mAbs exhibited stronger immunoreactivity toward the native scrapie brain homogenate than toward the denatured homogenate. Further, each mAb was characterized by immunoprecipitation assays. Of all the tested mAbs, 3B7 exhibited immunoreactivity against PrP in the brain homogenate of the scrapie-infected mouse, but not against that in the brain homogenate of a normal mouse. This result may confirm its specific immunoreactivity against PrPSc. This mAb also reacted with PrPSc in the brain of scrapie-infected hamster, but not with PrPSc in the brain of scrapie-infected sheep and cattle suffering from bovine spongiform encephalopathy. This result showed that there is conformational difference in the PrPSc of rodents (mice and hamsters) and ruminants (sheep and cattle).
Using the mAb 3B7, we examined the conformational transition of PrPSc during interspecies transmission. The immunoreactivity of the mAb 3B7 against PrPSc in the brains of scrapie-infected sheep and sheep scrapie-passaged mice (primary to 5th passage) was examined by an immunoprecipitation assay. No signal was detected from the PrPSc in primary and secondary passaged mice brains; this was also observed in the case of scrapie-infected sheep brains. However, the mAb 3B7 could react with PrPSc in mice brains after the 3rd passage. Based on the length of the incubation periods, prion adaptation requires at least 3 passage histories for interspecies transmission. This conformational change might be correlated with a decreased incubation period. Our data provides direct evidence for the conformational transition of PrPSc during prion adaptation.

S-28

TRANSMISSION OF BSE-301V FOLLOWING INFECTION FROM THE SMALL INTESTINE; A NEW MODEL FOR INVESTIGATING IATROGENIC TRANSMISSION RISKS FOR VCJD.

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The study explores the use of a novel challenge model designed to provide information on the levels of infectivity of tissues following infection from the small intestine. The model uses direct challenge via the small intestine to prevent the contamination of the oral cavity by the primary inoculum. Groups of VM mice (n=10) were inoculated with 100ul of 2% w/v BSE-301v infected brain homogenate directly into the small intestine and sacrificed at 3, 6, 9, 12, 15, 18, 21 weeks post-inoculation. Tissues including spleen, saliva, salivary gland, trigeminal ganglia, gingival margin, alveolar bone, dental pulp, posterior tongue, anterior tongue, brain were removed at the appropriate time points and re-inoculated (intra-cranially) into groups of mice (n=6) which were sacrificed at a clinical end point. Results to date indicate that brain and spleen from mice inoculated via this route became infectious very early in the course of disease progression and achieved maximum infectious titres well before clinical symptoms became apparent. Western blot analysis for the presence of PrP^{Sc} in the brain samples does not correlate well with the levels of infectivity and this is currently being investigated further. These results will be discussed in connection with our understanding of the iatrogenic transmission risks for (v)CJD via general surgery, endoscopy and dental practice.

S-29

REVERSIBLE OLIGOMERIZATION OF THE PRION PROTEIN UNDER PHYSIOLOGICAL CONDITIONS– EVIDENCE FOR 3D DOMAIN SWAPPING

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In prion diseases the □-helical prion protein PrP^{C} is converted into the aggregated and infectious isoform PrP^{Sc} which is rich in □-sheet. Using denaturing conditions *in vitro* the formation of □-sheet rich oligomers were shown to precede the formation of fibrils. Interestingly, the more unstable *ex vivo* prion oligomers were demonstrated to be the most infectious particles compared to larger prion aggregates. However, the mechanism of the first steps in the spontaneous oligomerization process under non-denaturing physiological conditions are still elusive.
It was proposed that 3D domain swapping play a role in oligomerization and aggregation of proteins. Domain swapping is a mechanism for forming homodimers and higher-order oligomers by the exchange of protein domains where the “swapped” domain can also be an element of secondary structure. For the prion protein 3D domain swapping including disulfide bridge shuffling was suggested as a possible first step in prion protein oligomerization. However, no disulfide-linked oligomers were found in PrP^{Sc}. Therefore, we investigated the oligomerization of different prion protein mutants under physiological non-denaturing conditions.
To our complete surprise we observed a reversible oligomerization of certain prion mutants with an unusual temperature dependence. It appears that the □-sheet in PrP^{C} plays an important role in this reversible oligomerization process suggesting that oligomerization is due to domain swapping.

S-30

GROWING 2D PRION CRYSTALS FROM POLYOXOMETALATE COMPLEXES

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Determining the structure of infectious prions at near-atomic resolution via electron crystallography has been constrained by the limited availability and quality of 2D crystals. Thus far, all 2D crystals of infectious, N-terminally truncated PrP^{Sc} (PrP 27-30) have been prepared from prion-infected brains by a well established density gradient purification procedure (Prusiner et al., Biochemistry 21:6942, 1982). We determined that in this procedure the 2D crystals form at the same time as the more commonly seen prion rods, during the limited digestion with proteinase K in the presence of sarkosyl. While we were able to increase the number of 2D crystals in our preparations by optimizing a range of buffer and protease digestion parameters, we failed to improve their size and order. We attribute this failure to the fact that, at this stage of the purification procedure, PrP 27-30 is only moderately enriched and numerous contaminating proteins and peptides interfere with crystallization. In order to overcome this problem, we decided to change our purification procedure completely and instead use sodium phosphotungstate (PTA), a polyoxometalate (POM) that selectively precipitates PrP^{Sc} and PrP 27-30 (Safar et al., Nature Med. 4:1157, 1998). This procedure allows rapid purification of PrP 27-30 as well as other protease-resistant and protease-sensitive conformers of PrP^{Sc}. Large and relatively well ordered 2D crystals could be found in some of these preparations, particularly after removal of PTA via dialysis. By employing various POMs (Lee et al., JACS 127:13802, 2005), we observed differences in the distributions between fibrillar prion rods and 2D crystals. Some POMs seem to favor the polymerization of PrP 27-30 into 2D crystals over the formation of prion rods. We may be able to identify conditions that allow the polymerization of PrP 27-30 into even larger, well ordered 2D crystals, suitable for high-resolution, cryo low-dose electron crystallography.
We acknowledge generous support from the Sherman Fairchild Foundation and the National Institute on Aging (Grants AG02132 and AG10770).

Poster Session

PRION STRAINS IN HUMANS AND ANIMALS

PR-01

MOLECULAR SIGNATURE OF ATYPICAL SCRAPIE AS OBSERVED IN FIELD ISOLATES AND AFTER TRANSMISSION IN AN OVINE TRANSGENIC MOUSE MODEL (TGOVPRP4)

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In this study we investigated a series of sheep and goats TSE isolates identified in France following active surveillance of scrapie between 2002 and 2004 which were identified as atypical cases of scrapie (Buschmann et al., 2004). We performed a detailed analysis of PrPsc biochemical signature (WB electrophoretic mobility, epitope mapping and PrPsc deglycosylation pattern) and transmission results to transgenic mice over-expressing the ovine PrP A_{136} $L_{141}R_{154}$ Q_{171} allele (TgOvPrP4 mouse line) (Crozet et al., 2001).
In all the field isolates we found an unique biochemical signature with five major bands including a characteristic band of low apparent molecular weight (≅ 10-11kDa). This unique pattern was shown to be undistinguishable from Norwegian Nor98 isolates and was fully conserved after transmission to ovine transgenic mice from a series of 11 different French isolates of different *PrnP* genotypes and species. Detailed analyses, in both small ruminants and ovine transgenic mice, strongly suggest that this unique pattern could originate from the presence of three different protease cleavage products, (i) a full length form of PrP, almost entirely protected against digestion by proteinase K, (ii) a 10-11kDa form cleaved in both N and C terminal ends of the protein (iii) and an 18kDa fragment probably N-terminally cleaved.
Interestingly, in our ovine transgenic mouse model, we made the observation that PrPc levels detected in the blood at the time of intracerebral inoculation strongly determined the incubation period of the disease with atypical scrapie isolates. Mice with low levels of PrPc in the blood had very prolonged incubation periods. This was not observed with classical scrapie isolates. However PrPc blood levels did not influence the nature of clinical signs or PrPres Western blot pattern.
These data have to be discussed with regard to other forms of prion diseases, including genetic (Gerstmann-Sträussler-Scheinker) and sporadic (CJD disease) forms in human. They raise essential questions regarding the aetiology of this recently described form of scrapie frequently observed throughout European countries.

PR-02

DISSOCIATION OF PATHOLOGICAL AND MOLECULAR PHENOTYPE OF VCJD IN TRANSGENIC HUMAN PRP 129 HETEROZYGOUS MICE

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All neuropathologically confirmed cases of variant Creutzfeldt-Jakob disease (vCJD), characterised by abundant florid plaques and type 4 disease-related prion protein (PrP^{Sc}) in brain, have been homozygous for methionine at polymorphic residue 129 of *PRNP*. The distinctive neuropathological and molecular phenotype of vCJD can be faithfully recapitulated in *Prnp* null transgenic mice homozygous for human PrP M129 but not V129 where a distinct prion strain is propagated. We have modelled in transgenic mice, susceptibility of 129MV heterozygotes, the commonest *PRNP* genotype, comprising 51% of the UK population. We show that, remarkably, propagation of type 4 PrP^{Sc} was not associated with characteristic vCJD neuropathology. Depending upon the source of the inoculum these mice can develop four distinct disease phenotypes after challenge with BSE prions or vCJD (human passaged BSE) prions. vCJD-challenged mice had higher attack rates of prion infection than BSE-challenged recipients. These data argue that human *PRNP* 129 heterozygotes will be more susceptible to infection with vCJD prions than to cattle BSE prions, and may present with a neuropathological phenotype distinct from vCJD.

PR-03

PET-BLOT ANALYSIS CONTRIBUTES TO BSE STRAIN RECOGNITION IN C57BL/6 MICE

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Identification of the strain of agent responsible for the bovine spongiform encephalopathy (BSE) can be made on histological basis, through the analysis of both distribution and intensity of brain vacuolar lesions after BSE transmission to mouse. Another useful way to distinguish the BSE agent from other prion strains is the study of the abnormal prion protein (PrP^{res}) distribution. For that purpose, here, the Paraffin-Embedded-Tissue-blot (PET-blot) method was applied on brains from C57Bl/6 mice infected with cattle BSE, experimental sheep-BSE or Feline Spongiform Encephalopathy (FSE) from a cheetah. The PrP^{res} distribution was comparable whatever the 3 BSE agent sources considered and was distinct from the PrP^{res} distribution in C57Bl/6 mice inoculated with a French scrapie isolate or with a mouse-adapted scrapie strain (C506M3). These data confirm a common origin of infectious agent responsible for the British and French cattle BSE. They also indicate that PET-blot method appears as a precise complementary tool in prion strain studies as it offers an easy and quick assessment of the PrP^{res} mapping. The advantages and limits of the PET-Blot method are discussed and compared to other established and validated methods of strain typing.

PR-04

AN EXPERIMENTAL PROTOCOL OF MALE AND FEMALE GENETIC SELECTION TO IMPROVE SCRAPIE RESISTANCE IN A SHEEP FLOCK

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The confirmation of BSE in a french goat on January 2005, raised the attention of European Union on prevention, diagnosis and control of scrapie in small ruminants population. Genetic susceptibility to Scrapie is related to polymorphisms at the Prion Protein gene (codons 136, 154 and 171). Animals with VRQ and ARQ alleles show the highest risk to develop scrapie, while the ARR allele is associated to low risk of disease. A local scrapie outbreak in goats raised in a marginal geographic area was stamped out in December 2004; the proximity of a sarda breed sheep flock worried local sanitary authorities that planned a gradual experimental genotyping programme in order to reduce ARQ allele frequency and to increase ARR allele never forgetting productive and phenotypic features too. All 510 animals were genotyped and only two males were ARR/ARR; to achieve our purposes in the flock and a good number of resistant males, it was therefore necessary to divide sheeps in two coupling-groups one of which was designed to carry on genetic selection. The newborn of this group were genotyped and results were used to decide lambs destination. Genotyping was performed by Real time PCR using a protocol of allelic discrimination and by a primer extension approach using a commercial kit. In one year of selection we obtained 6 more ARR omozygous males, a significant reduction of omozygous ARQ animals from 30% to 15% and a raised ARR frequency (omozygous from 18% to 22% and eterozigous from 50% to 61%). Therefore we can conclude that, in particular situations, the short time necessary to obtain a good increase of resistance to scrapie justify the additional work and costs related to female genotyping.

PR-05

INVESTIGATION OF HUMAN TSE STRAINS BY TRANSMISSION TO TRANSGENIC MICE EXPRESSING HUMAN PRION PROTEIN – THE 'HUMTRANS' NEUROPRION PROJECT

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For the purpose of human TSE surveillance it is beneficial to classify cases according to the clinical and pathological characteristics. If atypical forms of sporadic CJD or variant CJD occur this characterisation is important, as such cases may change the currently held ideas on the occurrence of such diseases. Bioassay in transgenic mouse lines is often used for such case analysis and therefore our three lines expressing human prion protein (with variation at codon 129) can be used for such work. We have used gene targeting to generate MM and VV genotype inbred lines that can be crossed to produce the MV genotype. They express physiological levels of human PrP^{C} and are more likely to develop clinical TSE after inoculation with sCJD than the wildtype line used for transgenic line production (129Ola). Inoculation of human transgenic mice with typical cases of vCJD and sCJD (all six Parchi / Gambetti types) and subsequent subpassage will produce a dataset for cross-comparison when investigating atypical cases. NeuroPrion funding for the 'HUMTRANS' project has allowed breeding stocks of these human transgenic mice to be produced in other European laboratories allowing for greater number of cases to be transmitted, together with cases specific to those countries. Preliminary strain investigation of sporadic CJD by transmission to these mice has shown that the most common type (MM genotype; PrP^{Sc} type 1) is the most efficiently transmitted. MM and MV genotype mice develop clinical TSE disease after similar incubation periods, and before the VV mice show symptoms. Further details of transmission experiments carried out by the UK IAH Neuropathogenesis Unit in Edinburgh will be presented.

PR-06

CHARACTERIZATION OF ATYPICAL BSE CASES OF THE H- AND L-TYPE IN GERMANY

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After the detection of BSE in the UK in 1986, it was assumed that BSE only occurred as a single strain. However, since 2003 BSE cases of two deviant kinds (L-type and H-type) have been reported in France and Italy in animals that were over eight years of age. Therefore we have re-assesed the 27 German BSE cases that were over eight years of age when the disease was diagnosed out of the 389 German cases that have been detected between November 2000 and December 2005. This characterization focused on the determination of the molecular mass (M_r) of the unglycosylated band as well as on the glycoprofile of the accumulated PrP^{Sc} by immunoblot analysis. We detected one L-type case which was characterised by a decreased M_r of the unglycosylated PrP^{Sc} and a unique glycopattern with almost equal percentages of the di- and monoglycosylated PrP^{Sc} fractions as well as one H-type case with a distinctly elevated M_r molecular mass of this smallest PrP^{Sc} fraction and an unaltered glycoprofile (using mab L42). Comparative analyses of these two cases together with French and Italian cases of the H- and L-types proved that these two cases indeed belong to the same groups. Both German isolates were inoculated into bovine PrP transgenic mice (Tgbov XV mice). The L-type provoked disease in these mice after very short incubation times of 183 days as compared to 230 days after challenge with classical BSE. In contrast, the H-type has not lead to any clinical symptoms in the mice after 300 days. Interestingly, the characteristic immunoblot profile of the L-type BSE was maintained after passage through Tgbov XV mice. In order to study the pathogenesis of these novel BSE strains in their natural host, transmission studies in 6 months old calves (i.c. route) have been initiated.

PR-07

CLASSIFICATION OF SPORADIC CREUTZFELDT-JAKOB DISEASE REVISITED

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Classification of the sporadic form of Creutzfeldt-Jakob disease (sCJD) is based on the molecular size of the unglycosylated isoform of the PK-resistant prion protein (PrP^{Sc}) and on the methionine (M)/valine (V) polymorphic genotype at codon 129. In one classification two types of PrP^{Sc} are recognized according to the relative molecular mass: ~ 21 kDa (type 1) and ~ 19 kDa (type 2). The PrP^{Sc} distinction into two types has supported the view that sCJDMM1, the most representative subtype, is a single entity. However, two other classifications subdivide sCJDMM1 into two different groups based on: 1. three (rather than two) molecular masse types of PrP^{Sc}, and 2. distinct phenotypic characteristics, primarily disease duration. To shed light on these divergences, we divided a group of twenty-two subjects with confirmed sCJDMM1 into two sub-populations according to their mean disease duration being 2.02 months (short duration sCJD) and 14.8 months (long duration sCJD). We first focused on the study of the migration in gel of the PrP^{Sc} prion protein under those conditions that had led to the detection of the two sCJDMM1 subtypes in the other classifications, under stringent pH conditions and making use of the high resolution gel electrophoresis. We studied the characteristics of PrP^{Sc} detected in the two sub-populations using two-dimensional immunoblotting, conformational stability immunoassay, and sucrose gradient fractionation. Moreover, clinical and pathological features were also investigated. The results showed no differences in gel mobility and conformation characteristics of the protease resistant PrP^{Sc} between the two populations when sample preparation for homogenization and PK digestion were performed under stringent pH conditions. Phenotypic manifestations were also homogenous except for the presence of more severe lesions in the long duration cases. Therefore, our finding suggests that 1) the variability in gel mobility of PrP^{Sc} associated with sCJDMM1 is largely due to the pH variations during tissue homogenization and that 2) the distinction of sCJDMM1 into two subgroups is not justified (Supported by NIH AG-08012 and AG-14359, Charles S. Britton Fund, and CDC UR8/CCU515004).

PR-08

ATYPICAL BSE ISOLATES IN CATTLE: IMMUNOHISTOCHEMICAL COMPARISON BETWEEN DUTCH AND ITALIAN CASES

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Unusual isolates of BSE have been reported in the past two years in different European countries and Japan. Two variant forms of BSE have been reported based on biochemical analysis of the molecular mass and glycoform ratio of the pathological prion protein (PrPsc): i) a type with a lower molecular mass of the unglycosylated isoform and a distinct glycopattern of PrPres (L-type or BASE) and (ii) a type with higher molecular mass of the unglycosylated isoform and a distinct glycopattern of PrPres (H-type). From a pathological point of view, a careful description was only available for the two Italian cases (L-type) where immunohistochemical analysis revealed an unusual PrPsc deposition pattern characterized by the presence of amyloid plaques particularly in the white matter of olfactory bulb, frontal and parietal cortex. Here we report the results of a comparative immunohistochemical study between an H-type BSE case identified in the Netherlands and the two Italian L-type or BASE cases, using a panel of four different antibodies (9A2, 94B4, 12B2, F99/97.6.1). The antibodies used recognize different linear and conformational epitopes that are dispatched along the PrP sequence. The study conducted at the level of the obex and pons indicates that F99/97.6.1 better discriminates the two different phenotypes.

PR-09

BANK VOLES ARE USEFUL BIOASSAYS FOR ARQ/ARQ SUFFOLK SCRAPIE INFECTION

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Scrapie is a transmissible spongiform encephalopathy (TSE) affecting sheep, goats and moufflon. The influence of genetic susceptibility in sheep is well described and the occurrence of natural and experimental scrapie has been linked to polymorphisms at codons 136, 154 and 171 of the PrP gene. The effect of such polymorphisms is also dependent on breed and in Suffolk sheep the ARQ/ARQ genotype is considered the most susceptible whilst the ARR/ARR genotype is most resistant to natural disease. The determination of levels of infectivity in tissues from scrapie affected sheep is fundamental to understand the pathogenesis, and the transmissibility of the disease. The most sensitive indicator of Suffolk scrapie infectivity is probably the parenteral challenge of ARQ/ARQ sheep. Such a system is however cumbersome and expensive. Detection of infection in laboratory mice is more rapid and economical, but is generally less sensitive, particularly in the case of primary isolation from Suffolk sheep. Recently, the bank vole (*Clethrionomys glareolus*) has been shown to be highly sensitive to different sources of natural scrapie. After intracerebral inoculation, bank voles succumb to disease following a short incubation time (around 200 days post inoculation) and also show high attack rates. With this study we established that voles are very susceptible to inoculation from ARQ/ARQ Suffolk scrapie, and that they succumb in 175 +/- 18 days after inoculation of 0.02 ml of brain homogenate.

PR-10

MOLECULAR CHARACTERIZATION OF THE POLYMORPHIC VARIANTS OF THE PRP D178N MUTATION EXPRESSED IN TRANSGENIC MICE

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Fatal familial insomnia (FFI) and a subtype of familial Creutzfeldt-Jakob disease (CJD^{178}) are clinically and neuropathologically distinct diseases linked to the D178N mutation in the gene encoding the prion protein (PrP). The disease phenotype is determined by the M/V polymorphism at codon 129 of the mutant allele: D178N/M129 segregates with FFI, while D178N/V129 is associated with CJD^{178}. It is hypothesized that conformational differences between the polymorphic variants of D178N PrP account for this phenotypic heterogeneity. We have generated transgenic (Tg) mice that express the mouse PrP homologues of the D178N mutants (D177N/M128 and D177N/V128). Analysis of the biochemical properties of PrP extracted from the brains of Tg mice indicates that both mutants are aggregated and partially resistant to low concentrations of proteinase-K, yielding protease-resistant fragments of similar molecular size. However, D177N/V128 PrP displays higher resistance to urea-induced dissociation than D177N/M128, suggesting that the two proteins possess different oligomeric states. To investigate possible differences in the secondary/tertiary structure, we are currently applying a conformation-dependent immunoassay, which compares the accessibility of antibody epitopes in the folded and unfolded forms of PrP.

PR-11

SHEEP-PASSAGED BSE ANALYSED IN BOPRP-TG110 MICE EXHIBITS BSE STRAIN FEATURES AND ACCELERATED DISEASE COURSE ALLOWING THEIR DISCRIMINATION FROM SCRAPIE

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We used transgenic mice expressing bovine PrP (BoPrP-Tg110) as a model to discriminate between BSE in sheep and scrapie. The transmission in BoPrP-Tg110 mice of prions from BSE infected sheep was examined and compared to the transmission of original cattle BSE and sheep scrapie prions. Our results indicate no transmission barrier for sheep BSE prions to infect BoPrP-Tg110 mice. In contrast, all three sheep scrapie isolates tested were only transmitted to BoPrP-Tg110 mice with an evident transmission barrier according to onset of disease and PrP^{res} scoring. The biochemical features observed in BoPrP-Tg110 mice inoculated with sheep-passaged BSE prions were similar to those rendered by the cattle BSE prions but different to those observed for the sheep scrapie isolates. These differences persisted after a second passage in these transgenic mice demonstrating that this bioassay is a valuable tool for distinguishing between BSE and scrapie in sheep.

PR-12

TWO-DIMENSIONAL ANALYSIS OF PROTEINASE K-RESISTANT PRION PROTEIN IN GSS P102L MUTATION

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The most common Gerstmann-Straussler-Scheinker (GSS) disease phenotype is associated with a point mutation at codon 102, which results in the proline-to-leucine substitution. In P102L mutation, immunoblot analysis of protease-resistant PrP, or PrP^{res}, shows the presence of a C-terminal 21kDa fragment, in addition to an internal 8kDa peptide, spanning positions 80-150. Protease-resistant PrP species encountered in P102L mutation are not limited to the above molecules but also include truncated fragments migrating at 17 and 14 kDa. With the exception of the internal 8 kDa fragment, the migration of the 21kDa core fragment and of truncated PrP species is indistinguishable from that observed in sCJD cases with type 1 PrP^{res}. However, when PrP^{res} species of GSS associated with P102L mutation were analyzed by 2D analysis novel C-terminal PrP^{res} variants were found. The present findings suggest that the composition of C-terminal variants observed in GSS is different from those found in sCJD, and this may accounts for differences in disease phenotypes.

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PR-13

CHARACTERIZATION OF AN ATYPICAL TYPE-H BSE CASE IN SWEDEN

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Bovine spongiform encephalopathy (BSE) is considered to be caused by a single strain producing a consistent and homogenous phenotype of disease in cattle. BSE had never been detected in Sweden until March 2006, when a 12-years old mixed-Charolais cow was screened positive using an ELISA test. The cow was in late pregnancy, had been recumbent and getting up repeatedly, was finally unable to rise and was euthanized. Western blot (WB) with Bio-Rad Bovine WB at SVA confirmed the case but showed that the unglycosylated band had a higher MW than in classical BSE. Further WB analysis was conducted in parallel at the VLA, AFSSA and CIDC-Lelystad. The VLA, applying mAb 6H4 in the hybrid, the OIE-SAF, and the NaPTA WB confirmed the results of SVA and also demonstrated binding of mAb P4 (specific for WGQGGSH), which is unusual for cattle. Image analysis estimated the difference of MW of being approximately 0.75-0.9 kD. Additionally, it showed more evenly distributed relative quantities of PrP^{Sc} in the di- and mono-glycosylated bands than in classical BSE. The AFSSA achieved similar results using a core antibody, Sha31, for the discrimination of apparent molecular masses and also showed binding with mAbs 12B2. CIDC-Lelystad showed excellent binding of 12B2 (specific for sequence WGQGG) and higher level of PK sensitivity, in contrast to classical BSE cases. IHC on brainstem, with mAbs F89 and P4 showed a characteristic widespread fine granular synaptic type of immunostaining of the neuropil throughout the grey matter in all the sections. The trigeminal tract nucleus and the solitary tract nucleus were more densely and intensively stained, and had also coarser granules. Perineuronal staining was observed in a few neurons. Notably, intracellular and glia-associated PrP^{d} types were not observed. In conclusion, the Swedish case shows similarities with previous descriptions of H-type of BSE.

PR-14

STRAINTYPING SHEEP BRAIN PRPSC BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Differential diagnosis of TSEs is particularly important because of the need distinguish scrapie from possible BSE infection in small ruminants. Until now, routine biochemical screening tests that can differentiate TSEs are based on Western blotting. However, the resolution of these tests is relatively low and known to be unable to differentiate BSE from some scrapie isolates such as CH1641.
A strategy has been developed at VLA for high resolution strain differentiation by sequencing the N-terminus of PrP^{27-30} by mass spectrometry. This approach has been progressed by incorporating quantification of N-terminal fragments against synthetic standards. For this purpose a new quantitative liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) method has been developed.
PrP^{27-30} was extracted from brain of naturally infected sheep by PK treatment and precipitation by sodium phosphotungstic acid (NaPTA) and/or centrifugation through a sucrose cushion (SCC). The resulting samples were reduced, alkylated and digested by trypsin under denaturing conditions.
The quantities of the various N-terminal tryptic peptides extracted were initially determined for pooled sheep brain. At least 10 picomoles of PrP^{Sc} could be extracted from 0.5 g brain of scrapie-infected sheep and that the dominant PK cleavage sites were at W84, H88 and W93. An extraction method which included NaPTA but not SCC proved to give highest recovery of PrP^{27-30}.
The outcome will be reported of application of this approach to confirm previous qualitative comparisons of mouse TSE strains. Brain PrP^{27-30} N-terminus profiles will be described for scrapie field cases (various breeds and genotypes) and for sheep inoculated with TSE isolates (CH1641 and SSBP/1) and BSE.

PR-15

LONG LASTING FAMILIAL CREUTZFELDT-JACOB DISEASE WITHOUT DEMENTIA: A CASE STUDY

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Creutzfeldt-Jacob Disease (CJD) is a fatal neurodegenerative disorder, often with a sudden onset and ultimately terminal, usually within a few months. The clinical characterization of CJD includes diverse neurological and psychiatric signs, but the major clinical symptom is a rapidly progressing dementia.
Here we describe an unusual familial case of a 72 years old woman that was diagnosed with CJD and died a few months later without signs of dementia or any psychiatric disturbances and with very minor cognitive deterioration. She was firstly tested in 1998 and retested in 1999 as healthy subject in a study related to preclinical signs in CJD (Gigi et al, 2005). In June 2004, she was hospitalized in the department of neurology with suspected CJD. Her clinical picture included dizziness and headaches, coordination disturbances in her left hand, motor speech difficulty and progressive weakness of all four limbs that reached full paralysis. Diagnosis was based on MRI, EEG, positive result for the CSF 14-3-3 test and positive PRNP E200K mutation. However, she showed only slight deterioration in cognitive tests performance in comparison to seven years before. Moreover, it appeared that her functioning in some of the tests were poor even in 1998.
This case is an unusual appearance of CJD without any dementia signs, and according to the tests results it is possible that she was ill for at least 7 years. The pathogenesis of familial CJD may be different from previous concepts.

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PR-16

MOLECULAR STRAIN-TYPING OF OVINE TSE DISEASES

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It is presumed that conformational differences in the PrP^{Sc} molecule impart the ability of TSE agents to exist as differing strain types which can be characterised by bioassay. Such three-dimensional structural variations will also dictate their interaction with enzymes and antibodies. At present, methods that detect Proteinase K (PK) resistant forms of PrP^{Sc} have been used to differentiate strain-dependent conformers by relative molecular mass, ratios of the different glycosylated forms and differential antibody binding in Western blot analysis and ELISA. However, these methods have not been able to reliably distinguish all variants of scrapie within sheep that have been revealed through bioassay. The present study aims to develop further molecular strain typing assays in order to provide complementary tests capable of differentiating BSE and a range of scrapie strains in sheep of differing genotypes. Rapid means of determining and characterising the different strains of TSE agents is essential for developing control and management strategies of prion diseases in animals and for assessing their risk to man. Here, we describe a novel method, using the thermostable protease thermolysin to digest PrP^{Sc}, which reveals PrP banding patterns on Western blots capable of differentiating ovine scrapie field cases and experimental ovine BSE. Such differentiation was apparent in all of the samples analysed, which included brain homogenates from scrapie affected animals of genotypes VRQ/VRQ, ARQ/ARQ, ARQ/VRQ and AHQ/AHQ compared to BSE affected animals with an ARQ/ARQ genotype. The application of such an assay to differentiate experimental scrapie strains is under investigation.

PR-17

TRANSMISSIBILITY AND CHARACTERISATION OF ATYPICAL SCRAPIE IN MICE

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The identification of atypical scrapie in sheep in several European countries has raised interest in the transmissibility of these agents to sheep and across species barriers. In the UK, atypical scrapie cases were detected as a result of on-going active and passive surveillance programmes for scrapie in sheep. In contrast to classical scrapie, the current characterisation of atypical scrapie cases includes: absence of vacuolation in the obex, restricted PrP^{Sc} distribution in the obex confined to the spinal tract of the trigeminal nerve, a low BioRad TeSeE ELISA signal, an extra ~12 kDa proteinase K resistant band in Western blots, and PrP genotype associated with resistance to classical scrapie. The purpose of this collaborative study is to examine whether atypical scrapie from sheep of various PrP genotypes are transmissible to transgenic PrP and conventional mouse lines, and to characterise any transmissible disease by neuropathological and biochemical means. To this end, brain homogenates from atypical scrapie sheep were inoculated into transgenic mice (Tg338, overexpressing sheep VRQ, and TgshpXI, overexpressing sheep ARQ) and wild-type mice (C57BL/6 and VM). Preliminary results have shown that transgenic mice inoculated with several atypical scrapie inocula have developed clinical signs of disease. Western blotting characterisation of brain samples from clinically affected Tg338 mice revealed Nor98-like profiles in the atypical cases examined to date (n = 10). Pathological analysis of brain samples from clinically affected Tg338 mice inoculated with atypical scrapie (6 cases) demonstrated lesion profiles indistinguishable from Nor98 and the French discordant cases, with incubation periods similar to Nor98 in Tg338 mice at ~200 days post-linoculation.

PR-18

STUDIES ON THE SECOND ATYPICAL BSE CASE IN A JAPANESE BLACK COW

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An ELISA (Plateria, BioRad) positive specimen of a 14 year-old Japanese Black cow (beef cattle) slaughtered in an abattoir was examined by western-blot (WB) and histological/immunohistochemical (IHC) analyses for the confirmation of BSE. Dysstasia had been reported as a clinical symptom. Histological examination of the medulla oblongata at the level of obex showed severe vacuolations in dorsal nucleus of the vagus, nucleus of the solitary tract and nucleus of the spinal tract. Granular and linear deposition of PrP^{Sc} was also detected in these areas by IHC analysis. Thus, histological and IHC data were compatible with the histopathology of the typical BSE. In the WB analysis, however, the amount of the di-glycosylated PK-resitant PrP^{Sc} was found to be at approx.35% of the total PrP^{Sc}, and the mono-glycosylated PrP^{Sc} was at approx.40%. The WB analyses showed that PrP^{Sc} distributed widely in the brain with the unchanged glycosylation ratio. Such a glycosylation-ratio is distinct from that of the typical BSE agent in which the di-glycosylated form is dominant (approx.70%) but, intriguingly, similar to that of the type-2 sporadic CJD agent. No DNA mutation was detected in the PrP coding region, except polymorphisms of the codons for Gln78 and Asn192 being determined as CAG and AAT, respectively. Judging from the glycosylation-ratio, BSE prion herein is different from the typical BSE prion, and the atypical BSE prion found previously in a Holstein steer in Japan (ref. 1). Instead, its molecular feature is close, if not identical, to PrP^{Sc} found in the cattle succumbed to bovine amyloidtic spongiform encephalopathy (ref. 2), and to the sporadic CJD-like PrP^{Sc} in the mice inoculated with BSE agent (ref. 3).

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PR-19

DISCRIMINATION BETWEEN SCRAPIE AND BSE IN SHEEP USING PORCINE PRP-TRANSGENIC MICE

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Previous work from our laboratory showed the existence of a strong transmission barrier for BSE prion in transgenic mice expressing porcine PrP (PoPrP-Tg). Here we study the transmission to PoPrP-Tg mice of prions from BSE infected sheep in comparison to the transmission of sheep scrapie prions. PoPrP-Tg mice were challenged with BSE infected sheep inoculum and with four different sheep scrapie isolates, in order to assess their susceptibility to the different inoculated prions. Intracerebral inoculations were performed with 10% brain homogenates. The results showed that scrapie isolates are not transmitted at all to PoPrP-Tg mice up to day (in some of them second passage is concluded). In contrast, 100% of the animals inoculated with sheep BSE inoculum succumbed to the primo-infection at approximately 450 days after inoculation. In conclusion, these data indicate a very strong transmission barrier in Po-PrP-Tg mice for sheep scrapie prions but very low for sheep BSE prions. On the other hand, these data reveal that this transgenic mouse model constitutes a valuable tool for the discrimination of BSE and scrapie in sheep.

PR-20

ATYPICAL SCRAPIE IN ITALY: IMMUNOHISTOCHEMICAL STUDY WITH A PANEL OF DIFFERENT ANTIBODIES

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Fifteen cases of scrapie obtained by active surveillance with unusual features have been diagnosed in Italy since 2005.
Immunohistochemical staining with our statutory antibody F99/97.6.1 showed a diffuse granular PrPsc pattern in the nucleus of the spinal tract of the trigeminal nerve and in the granular and molecular layer of the cerebellum.
The purpose of this work was to investigate PrPsc deposits by immunohistochemistry using a panel of 10 monoclonal antibodies recognizing different linear epitopes dispatched among PrP sequence from the N-terminal to the C-terminal part of the protein. This study was performed in 9 animals for which the whole brain was available and different brain regions were studied including telencenphalon, diencephalons, mesencephalon, pons, cerebellum and obex.
Results of this study provide comparisons between the different PrPsc deposit patterns detectable according to the epitope specificity of the antibody applied .
Immunohistochemical epitope mapping of PrPsc accumulation in the brain is useful for the characterisation of atypical TSE strains in the small ruminants population.

PR-21

TSES IN CATTLE: DISCRIMINATION OF THREE MOLECULAR TYPES OF PROTEASE RESISTANT PRION PROTEIN IN BRAIN DIAGNOSED WITH BOVINE SPONGIFORM ENCEPHALOPATHY.

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Transmissible spongiform encephalopathies (TSEs) exhibit strain specific properties by their behaviour in bioassays and in molecular analyses of the disease associated prion protein (PrP^{Sc}). However, bovine spongiform encephalopathy (BSE) of cattle appeared to be very homogeneous. Recently, a limited number of atypical isolates have been identified in several countries. This asks for a comparative study to find a firm molecular classification of these cases. Various conditions of digestion with proteinase K (PK) and PNGaseF, analysis by Western blot with a range of epitope-defined antibodies were explored. Analyses of the disease associated proteinase K resistant moiety of PrP (PrP^{res}), as described previously by Biacabe and Casalone, confirmed existence of two additional PrP^{res} variants, defined here as type-H and type-L following the higher and lower position of the aglycosylated PrP^{res} band in Western blots compared to that in BSE. This was further confirmed by varying the biochemical conditions like detergent addition, PK digestion, and deglycosylation treatment. The three molecular PrP^{res} types exhibited different migration patterns, glycoprofile, PK-susceptibilities and antibody binding. These properties appear independent of geographical origin suggesting the general occurrence of these two different PrP^{res} variants in cattle, possibly all over the world. A practical procedure for the recognition of these types in brain tissues has been worked out.

PR-22

DE NOVO GENERATION OF PROTEASE-RESISTANT PRION PROTEIN IN TRANSGENIC MICE MIMICKING FATAL FAMILIAL INSOMNIA

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Fatal familial insomnia (FFI) is produced by the aspartic acid to asparagine substitution at codon 178 (D178N) of the gene that encodes the prion protein (PrP). This mutation is coupled to methionine at codon 129 (129M), a common polymorphic site in the prion protein gene. We have analyzed the PrP characteristics together with the levels of expression of some other markers differently involved in FFI in a line of transgenic mice with the equivalent genetic background to that present in human FFI [Tg(MoPrP-D177N/128M) or Tg(FFI)]. We have found that, when compared to control animals [wt and Tg(MoPrP-128M)], Tg(FFI) showed a striking under-representation of the unglycosylated forms of PrP and resistance to digestion by proteases. The protease resistance was prominent in old mice, where the levels of resistance were comparable to those used for diagnostic procedures in human subjects. The size of the protease resistant core of PrP was 19 kDa, equivalent to that present in human FFI (type 2-PrP^{Sc}). The accumulation of abnormally-folded mutant PrP in Tg(FFI) is the most likely cause for all these features. Additionally, and as observed in human FFI patients, we noted an alteration of both the serotoninergic and orexinergic systems while the levels of parvalbumin were normal. The present findings suggest that Tg(FFI) reproduce at least some of the FFI pathogenetic mechanisms making Tg(FFI) a suitable tool for the study of this disease.

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PR-23

VARIANT CREUTZFELDT-JAKOB DISEASE WITH DETECTABLE PRPSC IN MULTIPLE ORGANS AND A NOVEL PK-RESISTANT PRPSC FRAGMENT IN THE BRAIN

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Variant Creutzfeldt-Jakob Disease (vCJD) is a prion disease thought to be acquired by the consumption of prion-contaminated beef products. Worldwide, nearly 200 cases have been identified mainly in the United Kingdom. Two case-patients have been detected in the United States, although both were born and raised in Great Britain where they presumably acquired their prion infection. We have characterized the neuropathology and brain PrP profile in the first US vCJD case-patient whose duration of illness was unusually long, 32 months. We have also studied the presence of PrP^{Sc} in peripheral tissues by applying modifications to the sodium phosphotungstic acid (NaPTA) precipitation, in the attempt to increase the recovery of PrP^{Sc}. In addition to PrP^{Sc} seen in other vCJD patients, a novel N-terminally truncated, PK-resistant fragment of PrP was detected in all the brain regions examined, independent of the number of plaques present. With high PrP^{Sc} recovery, we have been able to detect PrP^{Sc} not only in the lymphoreticular system and the gastrointestinal tract, but also in skin, lungs, adrenal gland, kidney, urinary bladder, ovary, uterus and liver. Extraneural PrP^{Sc} glycotype was similar to that present in brain. Our preliminary results significantly expand the spectrum of organs affected in vCJD. If confirmed by further studies, these results could carry important implications with regard to iatrogenic transmission. In addition, our results show the presence of a novel, PK-resistant PrP^{Sc} fragment that may shed new light on the mechanisms of PrP^{Sc} formation in this disease.

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PR-24

IN VITRO PROPAGATION OF PRION STRAINS

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Prions are unconventional infectious agents responsible for transmissible spongiform encephalopathies. Compelling evidence support the hypothesis that prions are composed exclusively by a misfolded form of the prion protein (PrP^{Sc}) that replicates in the absence of nucleic acids. It is well established that the prion infectious agent, like conventional micro-organisms, exhibit strain variation. Prion strains can be differentiated by their particular *in vivo* and *in vitro* characteristics, including differences in incubation period, lesion pattern of infected brains and biochemical properties of PrP^{Sc}, among others. Understanding how a single protein can provide the diversity to sustain the strain phenomenon has been a challenge for the prion hypothesis. We have recently described that PrP^{Sc} can be propagated indefinitely *in vitro* to generate infectious material using the protein misfolding cyclic amplification (PMCA) technology. Here we demonstrate that prion strains characteristics can be replicated *in vitro* by PMCA. Our results show *in vitro* amplification of various mouse strains including 301C, RML, ME7, 139A, and 79A. Moreover, wild type mice inoculated intracerebrally with *in vitro* generated PrP^{Sc} from 301C, RML and 79A strains show clinical signs, brain lesion pattern and biochemical characteristics identical to the animals inoculated with brain infectious material. Interestingly, the specific infectivity (infectivity per unit of PrP^{Sc}) was the same in both preparations. These results suggest that the strain characteristics of prions can be maintained by in vitro replication of PrP^{Sc}.

PR-25

CONJUGATED POLYELECTROLYTES – CONFORMATION SENSITIVE OPTICAL PROBES FOR STAINING AND CHARACTERIZATION OF PRION STRAINS

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It has been suggested that the prion strain phenomenon might be encoded in the conformation of the misfolded protein and that the conformation is the critical mediator of prion susceptibility. Hence, simple, sensitive and versatile tools that can be used to distinguish between different prion strains are of great interest. In previous studies we have shown that conjugated polyelectrolytes (CPs) can be used as conformation sensitive optical probes for the detection of conformational changes in synthetic peptides, amyloid fibril formation *in vitro,* and for histological staining of amyloid plaques in tissue sections. In contrast to small rigid fluorescent probes, such as thioflavin T (ThT) or Congo red, the conformational flexibility of CP probes allows direct correlation between the geometry of chains and the fluorescence from the probe. If conformational changes of proteins can lead to different conformations of the CP probe, an alteration of the color of the fluorescence from the probe is observed. Hence, CP probes can be used as conformation sensitive optical probes, providing a direct link between spectral signal and protein conformation. In this study, we have utilized fluorescent CPs as dyes for staining of prion deposits in brain tissue sections derived from a transgenic mouse model which overexpresses murine PrP (*tga*20 mice) to develop murine-adapted strains of BSE, sheep scrapie, and CWD. The technique reported in this article was also verified and compared against conventional staining techniques and biochemical techniques. The binding of the CPs to the three murine passaged ruminant strains was variable and the anionic CP, polythiophene acetic acid (PTAA) showed different spectral signatures when bound to different prion strain deposits, illustrating the ability of CPs being a useful complementary tool to conventional techniques for strain typing of prions. In conclusion, we present a novel technique to distinguish between prion strains in tissue sections.

PR-26

NOR98 SHOWS MOLECULAR FEATURES REMINISCENT OF GSS

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Molecular variants of PrP^{Sc} are being increasingly investigated in sheep scrapie and are generally referred to as “atypical” scrapie, as opposed to “classical scrapie”. Among the atypical group, Nor98 seems to be the best identified.

We studied the molecular properties of Italian and Norwegian Nor98 samples by WB analysis of brain homogenates, either untreated, digested with different concentrations of proteinase K, or subjected to enzymatic deglycosylation. The identity of PrP fragments was inferred by means of antibodies spanning the full PrP sequence.

We found that undigested brain homogenates contain a Nor98-specific PrP fragment migrating at 11 kDa (PrP11), truncated at both the C-terminus and the N-terminus, and not N-glycosylated. After mild PK digestion, Nor98 displayed full-length PrP (FL-PrP) and N-glycosylated C-terminal fragments (CTF), along with increased levels of PrP11. Proteinase K digestion curves (0,006-6,4 mg/ml) showed that FL-PrP and CTF are mainly digested above 0,01 mg/ml, while PrP11 is not entirely digested even at the highest concentrations, similarly to $PrP^{27\text{-}30}$ associated with classical scrapie. Above 0,2 mg/ml PK, most Nor98 samples showed only PrP11 and a fragment of 17 kDa with the same properties of PrP11, that was tentatively identified as a dimer of PrP11. Detergent solubility studies showed that PrP11 is insoluble in 2% sodium laurylsorcosine and is mainly produced from detergent-soluble, full-length PrP^{Sc}.

Furthermore, among Italian scrapie isolates, we found that a sample with molecular and pathological properties consistent with Nor98 showed plaque-like deposits of PrP^{Sc} in the thalamus when the brain was analysed by PrP^{Sc} immunohistochemistry.

Taken together, our results show that the distinctive pathological feature of Nor98 is a PrP fragment spanning amino acids ~ 90-155. This fragment is produced by successive N-terminal and C-terminal cleavages from a full-length and largely detergent-soluble PrP^{Sc}, is produced in vivo and is extremely resistant to PK digestion.

Intriguingly, these conclusions suggest that some pathological features of Nor98 are reminiscent of Gerstmann-Sträussler-Scheinker disease.

PR-27

HUMAN PRPSC "TYPING" PITFALLS ASSOCIATED WITH THE USE OF TYPE 1 SELECTIVE ANTIBODIES COMBINED WITH RELATIVE INEFFICIENT HYDROLYSIS OF PRPSC BY PROTEINASE K

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The bulk of current molecular CJD classification is based on the detection of either one of two protease-resistant PrP^{Sc} core fragments which differ in molecular mass and primary site of Proteinase K (PK) cleavage (i.e. 21 kDa and residue 82 for type 1, 19 kDa and residue 97 for type 2). Using antibodies recognizing an epitope between residues 82 and 96 (not detecting type 2), Polymenidou et al and Yull et al recently found at least some type 1 in all type 2 samples, and reached the conclusion that multiple PrP^{Sc} types regularly coexist in CJD. At variance, we explored the possibility that the "type 1-like"/type 2 co-occurrence detected by these selective antibodies represents, in most cases, an inefficient hydrolysis of type 2 rather than real, strain related, type 1. To verify this hypothesis we analysed the PrP^{Sc} core in 50 sCJD and 3 vCJD subjects using a wide range of PK activity, 15 cm long gels with high resolution, and both 3F4 and 12B2 (epitope 89-93) antibodies. When exposed to a relatively moderate PK activity (i.e. 1U/ml corresponding to 50 μg/ml when specific PK activity is 20 U/ml, for 1 h at 37°C, pH 6.9, 6 mg proteins/ml) both types 1 and 2 showed, in addition to the 21 or 19 kDa band respectively, a tight group of slower migrating bands. In type 2, 12B2 only recognized these bands which appeared as a single one, migrating similarly to type 1, in a standard 5-7 cm gel. The degree of PK resistance of these fragments ("type 1-like") was significantly lower than that of types 1 or 2 resistant cores. We conclude that the "type 1-like" signal detected by the 12B2 antibody in CJD type 2 is not a PrP^{Sc} resistant-core but instead matches the physicochemical properties of partially cleaved fragments with ragged N-terminus generated in both types 1 and 2 by relative inefficient hydrolysis.

PR-28

UNUSUAL MIGRATION PATTERN OF PRPRES FROM BOVINE BRAINSTEM TISSUE

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Confirmation of BSE may be difficult in rare cases especially when quality of brainstem is poor as in dead-on-farm animals. Experience from Western blotting (WB) has revealed that a low speed clarifying centrifugation can help to confirm a negative diagnosis by the removal of equivocal PrP-signals. However, we present here three cases - 2 autolysed cases from Poland and a case from a slaughterhouse in The Netherlands - which after centrifugation persistenly exhibit PK-resistant PrP-immuno reactive bands with an unusual migration. Confirmation was performed by OIE-SAF immunoblotting in the autolysed samples and by IHC in the case from slaughterhouse. The objective of this study was to further examine these cases for molecular properties of the PrP-bands. Studies carried out were: graded digestion with PK, deglycosylation with PNGaseF, precipitations, analysis by WB with a range of monoclonal antibodies. In comparison with BASE-like (or L-type) isolates, the present 3 examples exhibit PrP^{res} bands with lower MW_r. Antibodies 12B2 and 9A2, specific for the respective PK-resistant N-terminal regions aa residues 101-105 and 108-110 of bovine PrP, were not reactive, and removal of carbohydrates by PNGaseF indicated that the molecular entity of the PrP^{res} bands represented a glycosylated PrP-moiety encompassing an N-terminus located somewhere between aa residues 110 and 120 and the C-terminus of mature PrP. These observations call for a cautious approach especially when testing samples in a very advanced stage of autolysis. However, the finding of a similar case from a slaughterhouse indicates that all 3 represent natural *in vivo* phenomena rather than a consequence of autolysis.

PR-29

PRION DISEASE IN EXOTIC SPECIES

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Bovine spongiform encephalopathy (BSE) is known to be caused by a relatively promiscuous prion strain which appears able to infect other mammalian species readily by the oral route. In addition, BSE prions have resulted in subclinical prion infection, with no clinical dysfunction observed during the normal life span of the animal, in some animal models. During the epidemic of BSE in the United Kingdom not only cattle were affected by this disease but also gemsbok, nyala, American bison, scimitar-horned oryx, Arabian oryx, eland, greater kudu, cheetahs, puma, ocelots, African lions, tigers and domestic cats. These species had been exposed to contaminated meat and bone meal or bovine carcasses. Additionally, an African lion and a tiger from British zoos developed clinical signs suggestive of prion infection but without spongiform lesions in the brain. Transmission studies were performed to investigate if these animals were affected by a prion disease and whether it was caused by the BSE prion strain. Brain tissue homogenates from these exotic animals were inoculated intracerebrally into wildtype (FVB and RIIIS/J) and transgenic mice expressing human 129MM or 129VV genotype on a *Prnp*$^{0/0}$ background. Similar transmission characteristics as cattle BSE were seen and both neuropathology and Western blot analysis of PrP^{Sc} glycoforms showed a BSE-like profile. In several groups, mice showed subclinical prion infection. Molecular and biological strain typing are consistent with BSE aetiology for lion and tiger prion disease.

PR-30

PHENOTYPE IS PRESERVED FOLLOWING EXPERIMENTAL TRANSMISSION OF ATYPICAL SCRAPIE TO SHEEP

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Active surveillance for transmissible spongiform encephalopathies in small ruminants has been an EU regulatory requirement since 2001. A number of European countries have subsequently reported cases of atypical scrapie, similar to previously published cases from Norway (Nor98). These cases have pathological and molecular features which are distinct from classical scrapie. They display a relative absence of PrP immunolabelling in the diagnostic 'obex' region of the brainstem, with pronounced immunolabelling in the cerebellum, together with a distinctive banding profile on Western blot with a prominent low molecular weight band at approximately 12 kD. Most cases have occurred singly in flocks, and are associated with genotypes considered to be more resistant to classical disease (i.e. UK National Scrapie Plan groups 1-3). Experimental transmissibility of such isolates has been reported in certain ovinised transgenic mice, but has not previously been demonstrated in the natural host. This report presents the successful transmission, following intracerebral inoculation, of atypical scrapie (from an AHQ/AHQ fallen stock sample) to a recipient sheep of homologous genotype. Clinical end-stage was reached 378 days post inoculation, and the affected animal presented with weight loss, altered behaviour and compulsive circling, but no pruritis. There was full preservation of both the pathological and molecular characteristics of the donor. The transmission characteristics of this isolate in Tg338 mice are indistinguishable from those reported for Nor98. This finding strengthens the opinion that these cases result from a distinct and stable strain of scrapie agent which may require different regulatory controls.

PR-31

PHYSICO-CHEMICAL PROPERTIES OF PRPRES IN PERIPHERAL TISSUES OF EXPERIMENTAL TSES.

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The discovery of strain-related PrP-res physico-chemical properties has provided a potentially new approach for rapid strain typing in TSEs, but the lack of knowledge about PrP-res characteristics in peripheral tissues represents a significant obstacle. We studied PrP-res properties in brain and some peripheral tissues in: i) 20 mice orally exposed to the ME7 scrapie strain at different times after exposure, ii) 2 hamsters intracerebrally (ic) infected with ME7, 2 hamsters orally infected with 263K at terminal stage, and iv) 15 mice ic infected with 11 distinct scrapie and BSE strains, also at terminal stage. Because of the relatively low PrP-res content and the presence of non-specific signals, we used extraction procedures such as NaPTA precipitation and purification in Sarkosyl rather than crude homogenate analyses. The electrophoretic mobility of PrP-res showed a consistent pattern in all the TSE models analysed, unaffected by the type of tissue or stage of disease. As the only exception the 301C and 301V BSE strains in mice were distinguishable because of a faster gel migration. As shown in previous studies, PrP-res glycotype was highly heterogenous among different TSE strains and a quite distinctive feature in some of them. PrP-res glycotype was largely maintained in peripheral tissues in all the models analysed; as the only exception the hamsters i.c. inoculated with the ME7 strain showed a tissue-specific glycotype in the gut. The amount of PrP-res in peripheral tissues significantly differed among the TSE models analysed and never reached the amount detected in the brain. Our preliminary results indicate that, despite the limitations related to the low amount of PrP-res accumulation and the presence of a tissue-specific effect in some cases, biochemical strain typing can be also fruitfully applied to PrP-res extracted from peripheral tissues. Supported by EU contract QLG3-CT-2002-81030.

PR-32

TRANSMISSION OF CWD TO TRANSGENIC MICE

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Chronic wasting disease (CWD) is a lethal prion disease in deer and elk. Distinctive among the prion diseases, it is transmitted among captive and free-ranging cervids. To study the biology of CWD prions, we generated five lines of transgenic (Tg) mice expressing prion protein (PrP) from Rocky Mountain elk (Cervus elaphus nelsoni), denoted Tg(ElkPrP), and two lines of Tg mice expressing PrP common to mule deer (Odocoileus hemionus) and white-tailed deer (Odocoileus virginianus), denoted Tg(DePrP). At 550 days of age none of the Tg(DePrP) or Tg(ElkPrP) mice exhibited spontaneous neurologic dysfunction. After inoculation, brain samples from CWD-positive elk, white-tailed deer, and mule deer produced disease in Tg(ElkPrP) mice between 180 and 200 days and in Tg(DePrP) mice between 300 and 400 days. Tg(MoPrP)4053 mice overexpressing wild-type mouse PrP-A were susceptible to one of eight cervid brain inocula in ~540 days. Brains of diseased mice revealed abundant PrP amyloid plaques upon neuropathologic analysis. Serial passaging of brain homogenates from symptomatic Tg(ElkPrP) mice produced disease in 120–190 days in Tg(ElkPrP) mice. Contrary to Tg(DePrP) and Tg(ElkPrP) mice, Tg mice overexpressing human, bovine, or ovine PrP did not develop prion disease after inoculation with CWD prions from among nine different isolates after >500 days. These results suggest that CWD prions from white-tailed deer, mule deer, and elk can readily transmit among these three cervid species.

PR-33

BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERISATION OF THE UK ATYPICAL SCRAPIE STRAIN

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During active surveillance of the UK sheep population for TSEs, about half the suspect cases identified by rapid testing for abnormal PrP in the brain stem were found to be distinct from the classical form of scrapie. Theses cases appear to have PrP with a less biochemically stable core structure and under stringent proteolysis conditions PrP was no longer detectable by ELISA or Western Blot (Everest et al., 2006). Mild conditions of proteolysis, however, identified a molecular banding profile on Western Blot that was distinct from the classical scrapie samples and which bore similarities with Nor-98 and similar cases now found in Europe. In addition, this newly identified variant of scrapie appears to favour sheep carrying alleles for the AHQ and ARR (and $AF^{141}RQ$) genotypes. We have extended these studies to determine the biochemistry of the prion abnormality in atypical scrapie samples from a range of sheep genotypes by comparing the kinetics of proteolysis with those with prion protein from normal sheep brain and confirmed cases of classical scrapie. ELISA and Western Blot have been used to identify the molecular characteristics of the products of digestion. These studies will facilitate our understanding of the prion characteristics in the UK and underpin strategies for the control and eradication of TSEs in small ruminants. *This work is funded by defra, UK.*

Everest, S.J., L. Thorne, D.A. Barnicle, J.C. Edwards, H. Elliott, R. Jackman, and J. Hope. 2006. Atypical prion protein in sheep brain collected during the British scrapie-surveillance programme. J Gen Virol 87:471-7.

PR-34

PK-SENSITIVE OVINE PRPSC REVEALED BY CONFORMATION-DEPENDENT IMMUNOASSAY

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Detergent extraction and limited proteolysis of PrPSc results in fibrillar aggregates of a protease-resistant core (PrP27-30) that polymerise spontaneously into amyloid. Aggregated forms of PrP co-purify with prion infectivity, although infectivity does not always correlate with the presence of PrP27 – 30. Limited digestion with PK to discriminate between PrPC and PrPSc may underestimate the repertoire of conformations of PrPSc and their quantity within prion-infected tissues. We have developed a conformation-dependent immunoassay (CDI) utilising time-resolved fluorescence to study the conformers of ovine disease-associated PrP in the absence of PK-treatment to discriminate between PrPC and PrPSc. The CDI utilises a panel of either N- or C-terminal-specific anti-PrP monoclonal antibodies that recognise regions of the prion protein that are differentially buried or exposed. Epitopes buried in PrPSc show an increase in immunoreactivity after denaturation of the PrP molecule, whereas those that were solvent exposed do not change in their reactivity. PrPSc for analysis was precipitated from brain tissue of scrapie-positive sheep by detergent extraction, with or without the use of sodium phosphotungstic acid. Native and denatured PrPSc was subsequently subjected to a capture-detector CDI. PrPSc was readily detectable in homozygous VRQ and ARQ scrapie-infected sheep brains. The highest levels of PrPSc were found in homozygous VRQ scrapie-infected brains. The quantity of PrPSc was significantly reduced when samples were treated with PK prior to the CDI. These data show that the level of PrPSc in brain samples from cases of natural scrapie display genotypic differences and that a significant amount of this material is PK-sensitive.

PR-35

DIFFERENTIATION OF ANIMAL TSE STRAIN TYPES USING PROTEINASE K DIGESTION PATTERNS OF DISEASE-ASSOCIATED PRP

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Variation in the stability of the disease-associated prion protein (PrPd) associated with transmissible spongiform encephalopathies to enzymic proteolysis has recently been used to help identify different strains of sheep scrapie. The aim of this work was to determine if differences in PrPd proteolysis patterns could be used to identify cases of BSE in sheep. Using proteinase K digestion profiles obtained from SDS PAGE western immunoblot (number, size and intensity of protein bands at increasing digestion times), we compared the PrPd from brain homogenates of sheep experimentally infected with BSE and naturally incurred scrapie in sheep. Resistance to proteinase K (PK) digestion was measured by the binding of epitope specific antibodies to sample proteins extracted after prolonged digestion in modified immunoassays. Significant differences in susceptibility to PK digestion were observed between strains and host and banding patterns in western blots identified molecular characteristics of the digestion products. Similar methods are being employed to characterise scrapie field isolates. Funding: defra UK.

PR-36

COMPARATIVE INTER-SPECIES CARBOHYDRATE CHARACTERIZATION OF PRPSC BY LECTINS

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The key event in transmissible spongiform encephalopathies (TSEs) is the conversion of the soluble, protease-sensitive glycosylated prion protein (PrP^{C}) to an abnormally structured, aggregated and protease-resistant isoform (PrP^{Sc}). Both PrP isoforms bear two glycosylation sites, none of which is necessarily occupied, and thus in a typical western blot with an anti-PrP antibody three distinct bands appear, each corresponding to the di- the mono- or the unglycosylated form of the protein. The intensity and the electrophoretic mobility of each of the three bands is characteristic of each TSE type and has been used to discriminate between the various TSE strains and types. In the present study we examined the possibility that in addition to quantitative differences between the amounts of each glycoform, there are also variations in the sugars carried by PrP^{Sc} from different species, using lectins. Lectins are multimeric proteins, which can bind with high affinity to specific sugar moieties. PrP^{Sc} was purified from various TSE affected samples using a protocol based on differential guanidinium precipitation over a sucrose cushion, followed by proteinase-K treatment and salt precipitation. PrP^{Sc} was then analyzed by SDS-PAGE, electrotransferred on PVDF membranes and western blotted with a panel of biotinylated lectins and monoclonal anti-PrP antibodies. From this study were identified two plant lectins, namely Datura stramonium Lectin (DSL) and Ricinus communis Agglutinin I (RCAI), that bind PrP^{Sc} with high affinity. Furthermore, it was found that only a subpopulation of the PrP^{Sc} population is recognized by these lectins and that there are differences in the affinity with which these lectins recognize the PrP^{Sc} glycoforms in the various TSE types. Lectin staining of PrP^{Sc} could prove to be a useful tool for studying the effect of the host and the TSE strain based on glycotype profile and discriminating between the various types of TSEs.

PR-37

ALTERATION IN THE BIOLOGICAL CHARACTERISTICS OF BSE PRIONS WAS MONITORED BY THEIR INCUBATION PERIOD IN TRANSGENIC MICE

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Bovine spongiform encephalopathy (BSE) is a neurodegenerative disorder, which naturally affects cattle. However it also can be transmitted to a wide range of species including humans, where it appears as variant Creutzfeldt-Jakob disease. BSE prion has been transmitted to mice, but not to hamsters. Therefore, we analyzed the mechanisms responsible for species barrier in BSE prion by using mouse and hamster chimeric-prion protein (PrP^C) expressed transgenic (Tg) mice (MH2M and MHM2). The brain homogenate of BSE cattle or BSE-passaged mice was intracerebrally inoculated into MH2M, MHM2, tga20 and TgHaNSE mice and the incubation periods of the prions in each mouse were determined. The MHM2 and tga20 mice were susceptible to the BSE prion, but not the MH2M and TgHaNSE mice. The amino acid substitutions between MH2M and MHM2 showed that the species barrier of hamsters against BSE prion was attributable to PrP131-188 (I138L, Y154N, S169N). However, after a single passage to the wild-type mouse, the BSE prion gained transmissibility to all Tg mice. Although, the shortened of the incubation period of BSE prion in the wild-type mice required at least 3 passages, the incubation period of BSE prion in the Tg mice was not altered from mouse-passage history. The prion adaptation process for overcoming the species barrier might comprise several steps—one of these is associated with PrP^C while the other is not.

Furthermore, the characteristics of the BSE prion that was passaged in cattle PrP^C expressed Tg (TgBoPrP) mice were analyzed (BSE/TgBoPrP). The incubation period in the MHM2 with the BSE/TgBoPrP prion was approximately 220 days, which was different from the MHM2 with BSE prion (approximately 400 days). Although the mechanisms remained unclear, BSE/TgBoPrP showed distinctively different biological characteristics from the BSE of the cattle brain. Incubation time assay using a set of Tg mice could offer an effective parameter for monitoring the alteration of prion characteristics.

PR-38

CHARACTERIZATION PRPRES BY TWO DIMENSIONAL ANALYSIS IN ATYPICAL SCRAPIE CASES IN ITALY

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In the last few years, active surveliance system of TSEs in cattle and in small ruminants induced the detection of an increasing number of scrapie and BSE cases characterized by a PrP^{Sc} differing from that of typical cases. Most of these “discordant” cases were positive to ELISA (Biorad) screening test and negative to immunoblot because of the low resistance of PrP^{Sc} to proteases treatment. In this study, we obtained brain samples from italian scrapie cases of “Nor-98 like” in sheep and we carried out a biochemical characterization of PK-resistant PrP. One D-PAGE showed two different PrP^{Sc} banding patterns based on the anti-PrP antibody probed. In particular, by using mAb 4G11 directed to a C-terminal epitope (residues: 199-218) PrP^{Sc} was represented by three bands which migrated between 16-25kDa that were reduced to single band of 16kDa after deglycosylation. Conversely, P4 (residues: 89-104) detected a single band of 12kDa, that did not change in migration after glycans removal, likely representing N- and C-terminally truncated fragment. More interestingly, 2D-PAGE analysis showed that C-terminal and the internal fragments, recognized by mAbs 4G11 and P4 respectively, were clearly distinguishable because of to the opposite charges of this peptides. The 16 kDa C-terminal fragment had a pI ranging from 3.0 to 6.0 whereas the internal peptide of 12kDa migrated around a pH of 9.0. In addition, we compared 2D maps of the present cases to those obtained in previously described scrapie cases (Zanusso et al., J Gen Virol 2003). In conclusion, biochemical analysis of PrP^{Sc} in atypical scrapie cases showed that: (i) Italian cases show an electrophoretic PrP profile similar to those reported in other countries; (ii) the 16 and 12kDa fragments derive from two differently truncated fragments; (iii) PrP^{Sc} similarities, obtained by 2D-PAGE, between the present cases and previously reported scrapie cases suggest that the PrP^{Sc} pattern found in atypical cases might be frequently found in sheep population.

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Poster Session

PATHOGENESIS

PA-01

PRIONS ADHERE TO SOIL AND REMAIN INFECTIOUS

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An unidentified environmental reservoir of infectivity contributes to the natural transmission of prion diseases (transmissible spongiform encephalopathies, TSEs) in sheep, deer and elk. Prion infectivity may enter soil environments via shedding from diseased animals and decomposition of infected carcasses. We examined the potential for soil to serve as a TSE reservoir by studying the sorption of the disease-associated prion protein (PrP^{Sc}) with common soil minerals. We demonstrated substantial PrP^{Sc} adsorption to whole soils as well as clay minerals (montmorillonite and kaolinite) and quartz. We quantified the PrP^{Sc} binding capacities of each mineral examined. Furthermore, the PrP^{Sc} desorbed from montmorillonite (Mte) clay was cleaved at an *N*-terminal site and the interaction between PrP^{Sc} and Mte was strong, making desorption of the protein difficult. Despite cleavage and avid binding, PrP^{Sc} bound to Mte remained infectious. Results from our study suggest that PrP^{Sc} released into soil environments is maintained in a bioavailable form, perpetuating prion disease epizootics and exposing other species to infectious agent.

PA-02

PURKINJE CELLS DEGENERATION IN THE CEREBELLUM OF SHEEP AND GOATS NATURALLY AFFECTED WITH SCRAPIE

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The cellular prion protein plays an important role in the pathogenesis of transmissible spongiform encephalopathies (TSEs) such natural scrapie in sheep and goats. Purkinje cells are some of the largest neurons in the cerebellum, with intricately elaborate dendritic trees, characterized by a large number of spines. These send inhibitory projections to the deep cerebellar nuclei, and constitute the sole output of all motor coordination in the cerebellar cortex. The loss of Purkinje cells lead to motor disorders and ataxia. The purpose of this study was to evaluate qualitatively and quantitatively the degenerence of Purkinje cells in the cerebellum of small ruminants affected with natural scrapie. Six brains of Texel ewes and six brains of Alpine goats showing clinical signs of scrapie were examined in the cerebellum for the accumulation of PrPsc and Calbindin-28k protein, a "specific" marker of Purkinje cells. All the animals were confirmed as positive for PrPsc by Bio-Rad rapid test and/or immunohistochemistry. Three ewes (ARR/ARR) and three goats without neurological diseases were used as negative controls. Immunostaining of Calbindin-28k and PrP were performed using respectively a polyclonal anti-Calbindin-28k antibody (Swant, Switzerland) and a monoclonal anti-PrP antibody (8G8, CEA Saclay, France). Our results showed an important loss of dendritic trees of Purkinje cells in the molecular layer and a cell body degeneration in the cellular layer in scrapie animals. The decrease of Purkinje cells number in the cerebellum was estimated at 56% in sheep and 28% in goats comparatively to healthy controls. This major loss of Purkinje cells is correlated with the amounts of PrPsc evidenced by doublelabelling.
In conclusion, our results showed an important loss of Purkinje cells in the cerebellum of small ruminants naturally affected with scrapie and this degeneration could explain in part the motor disorders and ataxia usually observed in TSEs.

PA-03

PRIONS IN SKELETEL MUSCLE OF CWD INFECTED DEER

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The zoonotic potential of chronic wasting disease (CWD) has become a public health concern since the transmission of bovine spongiform encephalopathy (BSE) prions to humans resulting in variant Creutzfeldt-Jakob disease (vCJD). Studies in mice, sheep and humans indicated that PrP^{Sc} could be detected in the skeletal muscles. Since the most probable route of human exposure to CWD is through consumption or handling of meat from infected animals, it is important to assess whether skeletal muscle from affected cervids harbors prions. CWD-susceptible Tg(CerPrP) mice were intracranially inoculated with brain and matched skeletal muscle homogenates from moribund as well as non-infected control deer. Tg mice inoculated with either brain or muscle homogenates from CWD-infected deer developed clinical illness with characteristic prion disease symptoms and the brains of recipients accumulated cervid PrP^{Sc}. The mean incubation times for animals inoculated with brain material ranged between 231 and 283 days, whereas mice receiving muscle tissue had average incubation periods between 360 and 492 days. Tg mice inoculated with material from CWD-negative deer did not develop prion disease or accumulate PrP^{Sc}. Brain and muscle samples used to inoculate Tg(CerPrP) mice were analyzed for the presence of PrP^{Sc}. Brain samples producing the shortest incubation times had levels of PrP^{Sc} detectable by Western blotting in 25 μg total protein, whereas PrP^{Sc} was detectable only after sodium phosphotungstate (NaPTA) precipitation of 0.5 mg for isolates with the longest incubation periods. No protease-resistant material was detected in muscle when 50 mg total protein was precipitated with NaPTA and analyzed by Western blot. Although a possible role of prion strain variability cannot currently be dismissed, these results suggest variable prion titers in the CNS and skeletal muscle from different CWD-infected deer in the same phase of disease.

PA-04

ANALYSIS OF MURINE CNS PROTEOMES OF PRP KNOCKOUT AND WT MICE.

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The prion protein (PrP) is fundamental to TSE disease biology and its conversion from the normal, cellular form (PrPc) to a detergent insoluble and protease resistant isoform (PrP^{Sc}) appears to be a pre-requisite for disease progression. Aggregates that accumulate in TSE disease are strongly immunopositive for PrP^{Sc} leading to the suggestion that PrP^{Sc} aggregation may be responsible for neurodegeneration via a ‘gain of function' mechanism. However, in some TSE cases, extensive pathology can exist despite the absence of detectable levels of PrP^{Sc}. The role that PrP^{Sc} has in neuropathogenesis is therefore unclear and an alternate hypothesis suggests that the loss of PrP^{C} function during disease progression could be responsible for neurodegeneration. We hypothesis that PrP^{C} may function as a neuroprotective molecule and believe that mutations in the *PrnP* gene could initiate pathological disease due to impaired functioning of PrP^{C}. The normal biological role of PrP^{C} is still unclear and hence transgenic mice devoid of PrP^{C} ($PrP^{0/0}$) were developed in order to address this point. Knockout PrP mice do show subtle defects in synaptic transmission, mitochondrial function and circadian rhythm and an initial, collaborative, microarray based pilot study of wildtype (WT) versus $PrP^{0/0}$ mice uncovered several intriguing differences between them. Our ongoing work intends to build on this preliminary data and aims to define more specifically the temporal molecular changes in $PrP^{0/0}$ mice and establish whether mutant PrP, with a reduced neuroprotective function, can cause similar changes. Here we present preliminary steps to undertake proteomic analyses of WT and $PrP^{0/0}$ mice taken at different time points using traditional 2DPAGE techniques. Proteins solubilised from mouse hemisphere and cerebellum have been subjected to isoelectric focusing and then separated by SDS PAGE. Proteins have been visualised by silver staining and gel images digitised. Comparative analyses are currently underway and we aim to present our initial data. Using this approach we aim to confirm the microarray data by assessing if the changes seen in mRNA levels are replicated in protein expression changes. Furthermore, we intend to investigate whether similar changes can be seen in transgenic mice expressing mutant PrP molecules. Ultimately we hope to confirm and define a role for PrP^{C} in neuroprotection during ageing.

PA-05

GENE EXPRESSION RELATED TO NEUROPATHOGENESIS IN SHEEP WITH EXPERIMENTAL SCRAPIE

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Transmissible spongiform encephalopathy (TSE) diseases are also known as prion diseases and scrapie is the prion disease of sheep and goats. Prion diseases are recognised by the conversion of a cellular and soluble form of the prion protein (PrP^{C}) into a stable, insoluble form PrP^{Sc}. Expression of the normal form of the protein is necessary for propagation of the disease with pathological changes predominantly located to the brain. Different experimental models, both on the cellular and animal level, have recently given some insight into the neuropathology behind prion diseases, but much more data are needed to describe the damaging effects of PrP^{Sc} in detail. Here we present data from a study on experimental scrapie in sheep, and to our knowledge, this is one of the first studies on gene expression related to the neropathogenesis of scrapie in its original host organism under standardised and controlled conditions.
Two months old lambs were inoculated orally with homogenized scrapie infected brain tissues, the inoculation material carrying the same genotype as the experimental animal. The animals were kept isolated, and housed two or three individuals together, with video surveillance. At certain time points after inoculation, animals were killed and samples for gene expression analysis were collected. Real-time polymerase chain reaction (PCR) analysis on the expression level of selected genes was performed using SYBRgreen I on the LightCycler instrument. Levels of different genes measured at the level of mRNA amount in different parts of the brain were obtained and will be presented. Some of these genes are regulated by astrogliosis, a hallmark for prion diseases, and the cellular stress this pathological process has on astrocytes and neurons. Changes of expression of genes related to overall function of neurons will also be presented. All together, this information contributes to new important information of neuropathological effects of scrapie in sheep.

PA-06

ROLE OF GLYCOSYLPHOSPHATIDYLINOSITOL ANCHORS IN THE NEUROTOXICITY OF PRIONS

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There is increasing interest in the mechanisms by which the formation of PrP^{Sc} leads to neuronal dysfunction and ultimately neuronal death. While the changes in protein structure between PrP^{C} and PrP^{Sc} have been extensively reported, one consequence of the conversion of PrP^{C} to the PrP^{Sc} has been largely ignored; self-aggregation of PrP^{Sc} results in the clustering of glycosylphosphatidylinositol (GPI) anchors in lipid rafts that are enriched in signalling molecules. In this study we demonstrate that the addition of GPI anchors isolated from PrP^{C} or PrP^{Sc}, but not GPI anchors isolated from other proteins, to cultured neurones mimics some of the effects of PrP^{Sc}.
We previously identified phospholipase A_2 as a key enzyme in the process by which prions damage/kill some neurones. Here we report that phospholipase A_2 was activated by GPIs isolated from PrP^{C} / PrP^{Sc}. Immunoprecipitation studies showed that cytoplasmic phospholipase A_2 was associated with PrP^{Sc} in ScGT1 cells, but not with PrP^{C} in GT1 cells and that phospholipase A_2 activity was higher in infected than uninfected cells. The addition of these GPIs to neurones also caused synapse damage (reduced cellular synaptophysin content) and activated caspase-3, a marker of apoptosis. These activities of GPIs were lost following digestion with phosphatidylinositol (PI)-specific phospholipase C, the removal of acyl chains, the cleavage of the PI-glycan linkage, or following incubation with a monoclonal antibody that recognised PI. In competition assays, pre-treatment of neurones with the GPI analogue, glucosamine-PI reduced phospholipase A_2 activation in response to PrP^{Sc} and rendered neurones resistant to the otherwise toxic effects of HuPrP82-146 or PrP^{Sc} preparations. This neuroprotective effect was selective as glucosamine-PI treated neurons remained susceptible to the toxicity of arachidonic acid or platelet activating factor. We propose that PrP^{Sc} causes neuronal damage as a consequence of the clustering of specific GPI anchors at high concentrations within lipid raft membranes enriched with signalling molecules. More specifically aggregated GPIs activate phospholipase A_2 as an early event in prion-induced neurodegeneration.

PA-07

BSE AGENT IN A PRPARR/ARR SHEEP INFECTED BY PERIPHERAL ROUTE: COMPLETE PRPD IHC STUDY AND TRANSMISSION TO OVINE TG MICE

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The possible transmission of bovine spongiform encephalopathy (BSE) agent to ovine species is now considered for several years and demonstrated experimentally. More recently, the demonstration was made that after intracerebral challenge, the BSE agent was able to infect sheep believed as genetically most resistant to classical scrapie ($PrP^{ARR/ARR}$ genotype). Here, we report and describe in detail the disease associated prion protein (PrPd) immunohistochemical analysis of more than 160 pieces of the organs from a $PrP^{ARR/ARR}$ genotype sheep infected with the BSE agent by peripheral route. As PrP^{d} was detected in the brain in absence of any clinical symptoms, transmission studies were performed using a sensitive ovine transgenic mouse model (Tg(OvPrP4)) also acknowledged to be able to distinguish the BSE agent. Here we demonstrate that these PrPd deposits were associated with an infectious power. Besides, the occurrence of florid plaques in the infected transgenic mouse brains provided additional evidence for a link with the BSE agent. Altogether these results suggests interestingly that silent carriers of the BSE agent may exist among ARR homozygous sheep.

PA-08

PRPRES DEPOSITION IN SCJD AND GSS PATIENTS VISUALIZED WITH PARAFFIN EMBEDDED TISSUE BLOT AND IMMUNOHISTOCHEMISTRY

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The transmissible spongiform encephalopathies are characterized by vacuolization, gliosis and deposition of a misfolded and protease-resistant variant (PrP^{RES}) of the prion protein (PrP^{C}) in the central nervous system. Here, immunohistochemistry (IHC) and paraffin embedded tissue blot (PET-blot), performed with the anti-PrP MAbs 3F4 and KG9, were combined to study the deposition of protease resistant PrP^{Sc} in brain sections from patients with sporadic Creutzfeldt-Jakob disease (sCJD) and Gerstmann-Sträussler-Scheinker disease (GSS). Generally, there was a good correlation between the PrP^{RES}-depositions visualized with IHC and PET-blot. However, the PET-blot tended to show an increased sensitivity compared to IHC. A comparison of the appearance and morphology of the PrP^{RES}-depositions in selected regions of the brain in patients with sCJD and GSS is presented here.

PA-09

EVALUATION OF THE NEUROTOXIC PROPERTIES OF STABILIZED PRP82-146 OLIGOMERS

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Recent studies suggest that the neurotoxic effects of amyloidogenic proteins are not caused by mature fibrils but by small, soluble oligomeric assemblies. The characterisation of these species would require their isolation, as well as the evaluation of their stability. The first requirement is partly overcome by testing a mixture of few, possibly well defined, peptide families (as for example, for the quite heterogeneous A□ oligomers called A□ Derived Diffusible Ligands). Very few data are available on the neurotoxic properties of PrP oligomers. To this aim, we have used PrP82-146, an amyloidogenic prion peptide found in the brains of patients with Gerstmann-Sträussler-Scheinker disease (GSS), a familial prion disease. This peptide has been syntetized and used as an *in vitro* tool to investigate the physico-chemical features of PrP amyloid. Photo-induced cross-linking of unmodified protein (PICUP) technique was applied to PrP82-146 solutions to covalently stabilize metastable oligomers. SDS-PAGE analysis showed that, depending on the irradiation time, solutions contained small oligomers only (1-3mers), small and medium oligomers (1-6 mers) or mostly heavy oligomers (> 13mers). Electron microscopy analyses of these preparations showed that different oligomerization profiles are associated to different morphologies containing circular and spherical structures, and aggregates made up by spheres linked together in a rosary-like array.Oligomers preparations were separated by HPLC and their toxicity tested on N2a murine neuroblastoma cells. An inverse correlation between the size of oligomers and neurotoxicity was observed, suggesting that small oligomers are the toxic entities.

PA-10

COULD OESTRUS OVIS ACT AS VECTOR FOR SCRAPIE?

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Animal prion infections, such as scrapie and CWD, have shown a pattern of horizontal transmission in farm conditions. Previous studies on different ecto-parasites affecting domestic and wild ruminants, demonstrated the presence of PrPsc in fly larvae and pupae, speculating on the possible role of such parasites as vectors and/or reservoirs for TSE's. Oestrosis, the nasal myasis of sheep and goats, is caused by the larvae of Oestrus ovis that develop from the first to the third stage larva in nasal cavities and frontal sinuses of the host. To investigate the possible role of Oestrus ovis in the pathogenesis of scrapie infection, a large number of larvae was collected from the nasal cavities of positive and negative scrapie cases to be examined by Western blot. Furthermore, a panel of different organs including CNS, third eyelid and olfactory mucosa at the level of *labyrinthus ethmoidalis* was analysed by immunohistochemistry and Western blot methods. Here we report the presence of PrPsc in the larvae of Oestrus ovis found in three natural scrapie affected sheep coming from two different Italian outbreaks. Interestingly a linkage between positive larvae and positive olfactory mucosa was observed.

PA-11

WATER CHANNEL STUDY (AQUAPORIN 1 AND 4) IN BRAINS OF A MURINE BSE MODEL AND BSE AFFECTED CATTLE

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Aquaporins 1 and 4 belong to a family of membrane water channels involved in the water transport across the barrier between brain and other fluid compartments (blood and CSF). In the brain, AQP1 is described in the choroid plexus epithelial cells and ependimocytes. AQP4 is the most abundant isoform in the nervous tissue and it has been located mainly in astrocytes endfeet membrane. In CJD patiens and in the brains of BSE and Scrapie experimentally infected mice an increase of both isoforms has been described, assessed with western blotting (A. Rodriguez, 2005). These authors suggest a possible relationship between the aquaporin expression increase and the spongiform degeneration found in TSEs.
We have immunohistochemically studied the distribution of AQP 1 (Chemicon AB3065, 1:200) and AQP 4 (Chemicon AB3594, 1:200) in brains of a murine transgenic model of BSE (BoTg110), those mice overexpress the bovine cellular prion protein and have been intracerebraly challenged with a well characterized BSE inoculum (BSE1, TSE/08/59 originated from a pool of BSE cases from VLA-Weybridge, U.K.). To ascertain the reproducibility of the murine model, brains from field cases of BSE cattle diagnosed in Catalonia have been also included in this study.
In this poster we describe the immunostaining of both isoforms in the brains of uninfected animals and compare it with the ones affected of BSE.

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PA-12

COMPARATIVE PROTEOMIC ANALYSIS OF PRNP 0/0 MOUSE BRAIN HOMOGENATES

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The cellular prion protein (PrP^{c}) is a glycoprotein concentrated in neurons of the mammalian brain. Although its function is still elusive, its presence is necessary to generate the conformational pathogenic isoform (PrP^{Sc}) in human and animal transmissible spongiform encephalopathies, or prion diseases. The aim of this study is to examine protein changes in *Prnp*$^{0/0}$ (Zürich I.) transgenic mouse brain homogenates compared to wild-type homogenates determining the role of PrP^{c} in the brain. Two comparative proteomics techniques, fluorescence 2D difference gel electrophoresis (DIGE) as well as isotope-coded protein labelling (ICPL™) were applied. For the 2D gel analysis, twenty 2D-DIGE gels with two overlapping pH ranges (pH 4-7 and 6-11) of male, age matched (68 days), wild-type *vs. Prnp* knockout litter mates (n = 5 per group) were used. No statistical significant differentially expressed proteins ($p<0.05$, 2-fold change) were obtained by quantitative analysis using Decyder™ 6.5. The results were further confirmed by ICPL™ analysis, in which tryptic mixtures of ^{13}C-labelled *Prnp*$^{0/0}$ and ^{12}C labelled wild-type mouse brain samples or *vice versa* were studied by 2D-nanoLC-MS/MS (Thermo Finnigan LTQ). The data analysis using TurboSequest and ASAPRatio (Trans-Proteomic Pipeline software tools, Insilicos) revealed more then 100 identified ICPL-labelled proteins, but none of them were up- or down regulated by at least a two-fold change. These data agree with studies, in which behavioral alterations between *Prnp* knockout mice and their controls were only identified, if animals were subjected to abnormal baseline conditions, such as provoked seizures or acute stress.

PA-13

ACTIVATION OF NOTCH-1 SIGNALING PATHWAYS LINK PRPSC ACCUMULATION WITH DENDRITIC ATROPHY OF NEURONS AND HYPER-REACTIVE ASTROCYTIC GLIOSIS

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Neurodegeneration in prion diseases proceeds in a stereotypical set of steps beginning with accumulation of PrP^{Sc} in synaptic structures. Dendritic atrophy and presynaptic bouton degeneration start 2 wks later. Nerve cell death is a late event occurring at 1 to 3 months. During CNS development, activation of Notch-1 signaling pathways halts growth and development of dendrites and axons whereas other factors, such as □-catenin, stimulate their growth. We found a close association between PrP^{Sc} accumulation in synaptic regions, activation of Notch-1, and dendritic atrophy in CD1 mice inoculated with prions, but no change in □-catenin (Ishikura et al. PNAS 102:886-891, 2005). Evidence of Notch-1 activation included release of its intracellular transcription factor domain (NICD) and translocation of NICD to neuronal nuclei. We tested whether the increased NICD levels lead to increased expression of repressor Hes genes and regressive changes in neurons. We found significantly increased Hes5 protein in neocortical homogenates during prion disease relative to age-matched controls. In neurons, double-immunolabeling confocal microscopy revealed markedly increased levels of Hes1 and Hes5 (Hes1/5) proteins mostly in nuclei. In reactive astrocytes, virtually all Hes1/5 colocalized with GFAP in the cytosol, with little or none in nuclei. Nevertheless, NICD was elevated in both the cytosol and nuclei of reactive astrocytes, which correlated with progressive increase in GFAP mRNA expression. The latter argues that the embryonic Notch-1–GFAP pathway was activated in astrocytes, accounting in part for the hypergliotic state of prion diseases. Thus we propose that dendritic atrophy in prion diseases is caused at least in part by activation of Notch-1–Hes pathways. In contrast, reactive astrocytic gliosis is driven by activation of the Notch-1–GFAP pathway. Of equal importance, in astrocytes, sequestration of Hes proteins by glial filaments prevents the suppressive effect of the activated Notch-1–Hes pathway, which allows reactive astrocytic gliosis to develop. These results provide a molecular mechanism linking PrP^{Sc} formation and accumulation in the brain to the clinically relevant neuropathological changes in prion disease. (NIH support: AG02132, AG10770, and AG02160).

PA-14

PRP CONFORMERS EXPRESSED ON THE CELL SURFACE OF PBMCS ISOLATED FROM ANIMALS NATURALLY INFECTED WITH SCRAPIE DURING PATHOGENESIS OF DISEASE.

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Prior to invasion of the central nervous system, PrPsc is accumulated by follicular dendritic cells and macrophages in lymphoid tissue in the majority of scrapie infected sheep. Since peripheral blood mononuclear cells (PBMCS) ubiquitously express PrP on their cell surface, haematogenic distribution is a potential route by which PrPsc may disseminate through the lymphoreticular system and then ultimately to the CNS. Here, we have employed a panel of anti-PrP antibodies to various epitopes in attempt to distinguish between PBMCs isolated from sheep naturally infected with scrapie and age and genotype matched controls. Variations in staining profiles could imply the appearance of structural modifications of cell surface expressed PrP similar to those anticipated during PrPc to PrPsc conversion in the CNS. Briefly, PBMCs were isolated from blood samples taken from VRQ/VRQ (V/V) sheep in a high-incidence experimental scrapie flock. Cells were probed with PrP antibodies and then analysed using flow cytometry. The results were compared against age and genotype matched controls. Bloods samples were also taken from animals with scrapie and ovine BSE at the terminal stage of disease and analysed in a similar manner. Our results show statistically significant differences in antibody staining profiles between PBMCs isolated from control and scrapie

PA-15

IMMUNOCYTOCHEMISTRY AND IMMUNOLELECTRON MICROSCOPY OF PRP AMYLOID PLAQUES IN HUMAN PRION DISORDERS

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In human prion disorders, few studies have been addressed to immunoelectron microscopic investigations of prion protein-associated amyloid plaques, due to the lack of anti-PrP antibodies able to recognize pathological PrP in glutaraldehyde-fixed specimens.
We studied cerebellar sections from patients deceased from sporadic and familial human prion disorders characterized by amyloid plaques. Selected paraffin blocks, containing PrP amyloid plaques were deparaffinizzated, fixed in glutaraldehyde and embedded in Epon. PrP immunogold staining was obtained on semithin and ultrathin sections etched with potassium methoxide or sodium periodate, and treated with formic acid. By the use of different antibodies we compared the pattern of PrP deposition on paraffin sections with PrP staining in epoxy semithin and ultrathin sections.
Among several monoclonal anti-PrP antibodies we selected SA65 (previously obtained in our laboratory) that stained PrP in etched thin sections treated with formic acid.
In individual case, paraffin and epoxy thin sections stained with SA65 showed a similar pattern of PrP deposition. In immunoelectron microscopy SA65 selectively decorated amyloid fibrils without relevant background of gold particles. The PrP immunogold staining of amyloid plaques showed significant differences between genetic and sporadic human prion disorders.
These data confirm the validity of monoclonal antibody SA65 in PrP staining by the use of post-embedding immunoelectron microscopy methods. We describe the variety of patterns of PrP deposition in amyloid plaques of different human prion disorders. IEM methods complement immunocytochemical and biochemical studies of prion disorders and can help in understanding prion biology

PA-16

THE EFFECT OF AGE AT CHALLENGE ON SUBSEQUENT DEVELOPMENT OF BSE IN SHEEP

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Previous studies involving the oral dosing of sheep of susceptible PrP genotypes with BSE have shown wide variation of incubation period and less that 100% incidence of disease. Although these studies did not show any evidence of an age related effect on incubation, sheep were not challenged at less than six months of age. In very young lambs (one day) gut associated lymphoid tissue (GALT) is much more extensive than in animals of a few months or even weeks. The process of infection may depend on the extent of lymphoid tissue in the gut and so the greater the amount the better the chance of infection occurring. However, other factors may influence the ability of the young lamb to become infected following oral challenge. These include the reflex operation of the oesophageal-groove which allows lambs to ingest milk directly into the abomasums thereby aiding the direct absorption of larger protein molecules and possibly BSE. At this time too the level of proteolytic activity in the digestive tract is low to facilitate the transfer of maternal immunoglobulins into the lamb's circulation. This also might aid the absorption of BSE across the lamb's gut. The purpose of this study is to establish whether age at challenge has an effect on the incubation and disease incidence by dosing day-old lambs, as well as some older age groups. These included day-old lambs, 2-3 weeks (lamb still suckling), 12 weeks (weaning), 26 weeks (young adult) and 52 weeks (adult). The dosing regime also needed to reflect the relative size of animal at the time of dosing and for this reason different doses were used at each stage. These comprised 1.0g, 0.5g and 0.05g per animal, as well as 0.5g of control material (uninfected brain) for each age group. All age groups have been dosed and each have produced clinical cases, apart from the controls. From the results it is clear that the 14-21 day old lamb is most at risk from BSE by the ingestion of disease. All three dosing regimes in this age group have induced a high incidence of disease. There is a considerable number of survivors from the other age groups, which continue to be monitored.

PA-17

EARLY DEATH OF SCRAPIE INFECTED MICE AFTER INDUCTION WITH EAE

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During the years or decades of prion disease incubation period, the at-risk individuals are certain to encounter diverse pathological insults, such as viral and bacterial infections and other inflammatory processes. Whether prion disease incubation time, location and accumulation of PrP^{Sc} or otherwise the pathological profile of prions and other diseases are affected by the co-infection process is unknown. Experimental autoimmune encephalomyelitis (EAE) is an immune-mediated disease of the CNS, used extensively as the animal model of multiple sclerosis (MS), as well as a model for brain inflammation. EAE in mice is induced by immunization with several myelin proteins resulting in an inflammatory response comprising of mononuclear cell infiltrates around venules, leading to demyelination, axonal pathology and gliosis. Damage to the brain and spinal cord is mediated by CD4+ Th1 cells and inflammatory cytokines. In this work, EAE was induced in C57BL/6 mice, that were previously infected with prions (i.c. or i.p.), by immunization with myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide. Animals were observed daily for signs of EAE and scrapie or other neurological dysfunctions. EAE induction resulted in chronic paralysis of the tail and hind limbs. All control i.p. scrapie infected mice died after an incubation time of more than 200 days. Surprisingly, the co-infected animals died much earlier (90-181 days), following clinical signs combining scrapie infection and EAE. Similar results were obtained for i.c. inoculation of prions. Brain histological examination of the co-infected mice showed both immune cell infiltrates, as seen in EAE, and brain vacuoles, as seen in the scrapie controls. Western blot analysis did not show any detectable difference in the level of PrP^{C} or PrP^{Sc} in the brains of the co-infected animals, when compared to animals incubating scrapie alone for the same period of time. Immunocytochemistry experiments to deduce changes in PrP^{Sc} location are in progress. We conclude that co-infection of prions and inflammatory insults can result in an early fatal neurological disease. Whether these results have implications regarding the vCJD epidemic remains to be established.

PA-18

MECHANISM OF PRION PROTEIN RESCUE OF DOPPEL-INDUCED NEURODEGENERATION

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Doppel (Dpl) is a paralog of the prion protein (PrP). Dpl has been identified in all mammals including marsupials, suggesting ancestral gene duplication at least 140 million years ago. The normal functions of Dpl and PrP are not clearly understood, and their endogenous expression differs markedly. Dpl is predominantly expressed in the testis with little or no expression in the central nervous system (CNS), while PrP is predominantly expressed in the brain. Overexpression of Dpl in the brain, either as a consequence of targeted inactivation of the gene for PrP in certain PrP-deficient mouse lines, or by an exogenous transgene, gives rise to non-transmissible neurodegeneration. Coexpression of PrP in these Dpl-expressing lines has been demonstrated to rescue this phenotype. To identify the mechanism of PrP rescue of Dpl-induced neurodegeneration we developed novel *in vivo* and *in vitro* models. We previously showed that increasing Dpl expression in transgenic mice decreased the time to onset of disease. We now demonstrate that recombinant Dpl is neurotoxic to cerebellar granule neurons *in vitro* in a dose-dependent manner. Systematic *in vivo* and *in vitro* studies demonstrated a quantitative dose-dependent relationship for PrP-rescue of Dpl-induced neurotoxicty. This suggests a direct interaction of PrP and Dpl, which we were able to demonstrate *in vitro* by both surface plasmon resonance and immunoassay. While Dpl is expressed at low levels in the CNS, we could not exclude the possibility that microenvironment concentrations may be high, and that Dpl is involved in prion formation. We therefore developed mice in which Dpl expression was eliminated. These mice had identical incubation periods to wild type mice when inoculated with RML prions, conclusively proving that Dpl does not have a role in prion disease.

PA-19

ENFORCED DIMERIZATION OF CELLULAR PRION PROTEIN RESULTS IN PRION DEPOSITION AND VACUOLATION IN CULTURED CELLS

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Prion deposition and spongiosis are two recognized hallmarks of transmissible spongiform encephalopathies (TSEs) in humans and animals. Despite intensive research, these pathological features are still poorly understood. Since prion deposits are composed of aggregated cellular prion protein (PrP^C), an appealing hypothesis is that the first step of their production may consist in the induction of proximity between individual PrP^C molecules. Accordingly, enforcing dimerization or/and oligomerization of PrP^C could result in the formation of such deposits. We engineered fusion proteins between human PrP^C and one or two copies of an FK506 binding domain, Fv1. In the presence of AP20187, a homodimerizer ligand that binds Fv1, proteins containing one or two Fv1 modules are forced to interact and to dimerize or oligomerize, respectively. One or two Fv1 modules were inserted in the unstructured N-terminal domain of PrP^C to generate Fv1- and Fv2-tagged PrP^C, respectively. Addition of AP20187 to neuronal and non-neuronal cell cultures expressing Fv1-PrP or Fv2-PrP resulted in the release and deposition of extracellular prion aggregates resistant to proteinase K. Since similar results were observed in cells expressing Fv1-PrP and Fv2-PrP, we conclude that first, prion dimers spontaneously combine and form high-order aggregates that are released from the plasma membrane; and second, all the information responsible for PrP^C aggregation, release, and deposition is encrypted in the PrP^C molecule. Neuronal cells also undergo vacuolation. These findings strongly argue that dimerization of PrP^C is the initial molecular event in the pathology. Our study provides a basis for elucidating molecular mechanisms responsible for the neuropathogenesis of TSEs.

PA-20

DIFFERENTIAL GENE EXPRESSION STUDIES IN MURINE SPLENIC FOLLICULAR DENDRITIC CELLS DURING PRION INFECTION

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During pre-clinical infection PrP^{Sc} has been shown to accumulate in the secondary lymphoid tissues of infected humans and animals. PrP^{Sc} builds up in germinal centres and is strongly associated with follicular dendritic cells (FDCs). Additionally, studies have shown that mature FDCs are critical for PrP^{Sc} replication in lymphoid tissues, and dedifferentiation of the cells reduces scrapie susceptibility. Analysis of gene expression differences in lymphoid tissues may allow us to gain valuable insight into prion biology and the mechanisms of neuroinvasion. We have identified over 100 genes that are differentially expressed in the spleen during preclinical stages of the disease. We will pinpoint the cellular location of these gene expression changes in the spleen, in particular focusing on gene expression in FDCs during replication of infectious prion protein. Our objective is to use DNA microarrays to obtain gene expression profiles from FDCs, with the aim of identifying host factors involved in prion replication and also identifying preclinical biomarkers for diagnosis. Accordingly, mice were infected with scrapie and at the endpoint of infection the spleens were extracted. The spleens were then homogenized and a population of splenic FDCs were enriched. Total RNA was prepared from infected and non-infected cells and amplified using Eberwine linear amplification methods to generate enough target material for dual-color, competitive hybridization to a DNA microarray. The microarray platforms used in this project are custom made in our laboratory and comprised of over 17K genes of libraries made mostly from mouse CNS genes. The microarray data was analyzed using the latest computational statistical software available in order to identify differentially expressed genes during the course of the disease. Ultimately, the expression data will lead to the identification of specific biomarkers. Preliminary results have shown from studying non-infected mice that an enriched population of FDCs can be isolated from the spleen and their expression profiles are different from that of the whole spleen. We will now move on to studying the infected mice FDC population and compare their diseased expression profiles with what we have found with the non-infected FDCs.

PA-21

INTRANASAL AND AEROSOLIC PRION TRANSMISSION

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Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases of humans and animals. The underlying infectious agent, the prion, was shown to accumulate not only in the central nervous system (CNS) but also in secondary lymphoid organs of patients suffering from sCJD and vCJD. Prions can colonize hosts by a variety of extracerebral routes, including parenteral injection, transdermal administration after skin scarification, and oral administration. In contrast, prions are not generally considered to be transmissible by aerial routes. Here we have investigated the transmissibility potential of prions administered intranasally or by aerosols. Various transgenic mouse models (e.g. NSE-PrP; *tg*a20) expressing the cellular prion protein (PrP^{C}) in specific compartments or cells of the brain or the periphery (e.g. exclusively in the central nervous system) were investigated to identify the cellular and molecular mechanism(s) of prion invasion via the intranasal or aerosolic route. Moreover, we focused on elucidating if prions transmigrate directly into the brain via the olfactory bulb or if cells of the immune system control prion entry via the intranasal or aerosolic route. Results of this study identify prion aerosols or prions administered intranasally as a startlingly efficacious pathway of prion transmission, and call for appropriate revisions of prion-related biosafety guidelines.

PA-22

GRANULOMATOUS INFLAMMATION INDUCES PRION ACCUMULATION AND REPLICATION

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Prions colonize organs of the central nervous and the immune system, both in animals succumbing to prion diseases (e.g. scrapie; chronic wasting disease) and humans suffering from sporadic and variant Creutzfeldt-Jakob disease (sCJD; vCJD). Additionally, PrP^{Sc} and prion infectivity is present in muscle of sCJD patients and of elk and deer suffering from chronic wasting disease. We recently showed that chronic lymphofollicular inflammations alter the tropism of prions, thereby transforming organs previously believed to be devoid of prions (e.g. pancreas, kidney, liver, mammary gland) into sites of prion accumulation. However, lymphofollicular inflammation represents just one specific type of inflammatory morphology. Here, we investigated whether granulomatous inflammation, which is extremely common in ruminants and many other species, induces ectopic prion accumulation and replication. Granulomatous inflammation was induced subcutaneously in wild-type, PrP^{C} overexpressing (*tg*a20) and $Prnp^{o/o}$ mice prior to intraperitoneal administration of prions. Granulomatous nodules contained prominent immunohistochemical as well as molecular hallmarks of granulomatous inflammation similar to those found in humans, such as epitheloid macrophages, giant cells and upregulation of tumor necrosis factor. Surprisingly, levels of PrP^{C} expression in granulomas rose to levels comparable to levels found in spleen. In contrast to spleens and granulomas of peripherally infected $prnp^{-/-}$ mice that were devoid of prion infectivity, wild-type as well as *tg*a20 mice showed PrP^{Sc} and prion infectivity in all spleens and in up to 2/3 of all granulomas investigated. We found that prion accumulation and replication occurred despite the absence of morphologically and immunohistochemically recognizable FDCs. These data indicate that granulomatous inflammation can be an important site of prion replication and accumulation.

PA-23

GERMINAL CENTER B-CELLS ARE DISPENSABLE FOR PERIPHERAL PRION REPLICATION AND NEUROINVASION

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Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases. In most cases TSEs are initiated or else accompanied by replication of the infectious moiety, the prion, in the lymphoreticular system (LRS) of the host. The neuroimmunological synapse in the LRS was demonstrated as the entry point for prions and the relative distance between germinal center associated follicular dendritic cells (FDCs) and peripheral nerves was shown to control neuroinvasion. However, the exact mode of prion migration from the sites of prion replication to peripheral nerve endings as well as the role of other germinal center associated cells in peripheral prion pathogenesis remains elusive. Here, we investigated whether the absence of germinal center B cells (GCBs) influences the efficiency of peripheral prion replication or transport from prion replication sites to hot spots of innervation in the LRS. Mice conditionally depleted for GCBs were challenged intraperitoneally with various dosages of prions (RML). Kinetics of peripheral and central prion replication, distribution of the prion agent in the periphery and the CNS as well as terminal prion disease were analyzed. Interestingly, no differences as far as efficiency of prion replication, distribution and terminal disease were detected. Our results exclude GCBs as candidates influencing peripheral prion replication efficiency or neuroinvasion and point to other cells or paradigms of active or passive transport from the hot spots of prion replication to peripheral nerves.

PA-24

NEITHER BAX KNOCK-OUT NOR BCL-2 OVEREXPRESSION MODIFY BRAIN PATHOLOGY INDUCED BY SCRAPIE PRIONS IN TRANSGENIC MICE

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Although our understanding of prion diseases has greatly progressed over the last decade, the neurotoxic mechanisms triggered by prions in the mammalian brain remain unknown. The cellular prion protein is known to have antagonistic effects on mitochondrial apoptotic pathways by preventing pro-apoptotic BAX activation, thus mimicking the neuroprotective activity of BCL-2, a potent BAX inhibitor. By taking advantage of the strong tropism of the 22L scrapie prion strain for the cerebellum and the well known involvement of BAX and BCL-2 in several cases of apoptosis of cerebellar neurons, we analyzed the development of spongiform encephalopathy induced by the 22L strain of prion in the cerebrum and cerebellum of Bax knock-out mice, as well as in mice expressing a human Bcl-2 transgene (HuBCL-2). After infection with the 22L scrapie strain by either peripheral or intracerebellar routes, clinical illness developed in all cases and similar brain spongiosis profiles were displayed by both mutants and control wild-type mice at subterminal and terminal stages of the disease. In all mice, PrPsc immunohistochemical deposition patterns were similar throughout the brain including the cerebellum. Furthermore, no neuroprotective effects of Bax knock-out or of HuBcl-2 were revealed by immunohistochemical staining for the Purkinje cell marker Calcium binding protein, the synaptic marker synaptophysin or the neuronal marker NeuN in the brain of the mutants. Finally, a similar pattern of astrocytosis was observed throughout the brain of infected mutants and wild-type mice. These results demonstrate that neuronal death induced by the 22L prion strain occurs by a BAX-independent pathway that cannot be antagonized by increasing the expression level of the anti-apoptotic factor BCL-2. Sponsored by GIS Prions.

PA-25

EXPLORING THE METAL-TRIGGERED HYPOTHESIS IN SCRAPIE AND BSE

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Metal imbalances may trigger prion diseases. We measured selected metals in biological samples from healthy and prion-affected sheep and cows to detect possible differences supporting this hypothesis. Mn, Al, Cu, Zn and Se levels were quantified by atomic absorption spectrophotometry (Perkin Elmer 4100 and 5100) on blood/serum and CNS areas from animals from scrapie and BSE foci. Frozen tissues provided by IZS Turin after decontamination with sodium hypochlorite (2% chlorine) followed by autoclaving (134°C for 1h) underwent de-freezing at room temperature for 24 hours followed by dry thermal treatment (80°C for 2 h). After weighing and hydrolysis (48 h in nitric acid 60%, 0.5 ml, Ultrapur), bi-distilled water (4.5 ml) was added and the samples were kept in this solution for further 24 hours. Results were quoted in µg/g dry weight. Blood/serum samples were analysed after 1:10 dilution. We analysed 64 SNC samples from 6 healthy sheep, 41 SNC samples from 5 scrapie-affected sheep, 14 SNC samples from 1 healthy cow, 60 SNC samples from 5 BSE-affected cows (see poster data); Blood (b)/serum (s) samples: 110 ovine (108 healthy=H, 2 scrapie=Sc) and 9 healthy bovine samples. Metal blood-serum levels were as follows (arithmetic mean ± standard deviation; geometric mean): Mn-b (µg/l): H: 11.28 ± 7.04; 9.14 Sc: 11.15 ± 2,47; 11.01. Fe-b (mg/l): H: 386 ± 51; 382 Sc: 360 ± 39; 358. Cu-s (mg/l): H: 0.87 ± 0.32; 0.77 Sc: 1.01 ± 0.04; 1.01.Zn-s (mg/l):H: 1.10 ± 0.33; 1.05 Sc: 1.0 ± 0.47; 1.04. Al-s (µg/l): H: 9.13 ± 8.83; 7.45 Sc: 9.90 ± 2.55; 9.73. The study is ongoing to achieve a greater number of samples and thus provide significant results. To date no evident differences in metal levels are observed.
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PA-26

BSE IN INCUBATING BOVINE: SPREAD OF PATHOLOGICAL PRION PROTEIN VIA THE AUTONOUMOUS NERVOUS SYSTEM TO THE BRAIN WITHIN 24 MONTHS POST INFECTION

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To clarify the infection route and timing of the bovine spongiform encephalopathy (BSE) we performed an experimental oral BSE infection of 56 calves. Every four months 4-5 animals were euthanized and necropsied to collect numerous tissue and bodily fluid samples. Relevant tissues from the viscera, the lymphoreticular and in particular from the central and peripheral nervous system were selected to reveal the presence of disease associated prion protein (PrP^{Sc}) depositions. Highly sensitive immunhistochemistry and immunoblotting techniques were used, as well as rapid testing of the brain stem in selective cases. In the majority of bovines sacrified 12 months post infection and later PrP^{Sc} depositions can be seen in the Peyers patches of the distal ileum. Additionally PrP^{Sc} was already detected in the brainstem of a clinical unsuspicious cow 24 months post challenge, which is 8 months earlier than reported before. In regard to this cow we were able to demonstrate the most likely spread of the BSE prions during the incubation period: from the alimentary tract (distal Peyers patches) via the enteric autonomic ganglia to the corresponding parts of the spinal cord with a subsequent centripetal spread into the brain. While a simultaneous distribution of PrP^{Sc} by using the vagus nerve can not completely be ruled out, evidence for an involvement of the lymphoreticular system was not found. These results are important not only for the understanding of the BSE pathogenesis but also for developing new diagnostic strategies for this infectious disease.

PA-27

SIMIAN VCJD: COURSE OF THE DISEASE

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In the study referenced, we present clinical and laboratory data from a longitudinal study of 18 Cynomolgus monkeys dosed with BSE that ultimately developed simian vCJD. Animals (female, born in 1997, homozygous for methionine at codon 129 Prnp) were dosed with a brain homogenate mixture derived from British BSE cattle. Monkeys were either dosed intracerebrally or orally. The incubation period was defined as the time period from the inoculation time point until the first behavioural changes. The incubation period in monkeys intracerebrally dosed with 5 mg BSE each was 1837 dpi (median). In an ongoing study, the earliest time point of simian vCJD among the orally dosed monkeys was 1540 dpi. The first indication of ataxia occurred 1-4 months after the onset of behavioural changes. However, shorter incubation periods were obtained when the drop in body weight was used. Here, the onset of the disease occurred 60-140 days earlier. Interestingly, the drop in body weight was accompanied by the decline in blood cells expressing a 37kDa-non-integrin laminin receptor precursor. 14-3-3 protein-CSF samples were found during the period of CNS symptoms but not at earlier time points. Diseased monkeys were sacrificed 1 month after the onset of ataxia. At necropsy, the brain was taken and one hemisphere was fixed either in Carnoy's fixative or in buffered paraformaldehyde. Lesion profiles were determined in hematoxylin-eosin stained serial sections. Tissue samples from defined areas (medulla oblongata, pons, nuc. caudatus hypothalamus, thalamus and other areas) were taken from coronary sections of the other hemisphere, Varying amounts of PrP^{res} were found by western immunoblot using a number of different monoclonal antibodies against PrP. In conclusion, in simian vCJD, statistically significant changes occur long before brain damage is detectable. Body weight loss is a biomarker suitable for defining the onset of the disease. The work referenced was performed in partial fulfilment of the study "*BSE in primates*" supported by the EU (QLK1-2002-01096).

PA-28

EARLY EVENTS IN SCRAPIE PATHOGENESIS: THE ROLE OF PHAGOCYTIC CELLS IN SCRAPIE SUSCEPTIBLE AND RESISTANT SHEEP

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Resistance and susceptibility to TSEs in sheep is under genetic control, being clearly associated with specific alleles of the gene encoding PrP^{C}. However, the molecular mechanisms by which amino acid substitutions in the PrP protein result in resistance to infection are still unclear. They could have a direct effect of the stability and conformation of the protein, influencing the ease with which it can be converted to PrP^{Sc}, or produce indirect functional changes through interactions with other molecules. There is evidence that resistance to TSEs in sheep is associated with a failure to replicate in lymphoid tissues. In susceptible scrapie-infected sheep, PrP^{Sc} accumulates on follicular dendritic cells (FDC) in germinal centres of lymphoid tissues. Phagocytic cells, such as myeloid dendritic cells (DC), are thought to play a role in uptake and transport of the scrapie agent from the initial site of infection to FDC in the drainage lymph node, but may also digest and destroy the agent. In resistant sheep, failure to establish infection in lymphoid tissues could result from a) failure of uptake of the scrapie agent by phagocytes, b) more efficient degradation of the agent by phagocytes or c) inability of FDC to support replication. The main aim of this project is to determine whether resistance and susceptibility to scrapie in sheep is determined by the relative efficiency with which phagocytic cells take up and degrade the agent (represented by PrP^{Sc}) during the early stages of infection. Initially, we will isolate three different phagocytic cell types (neutrophils, monocyte-derived DC and monocyte-derived macrophages) the blood of scrapie-resistant and susceptible sheep. We will compare their ability to phagocytose and and degrade PrP^{Sc} *in vitro*. The relevance of *in vitro* findings will be confirmed by lymphatic cannulation of afferent lymphatics draining the site of inoculation in scrapie susceptible and resistant sheep, to identify cell types containing PrP^{Sc}. We will also compare the localisation and persistence of PrP^{Sc} in the drainage lymph nodes of susceptible and resistant sheep, focussing especially on the association with FDC.

PA-29

THE ROLE OF BRAIN SPECIFIC NF-kB ACTIVATION IN PRION DISEASE

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The Nuclear Factor-κB (NF-κB) pathway plays an essential role in immune responses involving induction of inflammation, proliferation and regulation of apoptosis. After scrapie-infection concomitant with first neuronal pathological changes NF-κB activity in the brain was shown to be enhanced *in vivo*. Furthermore the synthetic peptide $PrP^{106-126}$ activates NF-κB in microglial cells *in vitro*. Microglia, involved in the immune response of the brain, most likely contribute in the process of neurodegeneration caused by prions. The IκB kinase (IKK) signalosome, consisting of the IKKα and IKKβ catalytic subunits and the IKKγ (also known as NEMO) regulatory subunit, is essential in the NF-κB pathway and necessary for NF-κB activation through pro-inflammatory signals. An ideal model to study the influence of NF-κB activation on prion disease would be a mouse that lacks IKK dependent signaling. Unfortunately, mice with a non functional IKKβ or IKKγ subunit are not viable.
We investigate prion propagation after intracerebral inoculation (i.c.) in mouse models with a brain specific depletion of IKKβ and IKKγ. This is achieved through the Cre-lox system under the control of the brain specific promoter nestin.
Keywords: prions, IKK signalosome, NF-κB

PA-30

THE ORCHESTRA OF PATHOGENETIC EVENTS IN HUMAN PRION DISEASE: WHO IS THE CONDUCTOR?

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In sporadic Creutzfeldt-Jakob disease (CJD), distribution of affected brain regions is distinct between prion protein (PrP) genotypes at the polymorphic codon 129. Neuropathological studies have revealed different pathways of neuronal damage, like oxidative stress, apoptosis, complement activation, and involvement of the endosomal-lysosomal system. It is not clear which cell death pathways are important and whether neuronal death occurs randomly or in common vulnerable areas. Here we evaluated immunohistochemically the distribution of components related to different stages of cell death (BAX, PARP, C-Jun, Caspase 3 and 9, NfKB, BCL-2) in 30 well defined anatomical area in sporadic CJD subtypes. We performed double immunolabelling of the aforementioned and other components suggested to be important in disease pathogenesis, like the membrane attack complex (MAC) of the complement pathway, nitrotyrosine representing oxidative stress, cathepsin D lysosomal enzyme, and stress related protein Hsp72, using laser scanning confocal microscope. We observed variable distribution of cell-death related proteins within and between CJD subgroups. However in CJD subgroups we noted shared involvement of certain anatomical areas proposing common vulnerable areas. While upregulation and altered cellular distribution of cathepsin D correlates with caspase mediated cell death, MAC is controlled by complement regulatory proteins, and deposits only in severely affected areas. Furthermore, vulnerability of neurons depends on the capability of expressing cellular PrP preserving and cytoprotective proteins like Hsp72. In sum, neither investigated components proved to have an exclusive pathogenetic role in CJD, suggesting orchestral players who act without a conductor, in both con- and dissonance.

PA-31

SPONTANEOUS AGGREGATE FORMATION IN MAMMALIAN CELLS EXPRESSING CHIMERIC YEAST SUP35P/ MOUSE PRION PROTEINS

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A hallmark of prion diseases is the conversion of the cellular prion protein PrP^{C} into misfolded PrP^{Sc}, most probably the etiologic agent. A prion-like phenomenon has also been reported for the yeast, in which the translation termination factor Sup35p can adopt an altered conformation that is transmissible to daughter cells. Striking parallels make the well characterized yeast prions an ideal model to study principal mechanisms of prion formation. To investigate the importance of prion domains for prion aggregate formation fusion proteins of yeast Sup35p and murine PrP were expressed in mammalian cells. In yeast, prion formation of Sup35p or its prion forming domain NM is dependent on the existence of several identified co-factors. In mammalian cells, cytosolic NM was unable to aggregate, suggesting that NM is either unable to spontaneously aggregate in mammalian cells per se or that important co-factors needed for its conformational transition might be missing. However, fusion of NM to amino acid residues 90-230 of PrP (NM-PrP) rendered cytosolic NM capable of spontaneously forming aggregates that differed dramatically in size and frequency from the very small aggregates observed with control cytosolic PrP. Aggregate formation was also observed with N-PrP, arguing that the yeast M domain was dispensable for aggregation. Immunofluorescence analysis revealed that the chimera did not display typical characteristics of aggresomes. In conclusion, fusion of Sup35p prion domains to PrP leads to the spontaneous generation of chimeric aggregates with unique characteristics. Any potential amyloidogenic nature of Sup35p-PrP aggregates will be elucidated in future experiments.

PA-32

PRPSC DEPOSITS IN LUNGS AND MAMMARY GLANDS OF SHEEP EXPERIMENTALLY CO-INFECTED WITH SCRAPIE AND MAEDI-VISNA VIRUS.

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Scrapie, a fatal neurodegenerative disorder of sheep, is characterized by deposition of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system (CNS) and within the lymphoreticular system (LRS). Recent studies in mice transgenically engineered to develop organ specific inflammation demonstrated the co-occurrence of PrP^{Sc} in the inflamed organs (kidney, pancreas and liver). To test the possibility that ectopic PrP^{Sc} replication occurs in sheep affected with scrapie and chronic inflammations, Sarda breed lambs of three different *PrPnp* genotypes (136/154/171: ARQ/ARQ, ARQ/ARR and ARR/ARR, n=10 each) were inoculated with Maedi-Visna virus (MVV) intratracheally and intravenously, and with scrapie brain homogenate by the oral route (n= 10/group). Control lambs (n=10/group) were inoculated with scrapie brain homogenate only, or with mock brain (n=2/group). Nine of 10 ARQ/ARQ sheep inoculated with scrapie and MVV showed neurological signs, starting from 19 months post-inoculation. Eight sheep were diagnosed with scrapie by immunohistochemistry (IHC) and western blotting (WB) of brain and tonsil. Preliminary results demonstrated that 4 of the 8 scrapie affected sheep had PrP^{Sc} deposits in the inflamed mammary gland (lymphofollicular mastitis). Furthermore, 3 of these 8 sheep had a chronic progressive pneumonia and PrP^{Sc} accumulation as detected by IHC and/or WB.

PA-33

DIFFERENTIAL EXPRESSION OF BCL-2 FAMILY GENES IN CENTRAL NERVOUS SYSTEM OF SHEEP WITH NATURAL SCRAPIE.

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Neuronal loss is a salient feature of prion disease; however, its causes and mechanisms are unclear. Some experimental studies show that neuronal death could occur through an apoptotic process. In the present study this process was analysed in the Central Nervous System (CNS) of seven naturally scrapie infected sheep and five breed (Rasa Aragonesa), sex (female), age (3-5 years) and PRNP genotype (ARQ/ARQ) matched controls. In situ end labelling (TUNEL) and immunodetection of the activated form of caspase-3 were used to identify apoptotic cells in different brain regions from scrapie and control sheep. Both methods revealed a reduced number of stained glial cells and a very low amount of positive neurons with apoptotic appearance. These results could be expected as only a few neurons are supposed to die at a given time point in a chronic disease like natural scrapie. What is observable using these techniques may represent a minute proportion of all apoptotic events going on in TSE affected brains. The analysis of early apoptosis related markers could facilitate both, the identification of this process and the determination of the molecular pathways involved. We have investigated the existence of differences in transcript levels of genes involved in the mitochondria pathway (BAX, BCL-2, BCL-X_L, BCL-X_S, BCL-W, BAD, BAK, MCL-1, CYT-C, HSP-27 and APAF-1). When sheep sequences were known, specific primers were designed to amplify cDNA fragments. In most cases, heterologous primers were used to identify sheep genes and, after sequencing, to design specific primers. Gene expression was quantified using Real Time RT-PCR. PCR reactions were performed using the TaqMan and SYBR-Green assays. GAPDH, ACTB and 18S rRNA, three of the most common housekeeping genes, were used as internal controls against which all samples were normalized. Expression results indicate that genes belonging to the mitochondrial pathway are involved in the neuropathology observed in scrapie.

PA-34

SPORADIC CREUTZFELDT-JAKOB DISEASE: PRPRES IS CONSTANTLY PRESENT IN THE RETINA, AND RARELY IN THE OPTIC NERVE

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Creutzfeldt-Jakob disease (CJD) is marked by the presence of the protease-resistant prion protein (PrPres) in the brain. Studies of the retina and optic nerve in patients with CJD are scanty and on very small series of patients. We analysed ocular tissues of sporadic CJD patients (retina of 58 and optic nerve of 51), representing all combinations of PRNP codon 129 polymorphisms and PrPres types by Parchi, except VV1. Ocular tissue from 24 patients with other neurological diseases were used as controls. The ocular tissue was collected at autopsy and the samples were fixed in Carnoy solution or frozen. Before immunohistochemistry with 3F4 antibody, the sections were pretreated with proteinase K and guanidine thiocyanate. In all cases of sCJD the retina showed immunoreactivity for PrPres localized in the inner and outer plexiform layers, with a synaptic type of labelling. No difference in the pattern of labeling was detected between CJD patients with different PRNP codon 129 polymorphisms and PrPres types in the brain. In all cases with frozen retinal tissue available (n = 18), the immunoblot was positive for PrPres . Two out of the 51 sCJD showed the deposition of PrPres also in the optic nerve, corresponding to an immunostaining delineating stellate cells and associated with the presence of numerous CD68- and CD45-positive cells. Our results demonstrate the presence of the pathological form of prion protein not only in the retina of all sCJD cases analysed, but also in optic nerve in a small subset of sCJD patients, a finding previously described only in variant CJD and in experimental animal models. Moreover, our data suggest a correlation between the deposition of PrPres and inflammatory changes in the optic nerve in sCJD.

PA-35

NATURAL AND ORAL EXPERIMENTAL SCRAPIE INFECTION IN SARDA BREED SHEEP: A COMPARATIVE PATHOGENETIC STUDY ON ILEAL ENTERIC NERVOUS SYSTEM PLEXUSES

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The enteric nervous system (ENS) plays a key role in the early pathogenesis of sheep scrapie, but no information exists on the ENS cytotypes which are progressively involved during infection, nor on their morpho-functional changes in comparison with those affecting central nervous system neurons.

We investigated the ileal myenteric plexuses (MPs) and submucosal plexuses (SMPs) of 49 Sarda breed sheep carrying different PrP genotypes (ARQ/ARQ, ARQ/AHQ, ARQ/ARR, ARR/ARR), 33 of which had been orally dosed with scrapie at either 8 (28 animals) or 1.5 months of age (5 animals) and subsequently euthanized at definite time intervals post-infection (p.i.), while 16 undosed sheep served as normal healthy control animals. The ileal MPs and SMPs from 7 additional ARQ/ARQ Sarda breed sheep clinically affected with natural scrapie were also investigated.

PrP^{Sc} immunoreactivity (IR), along with neuronal protein Hu C/D, neuronal nitric oxide synthase, calbindin and glial fibrillary acidic protein IR, were evaluated by immunohistochemistry (IHC) and indirect immunofluorescence (IF) on paraffin sections and suitable *wholemount* preparations.

All 7 natural scrapie-affected sheep showed IHC and IF evidence of PrP^{Sc} in both their brain tissue (*obex* region) and ENS plexuses. The same was true, in experimentally infected animals, for 8 clinically healthy ARQ/ARQ sheep euthanized between 12 and 24 months p.i. and for additional 5 ARQ/ARQ (euthanized at 24 months p.i.) and 3 ARQ/AHQ (euthanized at 35, 36.3 and 39.5 months p.i., respectively) clinically affected sheep.

PrP^{Sc} deposition, mainly involving MPs in experimentally infected animals and SMPs in naturally affected sheep, was compatible with an involvement of enteroglial cells (EGCs) and, in natural scrapie-affected animals, with a simultaneous involvement of neuronal cells, the phenotype of which is currently being characterised.

On the basis of the above results, EGCs and neurons of ileal ENS plexuses are likely involved in the pathogenesis of natural and oral experimental scrapie infection in Sarda breed sheep.

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PA-36

CAN PRPSC BE DETECTED IN BLOOD - A WESTERN BLOT APPROACH?

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We have shown that TSE agents can be transmitted via blood using sheep models and in humans vCJD cases have been reported that were probably the result of infected blood transfusions. However, it is not known which blood components carry infection and using various approaches, PrP^{Sc} has not yet been conclusively detected in TSE-infected blood. It may be that PrP^{Sc} levels in TSE infected blood are low and the sensitivity of current detection methods insufficient. We have developed a sensitive immunoassay in an attempt to detect PrP^{Sc} in blood. NaPTA precipitation and a panel of novel monoclonal antibodies have been used to increase sensitivity. The method has been optimised using natural scrapie and experimental murine brain homogenates. To establish sensitivity limits for comparison with cellular components of blood SMB (scrapie-infected mouse brain) cells have also been tested. Assay sensitivity has been confirmed using scrapie-infected brain and SMB cell spikes. The methodology has been applied to buffy coat blood fractions from clinical scrapie-infected sheep. It was found that the overall sensitivity of the assay using novel antibodies combined with NaPTA concentration increased approximately 10-fold. The monoclonal antibodies tested have a broad specificity and two can detect PrP^{Sc} in the equivalent of 2 µg wet brain tissue and in approximately 6×10^4 SMB cells. This level of sensitivity was confirmed using SMB cells spiked into peripheral blood mononuclear cells (PBMC). We are currently applying the method to clinical buffy coat and plasma fractions from scrapie infected sheep. Although preliminary our results highlight the need for further assay development to achieve the required sensitivity for PrP^{Sc} detection in blood. By identifying the blood fractions that carry PrP^{Sc} we ultimately aim to improve our understanding of TSE transmission and pathogenesis. This work may also aid in the development of safer methods of blood transfusion in humans and also blood diagnostic tests.

PA-37

ACCUMULATION OF GAGS IN LISOSOMES DELAYS PRPSC DEGRADATION AND PROLONGS PRION DISEASE INCUBATION TIME

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Glycosaminoglycans (GAGs) and in particular, heparan sulfate (HS), have been shown to be associated both with prion disease pathology as well as with the metabolism of the prion proteins, and also to modulate prion infectivity. While addition of GAGs to scrapie infected cells increased PrP^{Sc} accumulation, degradation of these sulfated sugars reduced the content of PrP^{Sc} in cells.
Interestingly, reagents such as Tilorone and Quinacrine, which are known to cause chemical mucopolysaccharidosis (MPS) by inducing accumulation of GAGs in lysosomes, reduce the accumulation of PrP^{Sc} in ScN2a cells. However, Quinacrine had no effect on prion disease incubation time when administered to animals after prion infection. In this work we investigated whether pathological accumulation of GAGs can induce de-novo conversion of PrP^{C} to PrP^{Sc}, and prion infectivity. We also studied the effect of intracellular GAGs on the accumulation of PrP^{Sc} in cells.
We show here that PrP^{Sc} and prion infectivity were both absent from the brains of transgenic mice ablated for GAGs degrading enzymes, thereby suffering from MPS disease and accumulate GAGs in their lisosomes. However, addition of PrP^{Sc} to cells cultured in the presence of Tilorone resulted in sequestration of the prion protein in cells for a long period of time. Interestingly, when Tilorone was administrated to mice for weeks before infection with prions, incubation time for scrapie in these aninals was prolonged significantly. We propose that sequestration of PrP^{Sc}-GAGs complexes in the lysosomes, while resulting in reduced degradation of PrP^{Sc}, devoids the prion protein from its ability to convert new PrP^{C} molecules and subsequently infect cells, thereby resulting in prolonged incubation time for the disease and reduced PrP^{Sc} accumulation in ScN2a cells.

PA-38

TRANSMISSION OF BSE INFECTION, IN SHEEP, VIA BLOOD TRANSFUSION

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The possibility that vCJD may be transmitted by blood transfusion is an important public health issue. The involvement of lymphoreticular tissues in the peripheral pathogenesis of vCJD raises concerns that infectivity may enter the bloodstream in association with recirculating lymphocytes. We have shown that sheep infected with BSE by the oral route provide a suitable model to assess the potential of transfusion of blood components to transmit vCJD in man, as the distribution of PrP^{Sc} and/or infectivity in lymphoid tissues of sheep orally challenged with BSE closely resembles that of vCJD patients. We have previously demonstrated that both BSE and natural scrapie infection can be transmitted via transfusion of whole blood and buffy coat, taken from both pre-clinical and clinical donors. To date, 3 out of 5 recipients of BSE-infected blood, taken from donors at a clinical time-point, have developed TSE disease (approximate transmission rate of 60%). The equivalent transmission rate for recipients of BSE–infected blood taken from pre-clinical animals is lower. The transmission rate for natural scrapie is approximately 40-45%. We are currently setting up a new project to investigate the distribution of BSE infectivity in whole blood and separated components (plasma, platelets and erythrocytes), and the effectiveness of human leucodepletion filters in removing infectivity. The methods used for collection of blood and separation and filtration of components will follow as closely as possible those routinely employed for human blood by transfusion services, and have initially been developed and tested on uninfected sheep blood in the laboratories of SNBTS. A secondary aim of the project is to develop a bioassay for measurement of titres of infectivity in blood components, using transgenic mouse lines that over-express ovine PrP. The aim of these experiments is to determine qualitative and quantitative data on the changes in infectivity in blood and its clinically relevant components with time, as well as assessing the effect of leucodepletion of such products and the potential for secondary transmission by blood transfusion.

PA-39

NEURAL STEM CELL MODEL FOR PRION PROPAGATION.

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Study of prion transmission and targeting is a major scientific issue with important consequences for public health. Only few cell culture systems able to convert the prion protein, PrPC, into its pathologic isoform, PrPSc, have been described. We hypothesized that CNS neural stem cells (NSCs) could be the basis of a new cell culture model permissive to prion infection. CNS neural stem cells are the self-renewing, multipotent cells that generate neurons, astrocytes, and oligodendrocytes in the nervous system. Here, we report that monolayer cultures of differentiated fetal NSCs or adult multipotent progenitor cells isolated from mice were able to propagate prions. Moreover, orientation of cell differentiation experiments allowed us to demonstrate the large influence of neural cell fate on the production of PrPSc allowing the molecular study of prion neuronal targeting in relation with strain differences. This new stem cell based model, which is applicable to different species and to transgenic mice, will allow rapid and thoughtful investigations of the molecular basis of prion diseases and will open new avenues for diagnostic and therapeutic research.

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PA-40

EXPRESSION OF THE CELLULAR PRION PROTEIN IN THE RAT BRAIN AND CHARACTERIZATION OF ANIMAL MODELS OF FATAL FAMILIAL INSOMNIA

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The cellular prion protein (PrP^{C}) is a membrane-bound glycoprotein mainly present in the central nervous system of mammals. Prions (PrP^{Sc}) are conformationally-altered isoforms of PrP^{C} that are responsible for transmissible spongiform encephalopathies (TSE), a group of neurodegenerative diseases affecting both humans and animals. As the presence of PrP^{C} is necessary for the establishment and further evolution of these pathologies, we have mapped its regional distribution in the rat brain and studied the chemical nature of these neurons. We have also generated a line of transgenic mice (Tg) that mimics some of the most relevant biochemical features of fatal familial insomnia (FFI), a human TSE where the damage is primarily confined to the thalamus. Additionally, we have injected neuronal tracers into the rat thalamus in order to explain the pathogenic mechanism leading to FFI. The ubiquitous distribution of PrP^{C} throughout the rat brain, especially in areas that send projections to the thalamus, together with its neurochemical partners and the connectivity of the rat thalamus, suggests that there is a retrograde transport of prions in FFI. The characterization of Tg(FFI) validates it as a good model for FFI and reveals that our observations in rat can be further extrapolated to human subjects. With the combination of all these approaches, and based on this retrograde transport, we are now able to successfully explain some characteristic pathogenic features observed in FFI patients and to explain the selective vulnerability of particular subsets of cells in animal and human TSE.

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PA-41

PATHOGENESIS OF SSBP/1 SCRAPIE IN SHEEP OF SUSCEPTIBLE AND RESISTANT PRP GENOTYPES

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SSBP/1 is a source of experimental scrapie that causes clinical disease following subcutaneous inoculation in sheep of PrP genotypes encoding at least one VRQ allele. VRQ/VRQ animals have shorter incubation periods than the heterozygotes VRQ/ARR by around 100 days whereas animals of ARR/ARR genotype resist the infection and do not develop clinical disease. In this project a time course study was carried out for the first time to follow spread of SSBP/1 infection in the peripheral lymphoreticular system and at different sites in the brain of the sheep using PrP^{SC} as a marker, detected by immunohistochemistry using two different anti-PrP antibodies BG4 (N terminal epitope) and R145 (C terminal epitope). The time course starts at 10 days after infection and continues to terminal stages in the susceptible sheep. Differences were found in the patterns of disease-related PrP deposition between VRQ/VRQ and VRQ/ARR sheep and between the patterns detected by the two antibodies. The resistant ARR/ARR sheep were also been examined to detect any low levels of PrP^{SC} which would be indicative of subclinical infection . The results will be presented and the implications for diagnostic tests discussed.

PA-42

DEPOSITION OF PRP AMYLOID IN THE ABSENCE OF TRANSMISSIBLE DISEASE.

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The Prion hypothesis predicts that the aetiological agent of the Transmissible Spongiform Encephalopathy (TSE) diseases is an abnormally folded isoform of a host glycoprotein, PrP. This abnormal isoform (PrPSc) is partially proteinase K (PK) resistant, is deposited in infected tissue, and co-purifies with infectivity. Definitive diagnosis of TSE disease and identification of infectivity can only be obtained by transmission studies to mice or other mammals. However these experiments are expensive and time consuming. Current diagnostic methods are therefore based on the detection of the abnormally folded PK-resistant form of PrPSc in post mortem brain tissue, and its identification is taken as indicative of the presence of TSE infectivity. The relationship between PrPSc and TSE infectivity is however still unclear. If PrPSc is not associated with infectivity, its reliability as a diagnostic marker in all cases of disease may be questionable. We have produced transgenic mouse models of TSE disease in which the presence of misfolded forms of PrP and TSE infectivity do not correlate. In gene targeted transgenic mice inoculated with human Gerstmann-Sträussler-Scheinker P102L (GSS P102L) disease (diffuse and amyloid PrP deposition, and spongiform degeneration) or hamster 263K, low or undetectable levels of proteinase K resistant PrP are found in association with high levels of TSE infectivity. Conversely, 101LL transgenic mice inoculated with an atypical isolate of GSS P102L (diffuse and amyloid PrP and no spongiform degeneration) do not develop clinical TSE disease or TSE associated spongiform degeneration during their lifespan. They do however show PrP amyloid accumulation in the sub-callosal region of the brain. Disease observed in 101LL mice with low levels of PrPSc was transmissible to both 101LL and wild type mice, but mice showing only PrP amyloid deposition failed to transmit disease to either 101LL or wild type mice. However, several of the 101LL mice which received the amyloid inoculum were found to contain the same PrP amyloid deposition in the sub-callosal region of the brain. These data suggest that not all abnormal isoforms of PrP are infectious, and that PrP amyloid in particular does not cause a transmissible disease. The reliance on identification of PrPSc in TSE disease diagnosis may therefore be questionable.

PA-43

THE ROLE OF OXIDATIVE STRESS IN PRION PROVOKED NEUROTOXICTY

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There is a wealth of evidence implicating oxidative stress in the pathology of many neurodegenerative diseases. In prion diseases the accumulation of PrP^{sc} correlates with disease progression and coincides with the appearance of markers of oxidative stress in the brains of infected mice. This suggests that the disease-associated neurodegeneration may in part be due to oxidative damage. To investigate the role of oxidative stress in prion provoked neurotoxicity the influence of a dietary supplement on oxidative stress and disease progression was determined. Brains and body fluids were collected at various time points throughout the course of the disease and markers of anti-oxidant capacity, DNA damage and lipid peroxidation were measured. Scrapie infected mice displayed reduced total glutathione levels as compared to control mice. The appearance of decreased total glutathione levels relative to control was delayed in infected mice fed the anti-oxidant diet. Later stages of the disease were marked by an increase in 8-OHdG levels. The anti-oxidant diet caused a reduction in 8-OHdG levels, but they remained elevated relative to that observed in age-matched controls. Infection did not alter 4-HNE levels. The data demonstrates that oxidative stress is associated with prion disease well before clinical signs are apparent. Though the anti-oxidant diet may have ameliorated adverse effects resulting from the disease-associated oxidative stress the onset of terminal stage disease was not delayed.

PA-44

ANTIGEN PRESENTING CELLS FAIL TO MATURE AFTER IN VITRO EXPOSURE TO THE SCRAPIE AGENT BUT STOP SCRAPIE AGENT DEGRADATION AFTER TLR LIGAND-INDUCED ACTIVATION

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Following oral uptake of the scrapie agent (PrP^{Sc}) in mice, neuroinvasion occurs after a first phase of prion accumulation and replication in lymphoid organs such as the draining lymph nodes and Peyer's Patches. How the agent reaches respective sites of accumulation and if cellular and/or acellular components are involved in this process is under intense investigation. Since antigen presenting cells (APCs) are the sentinels of the immune system and migrate from peripheral sites to the draining lymph nodes (and therefore might carry infectivity as their cargo), the aim of our study was to analyze in vitro the role of APCs in PrP^{Sc} (RML strain) degradation and how degradation is affected by co-stimulation of APCs with several danger signals. We also investigated whether PrP^{Sc} itself represents a danger signal to bone-marrow derived dendritic cells (BMDCs) leading to an altered activation state that could ultimately contribute to the induction of tolerance to the prion protein. We found that both macrophages and BMDCs degrade PrP^{Sc} but stop degradation after Toll-like receptor (TLR) ligand-induced activation. Using several activation markers ($I\text{-}A^b$, $H\text{-}1K^b$, CD86, CD80) we could neither show any phenotypic change of BMDCs after exposure to PrP^{Sc}, nor altered activation by co-stimulation with TLR ligands indicating that BMDCs do not perceive the scrapie agent as a danger signal – or become attenuated by PrP^{Sc}. However, our data on reduced degradation of PrP^{Sc} after TLR ligand-induced activation indicate that inflammatory processes and/or co-infections during scrapie pathogenesis might enhance prion pathogenesis by impeding PrP^{Sc} breakdown by APCs.

PA-45

ADENOSINE A1 RECEPTOR EXPRESSION AND ACTIVITY IS INCREASED IN THE CEREBRAL CORTEX IN CREUTZFELDT- JAKOB DISEASE AND IN BOVINE SPONGIFORM ENCEPHALOPATHY-INFECTED BOVINE-PRP MICE

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Creutzfeldt-Jakob's disease (CJD) is the most prevalent human TSE, whereas scrapie and bovine spongiform encephalopathy (BSE) are the most common animal prion diseases. Adenosine function is mediated by adenosine receptors (ADRs), which are categorized into four types: A1, A2A, A2B and A3. A1Rs are G-protein-coupled receptors which induce the inhibition of adenylyl cyclase (AC) activity. In the present work, we have examined, by western blotting, the expression levels of A1Rs in the frontal cortex of 12 patients with CJD and 6 age-matched controls, and in bovine spongiform encephalopathy-infected bovine-PrP transgenic mice (BoPrP-Tg110 mice) at different post-incubation times to address modifications in A1Rs with disease progression. A significant increase in the expression levels of A1Rs was found, by immunohistochemistry and later by Western blotting, in the cerebral cortex in CJD, and in the murine BSE model at advanced stages of the disease and coincidental with the appearance of PrPres expression. In addition, the activity of A1Rs was analyzed by in vitro assays with isolated membranes of the frontal cortex in CJD. Increased activity of the receptor, as revealed by the decreased forskolin-stimulated cAMP production in response to the A1R agonists cyclohexyladenosine (CHA) and cyclopentyladenosine (CPA), was observed in CJD cases when compared with controls. These findings contrast with reduced signaling of group I metabotropic glutamate receptors in prion disaeses. Finally, mRNA A1R levels were similar in CJD and control cases, thus suggesting abnormal A1R turnover or disregulation of raft-associate signaling pathways in CJD. These results show, for the first time, sensitization of A1Rs in prion diseases.

PA-46

MICRO-RNA (MIRNA) EXPRESSION IN THE BRAIN DURING PRION INFECTION

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MicroRNAs (miRNA) are genome encoded RNA molecules approximately 22 nucleotides in length that recognize target mRNA(s) through base-pairing and thereby regulate their expression. MiRNAs have gained recent prominence as imporatant gene-regulatory molecules in the eukaryotic genome. Concurrently, a role for miRNAs in proliferative diseases has also been widely speculated upon as a result of abnormal expression. In this study, we investigated the expression of miRNAs during prion induced neurodegeneration in order to gain further insight into prion pathobiology. Employing a combination of both microarray technology and multiplex real-time PCR, we profiled the expression of miRNAs in whole brains of VM mice treated with mouse-adapted scrapie strain 22A. We identified miRNAs that showed marked differences in their expression when prion treated mice were compared to age-matched control mice. We determined the lineage specificity of some of these differentially expressed miRNAs and subsequently identified their potential mRNA targets. MiRNAs represent a new layer of regulatory mechanism for gene expression. Through binding of a minimal recognition sequence, miRNAs repress the expression of nearly a third of the metazoan protein-coding mRNAs. They are involved in a wide range of basic processes such as cell proliferation, development, apoptosis and stress response. The elucidation of how global miRNA expression responds to disease processes is imperative to gain further understanding of the disease so that novel molecular prognostic and diagnostic markers of the disease may be identified. Additionally, abnormally expressed miRNAs may themselves serve as potential areas of therapeutic intervention either through knockout or over-expression strategies. In this study, we showed evidence for the de-regulation of miRNAs during prion induced neurodegeneration and we proposed hypotheses for biological effects caused by changes in miRNA abundance.

PA-47

PRION CLEARANCE AND PLASMA LIPOPROTEINS

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Prions are composed solely of an alternatively folded isoform of the prion protein (PrP), designated PrP^{Sc}. The half-life ($t_{1/2}$) of PrP^{Sc} in the mouse brain is ~1.5 days and both protease-sensitive (s) and -resistant (r) conformers are cleared at the same rate. That the brain is capable of clearing prions so efficiently raises the possibility that PrP^{Sc} is normally made at low levels and continually cleared; thus, PrP^{Sc} may have a function in cellular metabolism; furthermore, this clearance mechanism improves prospects for effective therapy. Because such rapid clearance implies a powerful transport mechanism, we aimed to identify the proteins in plasma that bind and may carry prions from the brain. The similarities between PrP^{Sc} and plasma lipoproteins with respect to hydrophobicity and formation of polyoxometalate phosphotungstic acid (PTA) complexes led us to investigate whether these molecules bind to each other in human plasma. We found that prions from the brains of patients with sporadic Creutzfeldt-Jakob disease (sCJD) bind to very low-density and low-density lipoproteins (VLDL and LDL, respectively) but not to high-density lipoproteins (HDL) or other plasma components, as demonstrated by affinity assay and electron microscopy. Immunoassays showed that apolipoprotein B (apoB), which is the principal protein component of VLDL and LDL, also bound native PrP^{Sc} through a highly cooperative process; the apparent binding constants ranged from 28 to 212 pM. Furthermore, $HuPrP^{Sc}$ was detected in VLDL and LDL fractions, but not in the HDL or immunoglobulin fractions, of plasma collected from sCJD patients. These findings suggest that binding of human prions to plasma VLDL and LDL is an important factor in prion propagation. Whether detection of PrP^{Sc} in VLDL and LDL particles can be adapted into an antemortem diagnostic test for prions in the blood remains to be determined

PA-48

MECHANISMS OF NEURODEGENERATION IN TRANSMEMBRANE AND CYTOPLASMIC PRP

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Both effects in cis and in *trans* have been identified to influence the biogenesis of PrP leading to changes in folding and localization. One such example is an increase in transmembrane PrP (^{Ctm}PrP) seen in genetic and infectious prion diseases, the former by a mutation in *cis*, the latter by an effect of PrP^{Sc} in *trans.* Another example is the occurrence of cytoplasmic PrP (CyPrP) leading to disease, which may be a result of leaky ribosome scanning or retrograde transport from the endoplasmic reticulum. In this study we compared the cell biological impact of ^{Ctm}PrP and CyPrP expression in CHO cells. The mode of toxicity and the pathway of cell death in both cases was analyzed. Additionally we established an assay to investigate the degree of misfolding of each form by determining the proteasomal degradation profile and comparing it to the degradation profile of ^{Sec}PrP, the normal secreted form of PrP^{C} expressed predominantly by wild-type PrP. Our data leads to the conclusion that despite that the fact that both ^{Ctm}PrP and CyPrP trigger cell death by a common pathway, the proteasomal degradation profile is highly dissimilar. We interpret these findings to suggest that ^{Ctm}PrP is recognized by the quality control machinery as a bonafide physiological molecule; whereas CyPrP is recognized as a misfolded molecule that is subject to proteasomal degradation. This data supports our recently proposed hypothesis that ^{Ctm}PrP may be a physiologic form of PrP^{C} engaged to consummate an apoptotic program. Disease results only when ^{Ctm}PrP expression is deregulated, as we have demonstrated elsewhere.

PA-49

ANALYSIS OF SYNAPTIC DYSFUNCTION IN A TRANSGENIC MOUSE MODEL OF INHERITED PRION DISEASE

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Tg(PG14) mice express a mouse PrP homologue of a nine-octapeptide insertion associated with an inherited prion disease. These mice accumulate in their brains a PrP^{Sc}-like form of the mutant protein that is weakly protease-resistant and aggregated into small oligomers. As this form accumulates, Tg(PG14) mice develop a fatal neurological disorder characterized by dramatic atrophy of the cerebellum due to loss of synaptic endings in the molecular layer and massive apoptosis of granule neurons. Deletion of the proapoptotic gene *Bax* efficiently rescues cerebellar granule neurons, but does not prevent synaptic loss in the molecular layer and development of the clinical symptoms. Moreover, the activity of the calcium/calmodulin-dependent phosphatase calcineurin, which plays a pivotal role in regulating synaptic activity, is strikingly reduced in the cerebella of presymptomatic Tg(PG14) mice. These observations suggest that PG14 PrP causes neurological disease by disrupting the normal neuronal connectivity or function in the cerebellum. To characterize the synaptic dysfunction in the cerebellum of Tg(PG14) mice, we are carrying out morphological, functional and biochemical investigations in purified synaptosomal fractions. Specific methods have been setup to measure calcium-dependent neurotransmitters release, and investigate the functionality of the phosphorylation-dependent mechanisms governing the exo-endocytotic cycle of synaptic vesicles.

PA-50

IDENTIFICATION OF GENE EXPRESSION CHANGES IN NATURALLY SCRAPIE INFECTED SHEEP SNC USING A SHEEP CDNA MICROARRAY

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The pathogenesis of Scrapie and many neurodegenerative diseases is poorly understood. The identification of genes with differential expression in CNS of infected animals might provide clues to clarify the molecular mechanism that leads to neuronal loss, being useful for future therapies and to identify molecular biomarkers that might be the basis for new diagnostic tests. We present here an initial study on the transcriptional differences of cerebellum obtained from naturally infected Scrapie sheep using cDNA microarray hybridizations. We have used the sheep cDNA microarray generated in the CIDC of Lelystad (see the communication of Bossers et al. for details). Total RNA of cerebellum was isolated from 5 control sheep and 9 infected sheep. According to IHC characteristics on cerebellum (CER), spleen (SP) and mesentery lymph node (MN) of infected sheep their RNAs were grouped into 4 pools (1: +CER, +SP, +MN; 2: -CER, -SP, -MN; 3: +CER, -/+SP, -MN; 4: -CER, +SP, +MN). The remaining (5) pool was formed with the 5 controls. The five RNA pools were hybridized against a universal reference RNA, after cDNA synthesis and fluorescent labeling. We compared "*in silico*" gene expression of the 4 positive groups against the control group. One common clone was identified to be up-regulated within the 4 diseased groups and two clones identified as down-regulated. In the two groups with PrP deposit on cerebellum (1 and 2) we found 2 up-expressed clones and 4 down-expressed clones in common. The other two groups (2 and 4) shared 6 clones with significative expression increase and 3 clones with decreased expression. We also obtained other clones with significative differential expression compared with controls. To confirm these results we are analysing the expression of selected clones by Real Time PCR.

PA-51

AUTOPHAGY IS A COMMON ULTRASTRUCTURAL FEATURE OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

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Autophagy is a process by which subcellular constituents and organelles are targeted for degradation in lysosomes. In macroautophagy, proteins and organelles are sequestrated into a double membrane bound vacuole called autophagosome, formed by ER membranes, under the direction of various proteins including MAP-LC3 - a microtubule associated protein – light chain 3, and several Apg proteins. In addition to maintaining cellular homeostasis, autophagy may also contribute to cell damage. It is involved in autophagic programmed cell death, called programmed cell death type II. The role of autophagy in neurodegeneration is not only in removing protein aggregates but also in inducing the death of neurons. To date, electron microscopy has been the best reliable method for monitoring autophagy. The presence of autophagic vacuoles in experimentally induced scrapie, Creutzfeldt-Jakob disease and Gerstmann-Sträussler-Scheinker syndrome as well as in human prion diseases has been described. We studied the ultrastructural signs of autophagy in prion diseases: in brain biopsies from Creutzfeldt-Jakob disease and fatal familial insomnia patients and in experimental scrapie in hamsters. Ultrastructurallly, several steps of autophagy can be observed. Initially, a part of the neuronal cytoplasm is sequestrated by concentric arrays of double membranes; the enclosed cytoplasm appears relatively normal except that its density is often increased. Next, electron density of the central area dramatically increases. The membranes then proliferate within the cytoplasm in a labyrinth-like manner and the area sequestrated by these membranes enlarges into a more complex structure consisting of vacuoles, electron-dense areas and areas of normally-looking cytoplasm connected by convoluted membranes. Of note, autophagic vacuoles formed not only in neuronal perikarya but also in neurites and synapses. Finally, a large area of the cytoplasm is transformed into a collection of autophagic vacuoles of different sizes. We have also observed many multivesicular bodies which are involved in the process of microautophagy. Although the role of autophagy in prion diseases remains unknown, at least three hypothesises must be taken into consideration; 1) autophagy plays a role in removing protein aggregates 2) autophagy in one of the ways of neuron death in prion diseases and 3) it may participate in a formation of spongiform change.

PA-52

THE TOPOGRAPHICAL DISTRIBUTION OF PRION PROTEIN IN THE BRAINS OF SHEEP CHALLENGES THE CURRENT HYPOTHESIS OF NEUROINVASION

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Clinical signs in sheep scrapie usually appear after widespread accumulation of the disease-associated isoform of the prion protein (PrPd) in the brain. It is generally believed that, after oral exposure -often followed by lymphoid tissue replication-, neuroinvasion occurs via the autonomic nervous system and, as a result, the dorsal nucleus of the vagus nerve (DMNV) is the initial point of PrPd accumulation in the brain. Nevertheless, the topographical and temporal spread of PrPd to other brain areas during the preclinical period in relation to the route of exposure and other factors is not properly documented.

We examined by immunohistochemistry the brains of 35 sheep either exposed to natural infection (Shetland and Suffolk sheep from two flocks) or to experimental challenge; these last included scrapie by the oral, subcutaneous and intravenous routes in Cheviot and Suffolk sheep, and BSE in Suffolk, Cheviot and Romney sheep by the oral or intracerebral routes. All animals were studied at preclinical stage of scrapie either because they died from intercurrent conditions, were part of a sequential killing strategy or were culled after confirmation of infection by biopsy.

We found that initial PrPd accumulation occurred in most cases in the DMNV and in the hypothalamus, regardless of the breed of sheep, PrP genotype, TSE source and, more surprisingly, route of infection. Moreover, the topographical distribution and magnitude of PrPd deposition, with consistent involvement of the periventricular organs, suggest that the pathways of entry of the TSE agents in the brain might be different from those arising from the autonomic nervous system. Also, the pattern of PrPd spread within the brain seemed to be TSE strain-dependent.

These findings, and the apparently route-related susceptibility to TSE infection in sheep of some genotypes, argue for a review of the current hypothesis of TSE neuroinvasion route.

PA-53

PRPSC AND NICASTRIN DETERMINE THE RATE OF NOTCH-1 INTRACELLULAR DOMAIN (NICD) FORMATION IN PRION DISEASE

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Activation of Notch-1-Hes repressor pathways link PrP^{Sc} accumulation to dendritic atrophy in mice inoculated with prions (Ishikura et al. PNAS 102:886-891, 2005). Activation of Notch-1 involves at least 2 steps: (1) Ligand-directed cleavage of Notch-1's extracellular domain yielding the truncated transmembrane NEXT peptide and (2) binding of the nicastrin component of the □-secretase complex to NEXT followed by intramembrane cleavage of NEXT by Presenilin-1 (PS1) releasing its C-terminal intracellular domain NICD. A correlation of 0.94 between neocortical synaptosomal PrP^{Sc} and NICD levels suggested PrP^{Sc} may be the main factor controlling rate of NICD formation. From 30 to 60 days postinoculation (dpi) the PrP^{Sc} and NICD curves were congruent supporting that possibility. However, from 60 to 90 dpi the rate of NICD formation decreased significantly although PrP^{Sc} levels continued to increase exponentially. Then from 90 to 130 dpi the rapid rate of NICD formation returned. These data suggest that at least 2 factors control the rate of NICD formation. Of the key components of the □-secretase complex, only nicastrin was significantly increased above background levels at 90 and 130 dpi to account for the second increase in the rate of NICD formation. This mid-incubation period increase in nicastrin without an increase in its mRNA suggests decreased degradation of nicastrin. Confocal microscopy showed increased nicastrin in association with PrP^{Sc} within dystrophic-appearing aggregates of lysosomes that appear in neurons during mid-incubation period. We propose that PrP^{Sc} and nicastrin have different but complementary effects on the rate of NICD formation. PrP^{Sc} may act as a Notch-1 ligand triggering NEXT formation. From 30 to 60 dpi baseline nicastrin levels capture most of the NEXT peptide for transport to the □-secretase complex to release NICD. From 60 to 90 dpi more NEXT is formed as a result of PrP^{Sc}'s effect on Notch-1 than can be captured by baseline nicastrin levels. From 90 to 130 dpi, accumulation of large amounts of PrP^{Sc} in lysosomes significantly depress degradation of nicastrin increasing its level, which then can keep up with the PrP^{Sc}-generated NEXT levels and restore the rate of formation of NICD. (NIH support: AG02132, AG10770, and AG02160).

PA-54

PRION PROTEIN IN HEALTH AND DISEASE

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The normal function of the prion protein (PrP) remains unclear. Deciphering the normal function of PrP could be of vital importance to understanding the pathogenic dysfunction of misfolded PrP in prion diseases. Recent work from our laboratory has demonstrated a function for PrP in the self-renewal of hematopoietic stem cells and in promoting neural precursor proliferation in vivo. We have been examining PrP knockout mice for other phenotypes as a means to determine the function of PrP. Another aspect of our work focuses on understanding how the misfolding of PrP causes disease. We have inoculated mice deficient for heat shock factor 1 (Hsf1), a key stress responsive transcription factor. These mice have alterations in prion incubation time and PrP^{Sc} accumulation.

PA-55

TARGETED KNOCK-DOWN OF MRNA ESSENTIAL TO PRION PATHOGENESIS REDUCES SENSITIVITY TO PRP106-126 CYTOTOXICITY

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Due to the interspecies conveyance and the terminal disease caused by the infectious prion protein, there has been much interest in identifying key molecular players in disease progression. To date, the only known gene product essential to prion pathogenesis is the PrP protein, the product of the *prnp* gene. Previous studies involving PrP knockout mice (*prnp*$^{0/0}$) have shown that challenge with infectious PrP-containing homogenate fails to lead to disease, and that neurons derived from these knockout mice are resistant to the cytotoxicity of the PrP$^{106-126}$ peptide *in vitro*. These observations parallel experiments that demonstrate that over expression of the PrP protein in animals succumb to disease at a faster rate than those expressing normal levels of PrP. The knockdown of a factor non-essential for viability of the host, but required for existence of the pathogen or its capacity to initiate pathogenesis, will make cells less susceptible to pathogen mediated toxicity. In this study, we have used inhibitors specifically targeting *prnp* mRNA for knockdown to observe the protective effect significantly reduced levels of *prnp* mRNA gene expression confers upon neuronal cells exposed to the cytotoxic PrP$^{106-126}$ peptide. Cell populations were generated with the targeted inhibitors, which either lacked or possessed a modification that permitted enhanced *prnp* mRNA interaction as well as knockdown function. To determine whether the location targeted along the *prnp* mRNA plays a role in knockdown efficiency, four sites were chosen. These targeted inhibitor-containing cell populations were exposed to 80□M PrP$^{106-126}$ peptide and their resistance to PrP$^{106-126}$ induced cell death was determined by flow cytometry analysis. The results demonstrate that significant knockdown of *prnp* mRNA reduces neuronal cell sensitivity to PrP$^{106-126}$ exposure. Our results also demonstrate that the site along the mRNA targeted for knockdown does play a role in determining the efficiency of mRNA knockdown and therefore the extent of PrP$^{106-126}$ resistance.

PA-56

PRPSC ACCUMULATION AND STABILITY IN OVINE PRP TRANSGENIC MICE INFECTED WITH BIOLOGICALLY DISTINCT SCRAPIE STRAINS

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While multiple conformations of the abnormal prion protein (PrP^{Sc}) are thought to encipher prion strain diversity, how they feature in the variable incubation periods observed upon experimental transmission into mice is currently unknown.
In order to investigate the natural diversity of sheep scrapie agent, our laboratory has developed a new experimental model consisting of transgenic mice overexpressing the VRQ allele of sheep prion protein (tg338 line). Among 6 potential groups of natural scrapie strains, we have presently stabilised 4 strains that have been biologically cloned and shown to produce stable and distinct phenotypes, based on the incubation time (from 60 to ~200 days), the molecular profile of PrP^{Sc} and its regional distribution in the brain. Moreover these scrapie strains differ in their capacity to replicate in peripheral tissues, such as spleen. For each of these strains, we have examined i) the kinetics of PrP^{Sc} accumulation in both brain and spleen, ii) the sensitivity of PrP^{Sc} to degradation by either digesting the protein with proteinase K or pepsin in an vitro assay or in vivo by exposing brain homogenates containing PrP^{Sc} to primary cultures of macrophages, iii) the resistance to denaturation with increasing concentration of guanidine-HCl.
These data will be presented and their possible significance in term of strain biology will be discussed.

PA-57

HOST PRP GLYCOSYLATION INFLUENCES THE OUTCOME OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHY INFECTION

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PrP possessed two potential sites for N-linked glycosylation which together with the complexity of the added sugars allows for the generation of a very large number of different glycosylated forms of PrP. Numerous prion or transmissible spongiform encephalopathy (TSE) strains exist, but to date the underlying nature of these strains that gives rise to their different properties remains elusive. However, it has been proposed that the variation in PrP molecules arising from differential glycosylation may account for, or contribute to, the many TSE strains and their characteristics. Therefore to investigate this possibility we have generated three lines of gene targeted transgenic mice with mutations at the first (G1), second (G2) or both (G3) glycosylation sites thus preventing the addition of N-linked glycans at these sites. By using the gene targeting approach we can ensure the altered PrP gene is in the endogenous position in the genome and is thus under its normal control elements thereby eliminating the complication of overexpression and/or ectopic expression. We have established that despite differences in cellular location, mono and un-glycosylated PrP can support both clinical and pathological disease, albeit with varying degrees of susceptibility, following TSE challenge. Moreover different TSE agents have dramatically different requirements for glycosylation of host PrP. We have also demonstrated that TSE strain characteristics can be modified when passaged through hosts with altered PrP glycosylation. Therefore, we consider glycoform analysis should be used with caution as a means of defining the TSE strain infecting the host.

PA-58

NEUROPATHOLOGICAL CHARACTERISATION OF AN ATYPICAL SPANISH CASE OF SCRAPIE

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The brain of a cross breed sheep analysed within the TSE active surveillance program was referred to our lab (PRIOCAT, TSE regional reference lab in Catalonia) as an initial reactor to the first rapid test (TeSeE ELISA, Biorad). The ELISA test was repeated not only in medulla oblongata samples but also in the cerebellum. In parallel, a second rapid test, Prionics-Check Western Blott, was also performed. The cerebellum yielded a higher positive ELISA score than the medulla and both samples were negative to Western Blott. Immunohistochemistry on formalin fixed, paraffin embedded tissue samples confirmed the presence of prion protein deposits, which were more intense at the granular layer of the cerebellum, mild punctiform deposits where also observed at the medulla oblongata. The case was diagnosed as an atypical presentation of scrapie, similar to those described as Nor98 cases. A discriminatory test was also performed (western blotting with antibodies against the prion proteins' N-terminus/core: P4/6H4) and ruled out a possible case of BSE in sheep. The prnp was sequenced and the animal belonged to the $AA_{136}RH_{154}QQ_{171}$ genotype, thus belonging to NSP Group 3 (animals with little resistance to scrapie), moreover it had a heterozygous phenylalanine substitution in codon 141.
The whole brain of the animal was sampled and neuropathological characterisation was performed. Half of the brain was formalin fixed and paraffin embedded. Haematoxylin-eosin stained sections were used to evaluate the morphological changes: spongiform lesions where minimal but a non purulent inflammatory infiltrate constituted mainly by macrophages, lymphocytes and plasma cells was observed as perivascular cuffs located sub-ventricularly, at the leptomeninges and choroid plexuses. Remarkably, in the third ventricle, the formation of lymphoid germinal centres was observed. A morphological diagnosis of concomitant Visna encephalitis was made. Further slides where processed for PrP^{sc} IHQ to map the whole brain distribution of the prion protein deposition. The lymphoid organs tested, including third eyelid, palatine tonsil, retropharyngeal lymph node and the inflammatory infiltrate itself were PrP^{sc} negative. Other parameters evaluated were gliosis (GFAP), cellular death (C3A), extracellular matrix (WFA) and the calcium binding proteins parvalbumin and Calbindin D28K positive GABAergic neural population.

PA-59

GLYCOSYLATION OF PRP AND THE TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES SPECIES BARRIER

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Cross species transmission of transmissible spongiform encephalopathies (TSE) to the human population is of major public health importance. The molecular factors governing the spread of TSE between host species are not fully understood; although host encoded PrP is thought to be central. PrP is variably glycosylated at two sites *in vivo* (N^{180} and N^{196} in mice). These N-glycans may greatly influence TSE transmission between species. To address this issue, we have transmitted human and hamster TSE strains to PrP glycosylation deficient transgenic mice, produced by gene targeting (G1 T^{180}; G2 T^{196}; G3 T^{180} and T^{196}). These mice have an altered pattern of glycosylation of PrP but do not develop spontaneous disease. Here we present data showing a difference in the incubation period and disease incidence between the glycosylation deficient transgenics and control mice; demonstrating that PrP N-glycans do have a species barrier role. However, the nature of this role is dependent upon TSE strain and the site of host PrP N-glycan attachment. To further understand the relationship between PrP glycosylation state and cross species TSE susceptibility we have undertaken *in vitro* conversions to examine the underlying causes of the observed alteration of disease incidence and incubation period.

PA-60

GENE EXPRESSION ALTERATIONS OF THE CNS IN PRION DISEASES

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The pathogenesis of prion diseases in the central nervous system is so far insufficiently understood. In this study, we applied genome-wide gene expression technology to identify disease-associated alterations. To find common alterations in prion diseases, we studied post-mortem human brains with Creutzfeldt-Jakob disease (CJD) and scrapie-infected mouse brains. For the study on human brains, we collected frontal cortices from sporadic CJD patients and control individuals who had died of unrelated diseases, and analysed the gene expression using Affymetrix HGU 133A microarrays. For the expression profiling in mouse brains, we used C57/Bl6 female mice which were inoculated intracerebrally with 30 µl of a 10% brain homogenate of healthy mice or mice infected with mouse-adapted scrapie (ME7 and RML) and studied the gene expression in mouse brains at different time points after inoculation using Affymetrix MOE430A microarrays. A comparison between human control and sCJD frontal cortices identified 79 upregulated and 275 downregulated genes, suggesting a pronounced reduction of gene expression activity in CJD cortex. In scrapie-infected mouse brains, we identified over 400 genes that showed changes in expression over the time course of infection and observed predominant upregulation of gene expression as compared to controls. Although upregulation of genes encoding immune response factors were observed both in CJD and scrapie-infected mouse brains, the range of the involved genes and the degree of the increased expression levels were more pronounced in scrapie-infected mouse brains. In CJD brains, downregulation of genes encoding synaptic proteins was more prominent. These findings support the hypothesis that immune response including activation of complement proteins and inflammatory factors is an important pathogenic event and precedes neuronal dysfunction. We further compared our findings in human CJD with data derived from scrapie mouse brains and identified a number of overlapping genes including upregulated genes such as Abca1, cathepsins, metallothioneins and downregulated gene like ADAM 23. These genes showed altered expression in brains of both human and mouse prion disease. The findings of this study shed light on the complex molecular events that occur during prion disease and offer a data resource for the biomarker selection.

PA-61

A COFACTOR LIMITS THE PMCA CONVERSION REACTION

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Protein Misfolding Cyclic Amplification (PMCA) is an *in vitro* method of converting cellular prion protein (PrP^C) to its abnormal isoform (PrPres). This conformation change is associated with pathogenesis. We use PCMA as a model of *in vivo* conversion in order to identify cofactors in the conversion reaction. Our version of PCMA uses brain homogenate from healthy hamsters, diluted 1/10 in PBS, 1% Triton X-100, 4mM EDTA, with protease inhibitors, and sonicated repeatedly in the presence of a small amount of a homogenate prepared from infected hamster brain. Five seconds of sonication is alternated with 60 minutes of incubation at 37 ºC. We characterize reaction progress via ELISA and Western blot. Prions are probed with a biotinylated 3F4 antibody streptavidin-AP complex and visualized using BCIP/NBT precipitating dye. When analyzed by Western blot of non-denaturing polyacrylamide gels or native ELISA, the PrP^C concentration appears to decrease rapidly within the first 5 seconds of sonication. However, upon analysis by Western blot of denaturing polyacrylamide gels, the concentration of PrP^C does not change. This suggests a presence of an intermediate PrP^C conformer as a first step in the conversion process of PrP^C to PrPres by PMCA. We have also observed that the PCMA reaction is not limited by the supply of PrP^C. Conversion slows over time, eventually coming to a stop, even though nearly half the starting amount of PrP^C remains. This suggests PCMA requires interaction between PrP^C and other cofactors present in brain homogenate. The cofactors appear to be the limiting reagents that are consumed in the conversion process and not recycled. Addition of fresh brain homogenate restores the conversion. These findings provide a new set of tools for studying the cofactor dependent conversion process and understanding disease pathogenesis.

Poster Session

CELL BIOLOGY OF PrPc and PrPsc

CE-01

THE N-TERMINAL DOMAIN OF THE CELLULAR PRION PROTEIN IS NOT NECESSARY TO INDUCE NEURONAL DIFFERENTIATION AND NEURITE OUTGROWTH

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The cellular prion protein is a glycosylphosphatidylinositol-anchored cell-surface protein whose biological function is largely unknown. In order to develop *in vitro* models for studying prion protein-dependent pathways, we have first generated a novel PrP knockout cell line called $PrP^{0/0}$ ML, which does express neither the prion protein nor doppel. Here we show that the $PrP^{0/0}$ ML cell line is a unipotent neuronal precursor line which can specifically differentiate into mature neurons when cultivated under specific culture conditions. The role of the prion protein in the process of neuronal differentiation was then analyzed in $PrP^{0/0}$ ML cells reconstituted with either the full-length or an N-terminal deleted form of the prion protein. We show that prion protein expression induces neuronal differentiation and neurite outgrowth and that the N-terminal domain containing the octapeptide repeat region of the prion protein is not necessary for the activation of the signalling pathway underlying such events.

CE-02

IN-VIVO IDENTIFICATION OF FUNCTIONAL DOMAINS WITHIN THE CELLULAR PRION PROTEIN

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One of the fundamental questions in the field of prion research is whether brain damage during the course of the disease is primarily due to neurotoxicity of the disease associated prion protein (PrP^{Sc}), or whether it is due to a loss of function of the cellular form of prion protein PrP^{C}. Very little is known about the physiological function of PrP^{C}. Ablation of PrP in most mouse models does not induce any pathological phenotype. Expression of a prion protein variant lacking amino acids 32-134 (termed $PrP_{\Delta F}$) has been shown to induce neurodegeneration in PrP^{C}-deficient mice, which is rescued by full-length PrP^{C}. We now report that expression of a PrP^{C} variant lacking the core domain 94-133 ($PrP_{\Delta CD}$) induces a rapidly progressive, lethal phenotype with extensive central and peripheral axomyelinic degeneration. This phenotype was rescued dose-dependently by coexpression of wild-type PrP^{C}, or of PrP^{C} lacking all octarepeats. Expression of a PrP^{C} variant lacking residues 114-121 was innocuous in the presence or absence of wild-type PrP^{C}, yet enhanced the toxicity of $PrP_{\Delta CD}$ but not that of $PrP_{\Delta F}$. Therefore, deletion of the core domain generates a strong recessive-negative mutant of PrP^{C}, whereas removal of residues 114-121 creates a partial agonist whose polarity of action is context-dependent. These findings suggest that axomyelinic integrity is maintained by a constitutively active neurotrophic protein complex whose effector encompasses residues 94-133 of PrP^{C}.

CE-03

CHARACTERIZATION OF CELLULAR INTERACTORS OF THE PRION PROTEIN

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Although a variety of functions has been proposed for the cellular isoform of the prion protein (PrP^c), the definitive one still needs to be elucidated. Similarly, the mechanism finally leading to cell death in prion-infected cells is only roughly understood. One way to address these questions is to identify and characterize proteins interacting with PrP^c. To search for novel interactors of PrP^c we applied the yeast-two-hybrid system (Y2H), screening a neuronal cDNA library with the N-terminal part of PrP as a bait. The N-terminal portion of PrP has been implicated by us in subcellular trafficking and cellular quality control mechanisms (Nunziante et al., 2003; Gilch et al., 2004). Positive interactors found in Y2H were confirmed by co-immunoprecipitation assay and confocal microscopy in cell culture. One of the confirmed proteins was intersectin 1 (ITSN1). ITSN1, a cytosolic, 160 kDa multi-domain scaffold protein is expressed in most cell lines and is involved in different pathways. The interaction with PrP was demonstrated in different cell lines. Interestingly, in prion-infected cells ITSN1 binds preferably to the Scrapie isoform of PrP (PrP^{Sc}). In the same complex also Grb2, another scaffold protein and PrP^c interactor (Spielhaupter & Schätzl, 2001), was identified. Over-expression of ITSN1 did not alter PrP^{Sc} levels, but significantly reduced the amount of surface PrP^c. Prion-infected cells over-expressing ITSN1 showed a distinct phenotype, characterized by many vacuole-like structures, probably representing autophagosomes. The role of ITSN1 in autophagy has to be further investigated to see if there is a functional link between ITSN1, PrP^c/PrP^{Sc}, autophagy, and the resulting cell death following prion infection.

CE-04

RAFTS BUT NOT THE PROTEASOME ARE INVOLVED IN THE MISFOLDING OF A PRP MUTANT RETAINED IN THE ENDOPLASMIC RETICULUM

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Inherited prion diseases are neurodegenerative pathologies related to genetic mutations in the prion protein (PrP) gene, which favour the conversion of PrP^C into a conformationally altered pathogenic form, PrP^{Sc}. The molecular basis of PrP^C-PrP^{Sc} conversion, the intracellular compartment where it occurs and how this process leads to neurological dysfunction are not yet known.
We have studied the intracellular synthesis, degradation and localization of a PrP mutant associated with a genetic form of Creutzfeldt-Jakob disease (CJD), PrPT182A, in transfected FRT cells. PrPT182A is retained in the Endoplasmic Reticulum (ER), is mainly associated with Detergent-Resistant Microdomains (DRMs) and is partially resistant to Proteinase K digestion. Although an untranslocated form of this mutant is polyubiquitinated and undergoes ER-associated degradation, the proteasome is not responsible, suggesting that it does not have a role in the pathogenesis of inherited diseases. On the contrary, impairment of PrPT182A association with DRMs by cholesterol depletion leads to its accumulation in the ER and increases substantially its misfolding. These data support the previous hypothesis that DRMs are important for the correct folding of PrP and suggest that they might have a protective role in pathological scrapie-like conversion of PrP mutants.

CE-5

PRP- PLASMINOGEN INTERACTION IN HEALTH AND MASTITIC BOVINE MAMMARY GLAND: PRELIMINARY DATA

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Prion diseases involve conversion of a physiologic protein PrP(c) to the pathological isoform, PrP(Sc). Plasminogen (PG) has been shown to interact with PrP(Sc). The plasminogen activator system (Pas) is the most important in degrading extracellular proteins. PG crosses directly from blood to milk and it is converted to plasmin during lactation and mammary gland involution. The aim of this study is to evaluate the possible interactions between PrP/Pas on mammary tissue samples of health (n°50) and mastitic (n°150) cows collected in different range of lactation (0-1.5; 1.3-3; 3-7; 7-10; more than 10 months in lactation). The Pas in milk and mammary tissues was also investigated. Immunohistochemical and immunogold post-embedding techniques using different antibodies (12F10 (CEA) anti PrP Mab; 7336 (AbCAM) anti PG Pab and a new anti uPAR Pab) were performed on fixed samples. Bacteriological investigations were performed too. The milk fractions were analysed using a colorimetric assay for plasmin, PG and plasminogen activator. All methods revealed increased levels of Pas with advancing lactation and with mastitis. Any significative variations in the expression of uPAR were observed relatively to different isolated bacteria. The study on tissues showed the presence of PrPc anchored to the cell surface of the plasma membrane and into the cells. No PG immunoreactivity was observed. uPAR expression was limited to the cytoplasmic structures. Further studies are necessary, but it seems that there is no interaction, at mammary gland level, between PrPc and different components of Pas (PG, uPAR) also during mastitis, condition associated to an increased PG presence in the gland.
This work was supported by funds of MURST 2003 and Piemonte Region 2003-2004.

CE-06

CELLULAR LIFESAVER: EXPLORING THE ROLE OF THE CELLULAR PRION PROTEIN IN NEUROPROTECTION

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Several lines of *in vivo* and *in vitro* evidence suggest that PrP^C plays a role in protection of neurons from apoptotic cell death. Wild type PrP^C rescues transgenic mice from neurotoxicity induced by PrP(Δ32-134) and Doppel expression (Behrens, A. and Aguzzi, A. (2002) *Trends Neurosci*. 25(3): 150-4). Work in our laboratory has shown that PrP^C efficiently rescues *S. cerevisiae* from cell death induced by Bax (Li, A. and Harris, D.A. (2005) *J Biol Chem*. 280(17): 17430-4). Since the ability of PrP^C to protect cells from toxic insults has been demonstrated in transgenic mouse models and yeast, we explored *in vitro* systems previously described in the literature to establish a mammalian cell system to further investigate PrP^C-mediated neuroprotection. PrP^C was reported to rescue MCF-7 cells (Diarra-Mehrpour, M. *et al.* (2004) *Cancer Res*. 64(2): 719-27) and immortalized *Prnp*$^{-/-}$ hippocampal neurons (HpL3-4 cells; Kuwahara, C. *et al.* (1999) *Nature*. 400(6741): 225-6) from death stimulated by TNF-α and serum deprivation, respectively. TNF-α treated MCF-7 cells stably expressing human PrP^C displayed only a slight decrease in cell death compared with vector controls. Stable expression of PrP^C in HpL3-4 cells did not produce any consistent effect. In addition, the HpL3-4 cell morphology was not consistent with their being of neuronal origin. Quantitative RT-PCR analysis revealed that the HpL3-4 cell line did not express neurofilament light chain or GFAP. Surprisingly, these cells did not express Doppel, which was unexpected since they were derived from the Rikn *Prnp*$^{-/-}$ mice that ectopically express Doppel in the brain. In striking contrast, a PrP^C-mediated rescue effect was observed in cerebellar granule neurons (CGNs) that were transiently transfected to express mouse Bax. CGNs derived from *Prnp*$^{-/-}$ mice had increased sensitivity to Bax-mediated cell death compared with those expressing PrP^C. Introduction of Bax into cerebellar granule neurons constitutes a tractable system for investigations into the mechanism of PrP^C-mediated rescue.

CE-07

EVALUATION OF POTENTIAL ANTI-PRION DRUGS USING THE NEUROTOXIC HPRP90-231 PRION PROTEIN FRAGMENT(MINIPRION, RMP)

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In vitro and *in vivo* studies have been performed to identify the molecular determinant of the neuronal death induced by full-length PrP^{Sc} or PrP-derived peptides. We developed a completely new experimental model to assess PrP^{Sc} neurotoxicity, using the recombinant PrP-related polypeptide, encompassing the amino acid 90-231 of the human PrP sequence (rMP) that corresponds to the protease-resistant core of PrP^{Sc}. This study was aimed to evaluate the role of PrP structural configuration in prion-dependent neurotoxicity and to identify drugs able to interfere with the neurodegenerative pathways. Using a controlled thermal denaturation protocol, we set up an experimental model to convert rMP from a PrP^{C} like into a PrP^{Sc} like conformation. Thermal denaturation converted rMP into an isoform characterized by high content of sheet structures and partially resistant to Pk treatment. In virtue of these structural changes, rMP powerfully affected the survival of SH-SY5Y cells, inducing a caspase-3 and p38 dependent apoptosis. Conversely, in the native -helix-rich conformation, rMP did not show a significant cell toxicity. Thus we report a precise correlation between the toxicity of rMP and its three-dimensional structure. In order to identify drugs able to interfere with rMP toxicity, we determined the efficacy and the putative molecular mechanisms of minocycline (M) a second-generation tetracycline, and quinacrine (Q) a heterocyclic acridine derivatives compound, in inhibiting cell death induced by rMP in SH-SY5Y cells. We demonstrate that both Q and M are able to revert the rMP-induced toxicity. We identified two distinct mechanisms activated by these drugs: Q exerts its protective action by binding with rMP and preventing the formation of the ~rich toxic isoform, while M reverts the activation of p38, and the inhibition of ERK1/2 and AKT, induced by rMP. In conclusion we demonstrate, using rMP model, that quinacrine and minocycline are compounds able to block rMP neurotoxic effects, and we identify their possible cellular mechanisms of action. (Grant by PRIN 2004 to TF)

CE-08

A CELL LINE INFECTIBLE BY PRION STRAINS FROM VARIOUS SPECIES.

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We have previously shown that expression of ovine PrP renders rabbit epithelial RK13 cells permissive to the multiplication of ovine prions (Vilette et al., 2001), providing the first circumstantial evidence that efficient prion replication can occur in cultured cells from a nonneuronal origin and that the species barrier can be crossed *ex vivo* through the expression of a relevant PrP. We have now significantly extended our earlier observation by showing that prions adapted to different rodent species (mouse, hamster and bank vole) can be propagated in RK13 cells expressing the relevant PrP. RK13 cells expressing mouse PrP support multiplication of Fukuoka and 139A strains of murine prions while Sc237/263K strain multiply in RK13 expressing hamster PrP. In addition, BSE prions from cattle and sheep, adapted to bank vole, were found to propagate in RK13 expressing the vole PrP. Importantly, all these strains retained strain-specific banding patterns of PrPres after multiplication in cell culture. In summary, this work has identified a new cell model for hamster and mouse prions and, remarkably, the first one for BSE-derived prions. While the basis of cell permissiveness to a given strain remains enigmatic, multiplication of a range of prions from different species in the same cell model should facilitate elucidation of the molecular basis of prion diversity.

CE-09

EXPRESSION AND LOCALIZATION OF THE CELLULAR PRION PROTEIN IN THE COW BRAIN: STUDY OF THE AUTOCHTHONOUS PYRENEES BREED

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The appearance of bovine spongiform encephalopathy (BSE) in the United Kingdom had many economical implications in farmers. Additionally, the finding that it could be transmitted to human via consumption of BSE-contaminated beef products raised concerns on its safety for human consumption. Neuropathological examination of BSE-affected cattle has revealed a prominent alteration in the brainstem, suggesting that this is the primarily affected brain area. Based on the protein-only hypothesis, the presence of the cellular isoform of the prion protein (PrP^C) is necessary for the establishment of BSE. Thus, we have studied the expression and localization of PrP^C in fifteen brains of a breed of cows with a high incidence for BSE in Navarra (Spain). The brains of these animals were negative for prions, as confirmed by routinely-used diagnostic procedures for BSE screening. We have observed that the expression of PrP^C follows a rostrocaudal shift throughout the cow brain, being more abundant in rostral areas such us the cerebral cortex and hippocampus. Immunohistochemical staining also revealed that PrP^C is present in several areas of the brainstem such us the dorsal motor nucleus of the vagus, the hypoglossal nucleus and the inferior olives. The presence of PrP^C in the vagus and the hypoglossal nuclei might explain a retrograde spread of prions in cows from the gastrointestinal tract via vagus or pneumogastric (cranial nerve X) and hypoglossal nerves (cranial nerve XII) to those areas of the brainstem. From those nuclei, prions would spread through internal brain connections to rostrally-located structures within the brain of cows.

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CE-10

DISTRIBUTION OF NERVE FIBRES IN BOVINE AND HUMAN MUCOSAL ASSOCIATED LYMPHOID TISSUES.

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Prion cell tropism varies significantly among animal species, depending on both the agent strain and host-specific factors. For example, prions show high lymphotropism in scrapie infected sheep and vCJD, but little, if any, in sCJD or BSE. In particular, the BSE strain is associated with significant PrP-res accumulation in tonsils, spleen and appendix in humans, whereas it is largely confined to the nervous system in infected cattle. Therefore, at least in the case of BSE and vCJD, it appears that host properties can influence the accumulation of the infectious agent in lymphoid organs. Mature FDC play an important role in prion pathogenesis, since neuroinvasion following peripheral challenge is significantly impaired in their absence. The proximity between these FDC and sympathetic nerve endings is known to affect the speed of prion neuroinvasion.

In this study, we analysed the mucosal innervation and the interface between nerve fibres and FDC in bovine and human tonsils and in ileal and jejunal bovine Peyer's patches using a panel of antibodies observed by confocal microscopy. Since differences in the innervation of lymphoid organs depending on age have been reported, we analysed three categories of bovine ages (new born calves, calves less than 12 months old and bovines older than 24 months) and two categories of human ages (patients less than 5 years old and patients older than 25 years).

In both species, hypothetical ways of innervation by-passing germinal centre could be postulated: nerve fibres are widely distributed in antigens/cells traffic area (the lamina propria, the interfollicular zone, the suprafollicular dome in Peyer's patches and the lymphoepithelial area in tonsils). We pointed out that, only in ileal and jejunal Peyer's patches and in tonsils of bovines older than 24 months, nerve fibres are observed to be in contact with FDC. In contrast, in human tonsils, no nerve fibres established contact with FDC, whatever the age. Thus, innervation of germinal centres can be said to be an age-dependent dynamic process in bovines and a weak innervation of the secondary lymphoid organs could thus be a rate-limiting step to neuroinvasion in humans. This variation could influence the way of neuroinvasion and thus, the differences of susceptibility of bovines and humans to the BSE agent.

CE-11

ALTERNATIVE TRANSLATION INITIATION AT THE SIGNAL SEQUENCE CODING REGION CONSTITUTIVELY GENERATES A NUCLEOCYTOPLASMIC PRPC ISOFORM.

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Despite all advances, PrP^C function both in health and disease remains yet inconclusive in part due to its mysterious molecular diversity that has turned it into a multi-compartmentalized and multifunctional protein family. While PrP^C was initially characterized as a glycoprotein attached to the cell surface by a GPI anchor, PrP^C is indeed the sum of four basic forms generated at the ER translocation event (PrP^{Sec}, ^{Ntm}PrP, ^{Ctm}PrP and cytPrP). Out of these forms, cytPrP represents a minor intracellular subset of PrP^C which is constitutively populated by the nontranslocated nascent chains in a signal sequence-dependent process. Using SHaPrP signal sequence mutants containing single base insertions causing shift readings at different positions and cell-free translation assays we have found an alternate and minor translation start site at the AUG triplet coding for M15. The resulting synthetic product, HaPrP(15-254), is a novel isoform that accounts for an about 10% of the total PrP^C and partitions between the soluble and the membrane phases, in both phases being fully accessible to externally added proteases. In CHO and COS-7 stably transfectants, $HaPrP^C$(15-254) is found intracellularly distributing in a cell-dependent fashion between the nuclear and cytoplasm compartments as judged both by subcellular fractioning and confocal fluorescence microscopy experiments. Analysis of the nuclear population evidences several covalent modifications. The existence of this novel PrP^C isoform opens new pathways of function, as the cell cycle regulation.

CE-12

LOCALIZATION AND QUANTIFICATION OF PRION PROTEIN (PRPC) EXPRESSION IN SHEEP PLACENTA

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To increase our understanding of scrapie biology, prion protein (PrPC) expression was evaluated in near-term placentas of adolescent ewes fed at moderate (MOD; 100% of National Research Council [NRC] requirements) or low (LOW; 60% of NRC) diet intake during two periods of pregnancy (day 50-90 and day 90-130)], resulting in MOD-MOD, MOD-LOW, LOW-MOD, and LOW-LOW treatments. Gravid uteri (n=50) from singleton pregnancies were collected at day 130, and placentomes were perfusion fixed for immunohistochemical localization of PrPC. In addition, PrPC genotypes were determined (codons 136 and 171) using SNP assay. Most PrPC-positive cells were in the fetal placenta (trophoblast binucleate and mononucleate cells), with few localized in the maternal placenta (caruncular epithelium). PrPC protein expression (area of positive staining, graded from 1 [least] to 4 [greatest]) was less in MOD-MOD than in any other treatment ($P<0.05$). Overall, PrPC in maternal caruncular tissue was less ($P=0.003$) in MOD-MOD or LOW-MOD ewes compared to those receiving reduced nutrition in late pregnancy (MOD-LOW or LOW-LOW). PrPC was more uniformly distributed in caruncles of MOD-MOD than in any other treatment ($P<0.05$) and more uniform with MOD than LOW at day 90-130 ($P=0.007$). PrPC in fetal placenta was less uniformly distributed ($P<0.01$) in MOD-LOW ewes than in any other treatment. There were no genotype effects on PrPC protein expression. Thus, expression of PrPC protein is influenced by maternal nutritional level, with greatest expression in diet-restricted ewes late in pregnancy. Further study is needed to determine the role of PrPC in placental biology and scrapie transmission via the placenta.

CE-13

UNRAVELLING THE FUNCTION OF THE PRION PROTEIN : A PROTEOMIC APPROACH USING A 2D-LC TOOL

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The loss of function of the host prion protein (PrPc), following its transconformation into an pathogenic isoform, is the causative factor of the fatal neurodegenerative transmissible spongiform encephalopathy. A role in circadian rhythm regulation, synaptic transmission, ion currents, nerve fibre organization, copper ion trafficking, nucleic acid chaperoning, anti-apoptotic processes and antioxidant has been suggested for PrPc[1]. Interestingly, PrPc could play a role in cellular defence mechanisms against oxidative stress induced by Paraquat[2,3] but not against oxidative damage by redox active metals such as manganese[4]. The mechanism by which PrPc protects against paraquat injury but not against manganese toxicity is unclear. This raises questions as to the cellular defence pathways in which the prion protein could be implied and opens a new area of investigation.

In this work, we have used a proteomic approach based on a two dimensional liquid chromatography tool, the ProteomeLab™ PF 2D, in order to study the possible roles of the prion protein in cellular metabolism. Our proteomic technique separates proteins according to isoelectric point on an ionic exchanger in the first dimension and according to relative hydrophobicity on a non-porous reverse phase column in the second dimension. Proteins are detected by UV in the first and second dimensions. Data handling of whole proteomes, fractionated by this system, is realised using three software: 32karat, Proteovue and Deltavue[5]. These software display fractionated proteins along their pI and relative hydrophobicity on an artificially generated 2D map and differentially expressed proteins are visualised along a third artificial map, facilitating proteome to proteome comparisons.

Using this technique, we have fractionated and analysed the proteomes of three human neuroblastoma cell lines differing in their prion protein (PrP) levels of expression: SH-SY5Y, expressing basal levels of human PrP; wild type SH-SY5Y(wtPrP), expressing a murine PrP gene contained within a transfected plasmid[6]; and a negative control SH-SY5Y, containing the plasmid control without the murine PrP gene. The identification of differentially expressed proteins following paraquat and manganese injury will contribute to clarify a fundamental metabolic state in correlation with PrP expression level. Indeed, it is likely that a loss of prion protein function is the causative factor for transmissible spongiform encephalopathies.

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CE-14

CELL TYPE-SPECIFIC NEUROPROTECTIVE ACTIVITY OF UNTRANSLOCATED PRION PROTEIN IN PRIMARY NEURONS

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A cytosolic form of the prion protein (PrP) has been proposed to play a key pathogenic role in prion diseases. However, the consequence of PrP localization in the cytosol of neurons remains unclear, having either cytotoxic or anti-apoptotic effects been reported in different studies. The cellular mechanism by which PrP is delivered to the cytosol of neurons is also controversial, since both retrograde transport from the endoplasmic reticulum and abortive translocation have been proposed. In view of the physio-pathological interest of cytosolic PrP, we systematically investigated its biogenesis and effect on survival of cortical, hippocampal and cerebellar granule neurons cultured from newborn mice. We find that the proteasome inhibitors cause accumulation of an unglycosylated form of PrP in cortical and hippocampal, but not in cerebellar neurons. We provide evidence that this form contains uncleaved signal peptides, indicating that it corresponds to PrP molecules that have not co-transationally translocated into the endoplasmic reticulum lumen. Analysis of cell viability shows that neurons accumulating untranslocated PrP are more resistant to proteasome inhibitors’ toxicity than neurons that do not synthesize this topological variant. These findings indicate differences in the efficiency of co-translational translocation of PrP between distinct neuronal types, and underscore a potential physiological role of cytosolic PrP.

CE-15

EPENDYMAL CELLS IN SCRAPIE INFECTION.

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During the course of infection in the hamster scrapie model, the ependyma epithelium shows immunostaining of PrPsc in the subependymal layer and at the apical ependymal cell borders. In order to specify the interaction of ependymal cells with the scrapie agent, we used electron microscopy in combination with PrP immuno-detection in hamsters infected with the 263K scrapie strain. In this model we have previously shown that reactive astrocytes localized at the injection site appear to be the first cells propagating the agent in the brain. They contained lysosomal-like organelles positive for PrP. Interestingly, similar PrP-positive organelles were detected in ependymal cells suggesting an interaction between this cell type and the scrapie agent. In addition, PrP labelling associated with vesicular and multivesicular endosomal structures was observed in the ependymal cell cytoplasm close to the apical cell membrane. Examining the relationship between ependymal cell microvillosities and PrPsc, immunogold PrP labelling was found to be associated with either morphologically intact microvillosities and altered structures having a relationship with PrP fibrillar elements present in the intraventricular space. In contrast, ependymal cell cilia were completely devoid of PrP immunogold labelling. Following a careful morphological analysis, small electron-dense particles were observed in close association with the membrane of ependymal cell microvillosities and fibrillar elements. Particles could also be observed in subependymal plaque-like accumulations of PrPsc. These structures of about 15 nm in diameter showing a coma shape were present intracellularly in the apical zone of the ependymal cell cytoplasm but were never observed in ependymal cells of non-infected brains . They could represent a morphological aspect of the infectious agent in situ in the brain. These findings suggest a strong implication of the ependymal cells in the production of the scrapie agent in the central nervous system, indicating that this particular glial cell type is involved in the pathophysiological mechanisms of scrapie infection.

CE-16

STRAIN AND CELL-TYPE SPECIFIC RESPONSE OF STIMULATED INNATE IMMUNE CELLS TO PRION INFECTION

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The role of the innate immune system in prion infection has been extensively investigated during the last few years. It has been reported that application of CpG-motif containing oligonucleotides (CpG-ODN), which stimulate TLR9- expressing cells of the innate immune system, inhibit the progression of prion disease in mice after peripheral infection (Sethi et al., 2002). This prolongation of incubation is probably not caused by stimulation of immune cells, but by massive alterations in the architecture of the spleen, leading to a lack of follicular dendritic cells and therefore to an impaired peripheral propagation of prions (Heikenwalder et al., 2004). We used an *in vitro* model to study the effects of stimulation by CpG-ODN and LPS on transient prion infection of macrophages and microglia cells with different prion strains. Infection of J774 (murine macrophages) or BV2 cells (murine microglia) led to a different response dependent on the cell type and the prion strain used for infection. Furthermore, the cell surface expression of PrP^c, but not the amount of PrP mRNA, was increased in stimulated cells. Unspecific effects of CpG-ODN or LPS stimulation on prion infection were excluded by employing neuronal N2a cells. Our data indicate that stimulation of innate immune cells might support transient propagation of prions in certain cell types.

CE-17

DOES PRESENCE OF PRPSC IN LIPID RAFTS AFFECT SRC FAMILY KINASE SIGNALING?

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Since the conversion of PrP^{C} to PrP^{Sc} have been shown to occur in lipid rafts and that PrP^{C} might act as a signaling protein we hypothesize that misfolded PrP can cluster PrP^{C} together and initiate an uncontrolled signaling of intracellular signaling proteins e.g. the Src family kinase Fyn.
Lipid rafts are also known as detergent resistant membranes i.e. they are resistant to certain non-ionic detergents at low temperatures. This property is used to isolate lipid rafts as lipid raft complexes float to low density during sucrose gradient centrifugation. These fractions are further analyzed by western blot – immunoblotted with antibodies against Fyn and active Fyn. In vitro kinsae asssays are also performed on flotation fractions.
Preliminary results show an increased specific activity of Fyn kinase in scrapie-infected murine immortalized hypothalamic cells, ScGT1-1 compared to uninfected cells after immunoblotting with clone 28 (recognizes active Src and Fyn) immunoprecipitates of Fyn protein. Future studies will focus on the suggested colocalization of PrP^{C}/PrP^{Sc} and Fyn kinase in lipid rafts. Abnormal Fyn activation may be a key element in the neuropathology in prion disease.

CE-18

IINFECTION OF PRP KNOCK-OUT CELLS CONDITIONALY EXPRESSING MAMMALIAN PRION PROTEINS

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The prion protein (PrP) can be the cause of TSE disease, including mad cow disease (BSE), chronic wasting disease (CDW), and Creutzfeld-Jakob disease (CJD). These diseases are resulting from conversion of a cellular non-pathogenic to an infective pathogenic isoform. For understanding prion diseases the function of the normal cellular isoform is a fundamental must have.
We created a prion expression system in murine PrP knock-out cells. In these cells the stable transfected *prnp* gene was changed between different mammals, including human, chimp, crab eating monkey, cow and moose. In the different cell lines PrP expression is regulated by a tetracyline responding element (TRE). The regulation was achieved by using different tetracycline or doxycxline concentrations. The expression was analysed by western blot (Wb), FACS, immunfluorescence (IF) and circular dichroism (CD) spectroscopy analyses. After characterization of PrP expression, the cells lines were infected with different concentrations of a BSE stem brain homogenate. PrP propagation in the cells was observed after 48 h by using Wb, IF, and FACS analyses. The CD spectroscopy analysis of infected cells is still in process. In addition we will doe kinetic studies inculding proof of chronic infection. From our observations we conclude that PrP knock-out cells stably transfected with human, primate, and ruminant *prnp* can be infected with BSE. Currently, the molecular mechanism of prion protein propagation is examined more accurately by using 2-D gel electrophoresis (2-DE) analysis of infected and non-infected cell proteome.
This system can be used not only for infection studies instead of animal models. It can also be used for characterization of the molecular mechanisms of prion infectivity, e.g. the signal cascade of other proteins they are directly or indirectly involved in the prion protein expression and also PrP conversion.

CE-19

VARIOUS MOUSE-ADAPTED PRION STRAINS INFECTION OF A NOVEL MICROGLIAL CELL LINE

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Prion diseases are progressive, degenerative diseases of the nervous system. The histological characteristics of prion diseases are spongiform change of brain, neuronal loss, astrocytosis and increase of activated microglia. The relevance of microglia in the pathogenesis of prion diseases has been inferred by many studies, but the precise roles of microglia still remain largely obscure. To elucidate the roles of microglia in the pathogenesis of prion diseases, physiological and biochemical characterization of prion-infected microglial cells should be clarified.
In this work, we report a novel prion-infected microglial cell culture model. After exposure to Chandler scrapie, a microglial cell line (MG20 cell) established from the brain of neonatal *tga20* mice that overexpress murine prion protein, replicated disease-associated forms of the prion protein as well as infectivity. In addition, Chandler scrapie maintained biological properties in this cell culture. Furthermore, MG20 cells were susceptible to various prion strains, such as ME7, Obihiro scrapie and bovine spongiform encephalopathy agents. Thus, this cell line may provide a potentially valuable tool for the investigation of the molecular events in microglia that participate in the pathogenesis of prion diseases and prion strain determinants.

CE-20

EFFECTS OF NUTRITION AND GENOTYPE ON CELLULAR PRION PROTEIN (PRPC) MRNA EXPRESSION IN OVINE PLACENTAL TISSUES

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To determine the effects of nutrition and genotype on PrPC mRNA expression, singleton pregnancies from a single sire were established by embryo transfer to adolescent dams, which then were offered a moderate (M, n=22), high (H, n=20), or high-low (HL, with high to low switch at day (d)90 of gestation, n=10) diet intake. Whole placentomes, collected at d90 or 130 of gestation, were separated into maternal, (caruncle, CAR) and fetal (cotyledon, COT) tissues for quantitative real-time RT-PCR determination of PrPC mRNA expression. PrPC genotypes were determined for codons 136 and 171 using SNP assay. In CAR, across all treatments, PrPC mRNA was greater ($P<0.001$) on d90 than on d130, but in COT it was similar on both days. On d90, PrPC mRNA tended to be greater ($P<0.08$) in CAR than in COT, but on d130, PrPC mRNA was greater ($P<0.001$) in COT than in CAR. There were no effects of nutrition on d90 of pregnancy. However, on d130, for CAR and COT, PrPC mRNA was reduced ($P<0.01$) in H compared to M, but was similar in M and HL. PrPC mRNA expression was unaffected by codon 136 genotype. In CAR on d90 and in COT on d130, PrPC mRNA was greater in ewes with an R at codon 171 than in those with QQ (1.78±0.14 vs 0.98±0.25 for CAR, $P<0.02$; 1.64±0.10 vs 1.31±0.13 for COT, $P<0.06$). Thus, differences in PrPC mRNA expression in fetal and maternal placental tissues were influenced by nutrition, day of pregnancy, and genotype. Further study is needed to determine whether changes in PrPC mRNA expression affect scrapie transmission via the placenta.

CE-21

AMINO TERMINAL TRUNCATION OF PRION PROTEINS IN HUMAN, SHEEP, CATTLE AND MOUSE

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Prion diseases are fatal neurodegenerative disorders which affect both humans and animals. The pathogenic mechanism is associated with the conversion of normal prion protein (PrP^{C}) to a pathological isoform (PrP^{Sc}) followed by conformational change. The C-terminal domain of PrP^{C} (amino acids (aa) 125-231) is composed of three □-helices and two □-sheets and has two glycosylation sites. The amino (N-) terminal region (aa 23-124) containing the octapeptide region is unstructured with a flexible polypeptide chain. In physiological metabolism the N-terminal region can be forfeited by cleavage. Thus, it is supposed that cleavage may provide a mechanism for down-regulation of PrP^{C} activities. Truncated forms of 18 kDa were found by antibodies recognizing carboxyl (C-) terminal sequences after deglycosylation of the protein samples. In our study, however, we identified fragments of approximately 18 kDa even after immunoprecipitation without deglycosylation. We examined the forming of the C-terminal fragment and compared the expressions in human, cattle, sheep and mouse. Proteins of brain homogenates were immunoprecipitated and signals of PrP^{C} were quantified by densitometry. Interestingly, noticeable formation of the truncated forms differed among species. High levels of the C-terminal fragment were found in mouse brains and low amounts in sheep brains. Fragments of PrP^{C} in cattle were >3 kDa higher compared with sheep PrP^{C}. Moreover, cattle PrP^{C} were detected as a double band differing in the molecular mass of approximately of 2 kDa. Our data indicate that the C-terminal fragment is a major product of physiological PrP^{C} metabolism in different species, generated by cleavage and detectable by immunoprecipitation.

CE-22

ANTAGONISTIC CELLULAR FUNCTIONS FOR PRION PROTEIN AND ITS PARALOG DOPPEL

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The physiological function of the cellular form of the prion protein (PrP^{C}) has remained elusive. We recently described a function for PrP, by observing the effect of recombinant PrP to cultured primary neurons from the hippocampus of embryonic rats (E18/E19) (Kanaani, Prusiner et al. 2005). The assay showed that PrP plays a role in promoting both neuronal polarity and development. We have now established this assay in primary neurons cultured from the cerebellum of postnatal (P6) mice. In addition to confirming the findings of the rat model for PrP, we have elucidated a possible role for its paralog protein, doppel (Dpl). Our findings suggest that the two proteins may have an antagonistic relationship: PrP promotes cell survival and neuritogenesis, whereas Dpl induces apoptosis. Establishing the assay in postnatal mouse neurons has an important advantage over the rat model. Most importantly, the availability of a wide range of knockout and transgenic mouse models should provide a diverse source to dissect further the functions of PrP and its interaction with other cellular proteins and structures.

Reference

Kanaani, J., S. B. Prusiner, et al. (2005). "Recombinant prion protein induces rapid polarization and development of synapses in embryonic rat hippocampal neurons in vitro." J. Neurochem. 95: 1373-1386.

CE-23

PRION INTERCELLULAR PROPAGATION : AT THE FRONTIER OF MATHEMATICAL AND BIOLOGICAL APPROACHES

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The mechanisms involved in the misfolding of PrPsc, in its accumulation and in its intercellular propagation remain poorly understood. Moreover, the study of these phenomena at a single cell scale is difficult with the existing *in vitro* and *in vivo* approaches. At this level, complementary approaches can benefit from cellular automata mathematical theory which allows to investigate in what way a local parameter, related to a particular cell, can influence the global behavior of a system. Thus, we programmed a cellular automata to mimick prion propagation in a cell culture.
Our model allows us to set every wanted single cell-related parameter, as cell-cycle length, death rate, Prnp gene expression, level of infectivity. Depending on these parameters, some rules simulating the cellular metabolism of PrPc and PrPres (production, degradation, misfolding) have been implemented. These rules are based on the existing intracellular models of prion proliferation. They simulate prion propagation at a unicellular level. In addition, some rules concerning prion propagation from one cell to another one (by cell contact or by secretion of PrPsc in culture medium) have been added.
To estimate experimentally the different parameters included in our model, we used two cell lines, GT1-7 and SN56, chronically infected by Chandler prion strain. A magnetic cell sorting of infected cell lines, based on membrane expression of PrP, has been realized, that enriched the different cell line in infected cells. Thus, slight differences in PrP expression and in cell growth between infected and uninfected cells have been highlighted whereas no difference was observable before sorting. The different experimental observations are included regularly in the mathematical model to implement the parameters to be studied.
The influence of each hypothetic local rule (limited to one cell and its nearest neighboorhood) on the global behavior of the *in silico* culture is evaluated. The comparison with experimental data will allow to select the most realistic rules, and therefore investigate more precisely the cellular mechanisms potentially involved. Even if the model is still far from a real culture, such a multidisciplinar approach constitutes a new original perspective in the comprehension of prion infection.

CE-24

THE INVOLVEMENT OF GLYICAN-1 IN PRION FORMATION

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Conversion of the cellular prion protein (PrP^{C}) to the disease-causing isoform (PrP^{Sc}) may occur during endocytosis and/or recycling of PrP^{C}. The requirement of cellular cofactors involved in the conversion process has been suggested. Several lines of evidence have shown sulfated glycosaminoglycans like heparan sulfate (HS), heparin and pentosan sulfate to affect the metabolism of prions, and a cellular heparan sulfate has been shown to participate in prion propagation in scrapie-infected cells. Additionally, HS accumulates in prion amyloid plaques. Since PrP^{C} binds to HS and heparin, it has been proposed that sulfated glycans inhibits PrP^{Sc} formation by competing with the binding of PrP^{C} to a putative cellular HS proteoglycan. However, which HS proteoglycan is involved in PrP^{Sc} formation remains to be determined. One candidate is the glycosylphosphatidylinositol-anchored, raft-resident glypican-1 (Gpc-1) which is particularly expressed in the adult brain. During recycling of Gpc-1, degradation of HS takes place.
Recently, the metabolism of glycosaminoglycans was shown to be impaired in prion disease. Correspondingly, in this study, we show that the localization and processing of recycling Gpc-1 is dramatically altered in GT1-1 cells which have been prion-infected (ScGT1). Confocal laser scanning immunofluorescence microscopy shows that in ScGT1 cells, Gpc-1 assumes a punctuate distribution, in contrast to the even distribution in uninfected cells. Additionally, NO-catalyzed Gpc-1 HS degradation is increased, whereas enzymatic heparanase-dependent HS cleavage is decreased in ScGT1 cells. Our preliminary results have raised the hypothesis that Gpc-1 might be serving as a receptor for cellular uptake of prions, and/or as a cofactor for PrP^{C} to PrP^{Sc} conversion.

CE-25

CLEAVAGE AND SUBCELLULAR LOCALISATION OF THE OVINE PRION PROTEIN IN TRANSFECTED CELL LINES

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In normal brain and cell-culture, PrP^C is cleaved near amino acid 113 (ovine numbering), separating the unstructured N-terminal tail from the globular C-terminal two-thirds of the molecule. This processing is found in a wide variety of species indicating an important event in PrP metabolism. The cellular site and the functional purpose of the processing are still unsettled. Whether the processing is of relevance to the development and/or sensitivity to prion disease, analogous to the processing of the Alzheimer peptide amyloid beta, also remains to be investigated.
We have used several approaches to study the localisation of the major prion protein cleavage fragments: The murine (N2a) and humane (SH-SY5Y) neuroblastoma cells were transiently and stably transfected with the different ovine PrP-constructs. Constructs of the ovine PrP, with and without tags and GPI-anchor were made. To facilitate the studies of the short N-terminal cleavage product (9-10 kDa), a construct of PrP with a green fluorescent protein (GFP) in the N-terminal part was made. Other constructs had red fluorescent protein C-terminally with or without GFP N-terminally. By use of live imaging of fluorescent “tags” and immunohistochemistry with different monoclonal antibodies (Mabs), the full-size molecule and its two main cleavage products could be traced in the cell lines.
To test whether structural alterations in the conserved hydrophobic domain of PrP could influence the processing, we introduced a number of amino acid substitutions and deletion mutants by site directed mutagenesis.

CE-26

IMPACT OF THE HYDROPHOBIC CORE REGION ON PRPC TOPOLOGY AND METABOLISM

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The cellular prion protein (PrP^C) is a GPI-anchored cell surface protein. A small subset of PrP^C molecules, however, can be integrated into the ER-membrane via its transmembrane domain (TM), which also harbors one of the most highly conserved regions of PrP^C, termed the hydrophobic core.
A mutation in TM is implicated in Gerstmann-Sträussler-Scheinker Syndrome (GSS) resulting in an enhanced formation of a transmembrane form (^{Ctm}PrP), which has thus been postulated to be a neurotoxic intermediate in prion diseases besides PrP^{Sc}. Furthermore, a mutant of murine PrP^C, missing eight amino acids within TM, trans-dominantly inhibits accumulation of PrP^{Sc} in mouse neuroblastoma cells.
In order to elucidate a possible physiological function of the transmembrane domain, we created a set of different mutants, carrying micro-deletions from two to eight amino acids within the hydrophobic core of PrP^C between codons 114 and 121.
These mutations show a reduced formation of TM topology, which correlates with the reduction of the hydrophobic character of TM in the mutants. In addition, our mutants exhibited alterations in the formation of the C1 fragment, which is generated by □-cleavage during the normal PrP^C metabolism, *in vitro* and *in vivo*. The dependence of cleavage efficiency on the amino acid sequence of the hydrophobic core segment indicates that this region might function as recognition site for the proteases responsible for PrP^C □-cleavage. Interestingly, a mutant G113V, which corresponds to a prion disease associated mutation in humans, showed increased ^{Ctm}PrP topology as well as decreased □-cleavage in our *in vitro* assay.
The effect of decreased TM topology as well as decreased □-cleavage on PrP^C function is further investigated in transgenic mice expressing the deletion mutant 114-121.

CE-27

CELLULAR PRION PROTEIN IN MILK

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Cellular prion protein, PrP^{C}, is essential for the development of prion diseases where PrP^{C} is considered the substrate for the formation of a disease associated conformer, PrP^{Sc}. In sheep, the natural host of the prion disease scrapie, PrP^{C} is abundant in CNS tissue and is also found at lower concentrations in a range of non-neuronal tissues including skeletal muscle and mammary gland. Here, we demonstrate the presence of cellular prion protein in milk from both ovine and bovine sources. Milk PrP^{C} has increased electrophoretic mobility compared with brain PrP^{C} yet is recognised by antibodies directed to epitopes at the C- and N-terminal regions of the protein. Furthermore, milk PrP^{C} is apparently not membrane-associated; together these observations suggest absence of the C-terminal GPI-anchor. Milk PrP^{C} is present predominantly as three species that differ only in the extent of their N-linked glycosylation, with glycoform profiles varying among animals. These apparently full-length PrP^{C} species are also present in commercial homogenised/pasteurised bovine milk, although at reduced levels compared with unprocessed milk. Additional N-terminal fragments of PrP^{C} are also detectable in ruminant milk and commercial milk products. These findings represent the first evidence of the presence of PrP^{C} in milk and raise the question of whether disease-associated PrP species and prion infectivity can also be secreted into milk.

CE-28

ROLES OF CELLULAR PRION PROTEIN IN OXIDATIVE STRESS AND MITOCHONDRIAL FUNCTION

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Despite being the subject of many recent studies, the physiological function of the cellular isoform of prion protein PrPc remains largely unresolved. Several candidate functions have been discussed, including binding and internalisation of copper or other metals, superoxide dismutase-like activity, regulation of cellular antioxidant activities and signal transduction. We have focussed on the TM1 region of PrPc (codons 110-135), due to its high conservation and the neurotoxicity of peptides derived from this region. To elucidate the physiological role of the PrPc TM1 domain in the context of the full-length PrPc molecule, we have constructed a set of deletion mutants centred on codons 114-121 of PrPc and mice expressing Δ114-121-PrP in different genetic backgrounds (Prnp+/+, Prnp+/-, Prnp-/-). Our aim is to elucidate a possible role of PrPc in anti-oxidative defence in cultured cells. For this purpose we have studied for the effect of overexpression of wt-PrP or Δ114-121-PrP in N2A mouse neuroblastoma cells on the intracellular level of reactive oxygen species (ROS). Endogenous ROS production was significantly lower in cells transfected with either wt or Δ114-121 expression plasmids. Furthermore the mitochondrial membrane potential ($\Delta\Psi$ was significantly lowered in wt-PrP or Δ114-121-PrP transfected cells. As PrPc can bind copper via its octarepeat region, we also tested the impact of copper on ROS level and $\Delta\tilde{\Psi}$ Copper treatment leads to a decrease of $\Delta\Psi$in wt-PrP transfected cells, an increase of ROS level in Δ114-121-PrP transfected cells both under basal normal conditions and under exogenous oxidative stress. In order to perform analyses in a system closer to in vivo, we performed the same assays with primary brain cells derived from transgenic mice expressing Δ114-121-PrP in different genetic backgrounds (Prnp+/+, Prnp+/-, Prnp-/-). Our transgene seems to increase cellular sensitivity to oxidative stress. We conclude that PrPc plays a role in $\Delta\Psi$regulation, ROS level and oxidative stress sensitivity, mediated at least in part by the TM1 region.

CE-29

PURSUING THE BIOLOGICAL FUNCTION OF PRPC BY MEANS OF A NOVEL EXPERIMENTAL PARADIGM

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The prion protein (PrP) is a highly dynamic mammalian glycoprotein that presents in at least two different conformational isoforms. While it is accepted that the "scrapie" isoform (PrP^{Sc}) is the etiologic agent of prion disease, the physiologic role of the normal "cellular" conformer (PrP^{C}) is still elusive, despite the numerous functions proposed by *in vitro* and *in vivo* studies, including a role in signal transduction, cell adhesion and survival. Recently, however, accumulating data supports the notion that PrP^{C} serves in neurogenesis and in the differentiation process of neurons. Although these cells represent the model of election where to study PrP^{C} biology, the protein is nonetheless expressed at appreciable levels in other tissues, i.e. skeletal muscles. In this regard, using primary mouse myocytes, we have recently shown that the expression and metabolism of PrP^{C} change during *in vitro* myogenesis and in skeletal muscle fibres with different contractile properties (Massimino *et al.* 2006). This suggests that the protein may play an active role in skeletal muscle differentiation and in the maintenance of muscle functions. To explore this issue further, we have applied an *in vivo* degeneration/regeneration paradigm to wild-type (WT) and PrP-knockout (KO) mice, consisting in treating hind-limb *Tibialis anterior* with cardiotoxin, a myotoxin that provokes the specific degeneration of muscle fibres, while leaving intact nerves, blood supply and muscle precursor cells. The with-time muscle regeneration was followed by comparing several histologic and biochemical parameters of the two animal types, among which the maturation and dimension of fibres, and the expression of myogenic factors and muscle maturation markers. Taken together, our results show that a different kinetics of regeneration pertains to the two mouse lines. Being the process delayed in KO with respect to WT mice, these observations suggest that PrP^{C} may indeed act as a differentiative molecule in skeletal muscles.

(Massimino M.L. *et al.* (2006) Heterogeneous PrP^{C} metabolism in skeletal muscle cells. FEBS Lett. 580, 878-884.)

CE-30

GFP-TAGGED MUTANT PRION PROTEIN FORMS AGGREGATES IN AXONS OF TRANSGENIC MICE

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Mutations in the prion protein (PrP) are associated with several familial prion diseases. Tg(PG14) mice express a mutant PrP (PG14) which contains an insertion encoding nine additional octapeptide repeats. These mice develop a neurological disorder characterized clinically by ataxia and neuropathologically by astrogliosis and PrP deposition. However, buried epitopes within PG14 PrP hinder effective antibody recognition of the protein in immunocytochemical experiments. To study PG14 PrP cellular distribution *in vivo*, we have generated transgenic mice expressing a PG14 PrP-EGFP fusion protein that allows direct visualization of the mutant PrP. Tg(PG14 PrP-EGFP) mice display clinical and neuropathological phenotypes similar to Tg(PG14) animals. Control mice expressing wild type PrP-EGFP (WT PrP-EGFP) remain asymptomatic. Biochemically, PG14 PrP-EGFP is distinct from WT PrP-EGFP, but similar to the infectious form of PrP (PrP^{Sc}), in that it is detergent insoluble, weakly protease resistant, and PIPLC resistant. In cerebellar granule cells cultured from transgenic mice, PG14 PrP-EGFP forms aggregates within neurites and shows decreased cell surface expression compared with WT PrP-EGFP. Confocal microscopy studies show that PG14 PrP-EGFP aggregation also occurs *in vivo* in neuropil regions throughout the brain. Aggregates are found at high densities in axon-dense regions, specifically in the hippocampus, striatum, and molecular layer of the cerebellum. These aggregates do not colocalize with markers for dendrites nor organelles in the cell soma, including the ER, Golgi apparatus, and lysosomes. In contrast, WT PrP-EGFP is uniformly distributed along the axonal cell surface and does not form aggregates. Axon-specific PG14 PrP-EGFP aggregation suggests that disruption of axonal transport systems may contribute to the disease phenotype. Further examination of PG14 PrP-EGFP trafficking in primary neurons will clarify what role axonal pathogenesis may play in the inherited prion disorder.

CE-31

POSSIBLE PREVENTION OF CYTOSOLIC PRION PROTEIN-INDUCED NEUROTOXICITY BY HSC70-MEDIATED TRANSLOCATION OF CYTOSOLIC PRION PROTEIN INTO EEA1-POSITIVE VESICLES

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The molecular mechanisms of prion-mediated neurodegeneration are not yet fully understood. In the recent past, it has been proposed that neuronal death might be triggered by cytosolic accumulation of misfolded cellular prion protein (PrP^C) due to impairment of proteasomal degradation. Cytosolic PrP^C could result from either retrotranslocation via the endoplasmatic reticulum-associated degradation system (ERAD) or abortive translocation of PrP^C into the ER. Indeed, expression of cytosolic PrP^C both *in vivo* and *in vitro* was shown to be neurotoxic. However, conflicting results on cytosolic PrP^C-mediated neurotoxicity in cultured cells have been reported. In order to investigate the molecular mechanisms of cytosolic PrP^C–mediated cytotoxicity, a process which may play a central role in the pathogenesis of prion diseases, we performed a detailed analysis of N2a cells conditionally expressing cytosolic PrP^C (Cy-PrP(aa23-231)). We found that Cy-PrP expression *per se* is not sufficient to trigger cytotoxicity in N2a cells independently of proteasome inhibition. Furthermore, we show that Cy-PrP is degraded with kinetics resembling the degradation of cell membrane-anchored full length PrP^C and that the 20/26S proteasomal system is responsible for Cy-PrP degradation but not for that of full length PrP^C. Interestingly, Cy-PrP accumulates in fine foci when expressed at high levels and colocalises with the cytosolic chaperone Hsc70 in EEA-1 positive endocytic vesicles. We therefore propose that the chaperone Hsc70 acts as a regulator for the controlled formation of amorphous Cy-PrP aggregates and their transport to endosomal/lysosomal vesicles. This Hsc70-dependent mechanism may confer protection to N2a cells against cytoplasmatic toxic accumulation of Cy-PrP.

CE-32

EUKARYOTIC CELLS EXPRESSING HETEROLOGOUS PRPC AS A PUTATIVE TOOL IN THE DIAGNOSIS OF PRION DISEASES

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Currently, several different strains of pathologically folded prion protein (PrP^{Sc}) are recognised in sheep and cattle. Different strains are ultimately defined by bioassay, but can also be determined by more rapid methods like migration behaviour of the pK resistant fragment of PrP^{Sc} or by interpretation of the PrP^{Sc} distribution in the brain of infected animals. The research presented here focuses on enhancing the ability to discriminate between strains using eukaryotic cells expressing different variants of the cellular form of the prion protein (bovine and several allelic variants ovine).
Several isolates of scrapie and BSE have been used for in vitro infection of the PrP^C expressing cells available. Cells were serially passaged weekly for several weeks and accumulation of PrP^{Sc} has been determined using Western blot. Distribution of PrP^{Sc} was studied using immunoperoxidase monolayer assay (IPMA) and fluorescence microscopy.
Persistent in vitro infection, as demonstrated in ROV and MOV cells (Vilette *et al*, 2001, Archer *et al*, 2004), could not be detected in our own cells. Transient infection was seen in Western blot, fluorescence microscopy, and IPMA, but did not allow the discrimination between different strains. Whether confocal laser scanning microscopy can enhance the resolution is currently being investigated.

CE-33

PRION INFECTION INTERFERES WITH THE SEROTONERGIC DIFFERENTIATION OF THE MURINE 1C11 NEURONAL PROGENITOR

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Prion-propagating cell lines represent valuable tools to investigate into the biogenesis, conversion and trafficking of the scrapie prion protein PrP^{Sc}, and have been exploited for diagnostic and therapeutic assays. In contrast, relatively little information has been gathered on the impact of PrP^{Sc} accumulation on cellular functions. We took advantage of the 1C11 murine neuroectodermal progenitor, able to acquire upon induction the overall functions of serotonergic neurones ($1C11^{5\text{-}HT}$ cells), to assess the impact of prion replication on the implementation of a defined neuronal differentiation program. The EC-derived 1C11 clone behaves as a neuronal stem cell. Exposure of 1C11 cells to dbcAMP triggers the synchronous and homogenous onset of pan-neuronal and neurotransmitter-specific markers, reaching a complete serotonergic phenotype, including synthesis, storage, catabolism and uptake, within four days. The 1C11 cell line endogenously expresses the cellular prion protein PrP^{C} and has been instrumental in defining PrP^{C} as a signalling molecule. 1C11 undifferentiated cells were incubated with brain homogenates from prion-infected mice and tested for their capacity to accumulate proteinase-K resistant PrP. Significant PrP^{res} accumulation could be detected in 1C11 cells exposed to the Chandler, 22L and, most notably, Fukuoka, strains. Several clones (1C11-Fk) with various levels of PrP^{res} production were derived from the Fukuoka-infected cell population. Inoculation to tga20 mice allowed us to establish that prion-replicating 1C11-Fk clones carry infectivity. 1C11-Fk infected cells were then tested for their ability to differentiate into serotonergic neuronal-like cells upon induction. From a morphological point of view, prion infection interferes with the proper onset of neuronal polarity. While $1C11^{5\text{-}HT}$ day 4 cells display bipolar extensions, $1C11\text{-}Fk^{5\text{-}HT}$ day 4 cells form a heterogeneous population with over 70% cells being flat and bearing short neurites. In view of the proposed role of PrP^{C} in neurite outgrowth, this aberrant morphology could be accounted for by an alteration of PrP^{C} normal function due to prion replication. A drastic impact of prion infection could further be measured at the level of serotonin-associated functions. Indeed, $1C11\text{-}Fk^{5\text{-}HT}$ day 4 cells exhibit significantly lowered rates of serotonin synthesis and an altered serotonin metabolism.
The 1C11 cell system provides a novel model to decipher the impact of PrP^{Sc} accumulation on PrP^{C} normal function at a molecular level, notably with respect to PrP^{C} signalling activity. It may help design strategies aimed at abrogating prion replication while preserving PrP^{C} biological activity.

CE-34

STUDY OF SUBCELLAR LOCALIZATION OF PRPC USING CELL LINES TRANSFECTED WITH PRNP GENES.

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PrP^{c} is found predominantly on the surface of neurons, attached by a glycoinositol phospholipids anchor, while PrP^{sc} has been seen accumulating in intracellulary cytoplasmic vesicles. Previously we showed that PrP has been detected in the nuclei of uninfected cells. In this study PrP^{c} was detected more in detail using Prnp transfected cell lines. Cell lines used in this study were, type 2 $PrP^{-/-}$ cell line (HpL 3-4), and mouse or bovine PrP gene transfected cell lines, designated HpL3-4 mPrP or HpL3-4 bPrP. Type-1 $PrP^{-/-}$ cell lines (HpL2), and mouse or bovine PrP transfectant, NpL2 mPrP or NpL2bPrP cell lines were also used. Wild-type mouse neuronal cell line NB3-2 was used as a control.

Anti-PrP monoclonal antibodies (mAbs) T2 and 1D12 were used to detect PrP. Fluorescence were observed with a fluorescence microscope and confocal microscope.

T2 mAb detected immunoreactivity of PrP predominantly on the surface of HpL3-4 mPrP, HpL3-4 bPrP, NpL2mPrP, NpL2bPrP and NB3-2 cells. On the other hand 1D12 mAb detected immunoreactivity of PrP on the surface and nuclei of HpL3-4 bPrP, NpL2 mPrP and, NpL 2bPrP when fixed with acetone. NB3-2 and HpL3-4 mPrP did not show fluorescence in this condition. However several wild type neural cell lines showed intranuclear positive fluorescence using 1D12.

T2 mAb showed reactivity to mouse and bovine PrP on the cell surface. 1D12 mAb showed reactivity to bovine PrP on the cell membrane and organelle inside the nuclei. Role of PrP^{c} in the nuclei using NpL2mPrP could be studied during the course of infection using mouse adapted scrapie agent.

CE-35

THE 37 KDA /67 KDA LAMININ RECEPTOR: A RECEPTOR FOR INFECTIOUS PRIONS INHIBITED BY POLYSULFATED GLYCANES

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Recently, we demonstrated that the 37 kDa/67 kDa laminin receptor (LRP/LR) (1) acts as the receptor of the cellular prion protein (2). Here, we analyzed the binding of the infectious mouse scrapie prion protein (moPrP27-30) to BHK cells with the Semliki Forest virus (SFV) system. The enhanced binding of moPrP27-30 to BHK cells hyperexpressing moLRP::FLAG was inhibited by the LRP/LR specific antibody W3 suggesting that LRP/LR acts as a receptor for PrP^{Sc} (3).
The finding that LRP/LR acts as a receptor for infectious prion proteins was confirmed by a parallel study showing that bovine prions are internalized by human enterocytes, the major cell population of the intestinal epithelium, via LRP/LR (4).
The heparan sulfate mimetics HM5004 and HM2602 reduced PrP27-30 binding to moLRP expressing cells at a concentration of 10 µg/ml to approx. 30% and 20%, respectively, whereas pentosan polysulfate (SP54) and phycarin sulfate (PS3) both reduced the binding at a concentration of 100 µg/ml to approx. 40% (3).
We suggest that the previously reported inhibition of PrP^{Sc} synthesis and prolonged incubation times in rodent models by these polysulfated glycans might be due to the inhibition of the LRP/LR dependent binding of the scrapie prion protein to the target cells (3).
(1) Rieger, et al. (1997) Nat. Med., 3, 1383-8, (2) Gauczynski, et al. (2001) EMBO J., 20, 5863-75, (3) Gauczynski, Nikles, et al. (2006) J. Infect. Dis. in press (4) Morel et al. (2005) Am. J. Pathol. 167(4):1033-1042

CE-36

CELLULAR LEVELS OF PRPSC ARE AFFECTED BY INTERVENTIONS WITH THE MEK1/2 PATHWAY IN PRION-INFECTED GT1-1 CELLS

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The conversion of the normal cellular prion protein, PrP^{C}, to PrP^{Sc} may be facilitated by various factors, which remain to be further characterized. In the present study we demonstrate that brain-derived neurotrophic factor (BDNF), a growth factor of the neurotrophin family, stimulates formation of PrP^{Sc} in a gonadotropin-releasing hormone-secreting neuronal cell line (GT1-1 cells). We also found that the prion-infected cells can be completely cleared from PrP^{Sc} by treatment with inhibitors of MEK1/2, a component of one of the intracellular signaling pathways activated by BDNF. The MEK1/2 inhibitors were also efficient in clearing PrP^{Sc} from prion-infected GT1-1 cells stimulated to accumulate high levels of PrP^{Sc} by enhanced serum concentrations in the medium or by the use of the serum-free, neuron specific neurobasal medium. The neurobasal medium contains Zn and we observed that Zn increases the amounts of PrP^{Sc} in scrapie-infected GT1-1 cells. Zn acts as a co-factor for protein structure (zinc-finger proteins), serves as the catalytic site of many metalloproteins, increases aggregation of amyloid and modulates synaptic activities, but it can also activate the MEK pathway.
We conclude that factors, which can activate the MEK1/2 pathway such as BDNF and Zn, may increase levels of PrP^{Sc}, and, conversely, that inhibitors of the MEK1/2 pathway can clear PrP^{Sc} from prion-infected cells. The MEK1/2 pathway may therefore be a potential target for therapeutic intervention in prion diseases.
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CE-37

INFECTION STUDIES TO DETERMINE THE SUSCEPTIBILITY OF CELL LINES TO DIFFERENT TSE-STRAINS

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Cell culture models are an essential part of the prion research and may facilitate a better understanding of the cellular and molecular processes leading to the formation and accumulation of the disease marker protein (PrP^{res}) and to the infectious agent itself. Cell lines, susceptible to prions and replicating PrP^{res}, provide a basis for these studies. Unfortunately, only a limited number of these cell lines exist until now and the susceptibility of these is strictly limited to certain, mainly mouse adapted prion strains.
In our study, we examined a variety of cell lines from different eukaryotic species, tissues and developmental stages for their susceptibility to several prion strains. Moreover we generate a number of transgenic cell lines, expressing PrP^{C} of heterologous species under the control of different expression systems and investigated, whether this expression leads to an increased susceptibility. We analysed to what extend a successful infection of cell lines is determined by culture conditions or infection protocols and several detection methods were compared to verify even low levels of PrP^{res} accumulations. Infected cell lines were analysed and characterized in regard to strain- and species barriers, the feasibility to increase the PrP^{res} production by selection as well as to the stability of the infection under different conditions.
A large number of cell lines could not be infected or could be infected and lost PrP^{res} after a very short period of time. Nevertheless, we were successful in infecting some of the transgenic cell lines with RML and one not transgenic bovine cell line with natural sheep scrapie. In the majority of cases the susceptibility to prions was limited to a certain species, but not necessarily to certain strains. Cell lines successfully infected with TSE were stable, the infection was repeatable and the accumulation of PrP^{res} could be increased by selection.

CE-38

CONDITIONS OF ER STRESS FAVOUR THE ACCUMULATION OF CYTOSOLIC PRP

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After signal sequence-dependent targeting to the endoplasmic reticulum (ER)[1], PrP undergoes several post-translational modifications, including glycosylation, disulfide bond formation and addition of a GPI anchor. As a result, multiple isoforms are generated. Owing to the intrinsic weakness of the PrP signal sequence, a fraction of newly synthesized molecules fails to translocate and localizes to the cytosol. The physio-pathologic role of this cytosolic isoform is debated. Here we show that in both cultured cell lines and primary neurons, ER stress conditions weaken PrP co-translational translocation, favouring accumulation of aggregation-prone cytosolic species, which retain the signal sequence but lack N-glycans and disulfides. Inhibition of proteasomes further increases the levels of cytosolic PrP. Over-expression of spliced XBP1 facilitates ER translocation, suggesting that downstream elements of the Ire1-XBP1 pathway are involved in PrP targeting. These studies reveal a link between ER stress and the formation of cytosolic PrP isoforms potentially endowed with novel signalling or cytotoxic functions.

CE-39

PRION PROTEIN CO-LOCALIZES WITH NICOTINIC ACETYLCHOLINE RECEPTOR B4 SUBUNIT IN CENTRAL NERVOUS SYSTEM AND GASTROINTESTINAL TRACT.

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PrP^{C}, the cellular isoform of prion protein, is widely expressed in most tissues including brain, muscle and the gastrointestinal tract. Despite its involvement in several bioprocesses such as copper metabolism, neuroprotection and signal transduction, PrP has still no apparent physiological role. During propagation of Transmissible Spongiform Encephalopathies (TSE), prion protein is converted to the pathological isoform, PrP^{Sc}, in a process believed to be mediated by as yet unknown host factors. The identification of proteins associated with PrP may provide information about both the biology of prions and the pathogenesis of TSE. So far, PrP^{C} has been shown to interact with synaptic proteins, components of the cytoskeleton and intracellular proteins involved in signalling pathways. Here, we describe the association of PrP with the β4 subunit of nicotinic acetylcholine receptor (nAChR) as indicated by co-immunoprecipitation assays and double-label immunofluorescence. The interaction between prion protein and native β4 subunit was further studied by affinity chromatography, using immobilized and refolded recombinant PrP as a bait and brain homogenates from normal individuals. Additionally, the participation of β4 subunit in the pathogenesis of TSE was studied by *in vivo* assays. $\beta4^{-/-}$ mice were challenged with the infectious agent and displayed altered incubation times compared to $\beta4^{+/+}$ animals. Our results suggest that PrP^{C} is a member of a multi-protein membrane complex that participates in the formation and stabilization of α3β4 nAChRs. These receptors may play a role in the uptake of the infectious agent and the pathogenesis of prion diseases.

CE-40

METHODOLOGICAL APPROACHES TO CELL CULTURE MODELS OF TSE: SLOWING OF CELL GROWTH INCREASES PRION REPLICATION?

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The ability of prions to replicate *in vitro* is restricted to a few cell lines. Cultures exposed to prions produce only low levels of infectious prions, apparently because only a small percentage of cells become infected. A limiting factor in obtaining a successful infection of cells could be the high rate of cell division, which doesn't allow the prion enough time to replicate itself.
In the present investigation, N2a58 (a mouse neuroblastoma cell line overexpressing mouse PrP), Rov (a rabbit kidney cell line expressing the ovine VRQ allele of PrP in a doxycycline-dependent manner) and Mov cells (immortalized neuroglial cells isolated from mice expressing ovine PrP) were cultivated in soft agar in order to slow their growth and allow a more efficient prion infection. Different culture media were tested.
Growth curves showed a longer period for cell proliferation. This finding has potential implications in terms of designing new cell systems more permissive to prion replication *in vitro*.

CE-41

DEVELOPMENT OF A HIGH THROUGHPUT AUTOMATED SYSTEM FOR SCREENING OF PRION INFECTIVITY

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Until recently, animal models or 'bioassays' have been used to study prion infectivity, which are long, costly experiments with inherent variability. In 2003 a novel approach to assaying infectivity was developed using cells rather than animals[1]. The Scrapie Cell Assay (SCA) is 10 times faster and 100 times cheaper than bioassay. However, as originally developed, the SCA is a standard manual procedure and has an intrinsic technical complexity that does not allow the processing of a large number of samples and lacks good reproducibility. Therefore our aim was to automate the SCA for high throughput screening of RML prion infectivity and reduction of variability in readout. We used a Beckman Coulter Biomeck FX robot due to its suitability for 96 well-plate liquid handling. The SCA posed two crucial challenges to a robot. First, it is cell based and the results depend absolutely on the viability of the cells and the maintenance of their susceptibility to prion infection. Second, the quantification of infectivity requires complex technology – the 'Elispot' technique - for the visual readout of prion positive cells, the units by which infectivity is measured. We first taught the robot how to manipulate cells without increasing the death rate or lose their susceptibility to prions, and we and have now successfully overcome the main obstacles to automated Elispot analysis. The automated scrapie cell assay (ASCA) has been now available in the MRC Prion Unit for the last 10 months. We can run up to 600 samples per week at relatively low cost. We also have introduced a bar code system for sample tracking and data base analysis. The automation of the SCA is a technological breakthrough and will be critical in understanding fundamental unresolved issues of prion diseases. The automation of the technique will also provide a platform for high throughput diagnosis when cell lines susceptible to human prions become available.

1. Klohn, P. C., Stoltze, L., Flechsig, E., Enari, M., & Weissmann, C. A quantitative, highly sensitive cell-based infectivity assay for mouse scrapie prions. *Proc.Natl.Acad.Sci U.S.A* 100, 11666-11671 (2003).

CE-42

PROBING FOR PRPC AMINO ACID RESIDUES INVOLVED IN PRP-PRP INTERACTIONS.

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To understand the underlying mechanism of prion diseases the interaction between PrP^{C} and PrP^{Sc} is being investigated. We previously demonstrated that disease related polymorphisms do not modulate the initial binding of PrP^{C} to PrP^{Sc} and that a subsequent step in the conversion process should be responsible for determining differential conversion efficiencies. Because it is not clear whether the primary binding site of PrP^{C} to PrP^{Sc} is also the site where the actual conversion is initiated (nucleation site) or whether a separate nucleation site is present in PrP, residues involved in protein-protein interaction need to be determined. This may reveal the site(s) responsible for binding and/or initiation of conversion. In order to determine which residues are capable of interacting with PrP^{C} an synthetic solid-phase array containing a complete set of overlapping 15-mer PrP-peptides was probed with wild type (ARQ) sheep PrP^{C} fused to maltose binding protein (MBP-PrP^{C}) and bound MBP-PrP^{C} was detected by indirect ELISA for MBP. The results revealed two distinctive high binding areas the first covering the ovine PrP amino acid residues 30-108, comprising the N-terminal octarepeats, the second covering residues 128-197, encompassing the disease associated polymorphisms in sheep PrP. Based on these results several antibodies and peptides were tested for their capacity to block the binding of MBP-PrP^{C} to the peptides. Parallel to that, the peptides are currently under investigation for their capacity to block PrP^{res} formation in our in vitro conversion assay. Furthermore, binding of MBP-PrP^{C} to the peptides has spurred investigation into direct detection of PrP^{C} and/or PrP^{Sc} in brain homogenates.

CE-43

PROTEOMIC ANALYSIS OF THE PRION PROTEIN IN VIVO

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PrP is thought to be a signaling molecule, yet none of the proteins mediating its signals have been identified. Also, the difficulties in achieving prion replication under chemically defined conditions suggest a role for unidentified host components. We have developed transgenic mice expressing a PrP-mycTag fusion. PrPmyc rescues Shmerling`s syndrome in mendelian crosses, suggesting that it is physiologically functional, and supports prion replication. We are isolating the protein complexes that co-precipitate with PrP^{c} and/or PrP^{Sc}. Towards that goal, we have generated antibodies, POM2 and POM3, with extremely high affinities to well-defined PrP epitopes. POM2 and POM3 discriminate between PrP^{c} and PrP^{Sc}, and are specifically eluted by distinct epitope-mimetic peptides. PrP^{c} and PrP^{Sc} are then recaptured with antibodies to the myc tag, and eluted with myc-mimetic peptide. These unique proteomic tools allow us to confirm the identified interactors and to avoid the risk of losing possible interactions that occur in the same region of antibody-epitope. PrP-associated proteins were purified and are currently analyzed by isotope-coded affinity tags, 1D SDS-PAGE and mass spectrometry.

CE-44

REGULATION OF PRPC TOPOLOGY IS DEVELOPMENTALLY DEPENDENT

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We previously reported that temporal dysregulation of PrP^{C} expression in inducible transgenic mice leads to upregulation of transmembrane ^{Ctm}PrP and severe neurodegeneration. Further analysis suggested that the upregulation of ^{Ctm}PrP may be developmentally dependant. We now extend our previous findings and observe a close developmental correlation between the time point of PrP induction, the degree to which ^{Ctm}PrP is upregulated, and the level of cell death observed. Our data identifies the developmental window between birth and postnatal day 3 to be crucial for *trans*-mediated changes that lead to the dramatic induction of ^{Ctm}PrP and concomitant neurodegeneration. Furthermore, our data substantiates previous finding that ^{Ctm}PrP is a trigger of cell death by a caspase 3-mediated apoptotic pathway. Most importantly, our data prove that wild-type PrP^{C} has the intrinsic capacity to be expressed as either ^{Ctm}PrP or ^{Sec}PrP, which as we have previously shown have distinct functional roles as pro- and anti-apoptotic mediators of cellular survival, respectively. This model system may prove valuable to identify crucial trans-acting factors that may also participate in the aetiology of infectious or sporadic prion disease forms that are suggested to be *trans* mediated.

CE-45

AGGREGATION AND DEGRADATION OF CELLULAR PRION PROTEIN BY NOVOBIOCIN

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A coumarin antibiotic, novobiocin, is known either as a drug depleting the cellular prion protein (PrP^C) from cells or as an inhibitor of the C-terminal chaperone domain of heat shock protein 90 kDa (Hsp90). To elucidate the molecular mechanism of novobiocin-induced depletion of PrP^C from cells, we investigated the interaction between PrP^C and Hsp90 *in vitro* and in cultured cells. Incubation of soluble monomeric recombinant prion protein (rPrP) with more than 10 μM novobiocin induced its misfolding and aggregation *in vitro* in a manner irrespective of the involvement of Hsp90. Novobiocin-induced rPrP aggregates were resistant to limited proteolytic digestion and had higher molecular weight in sucrose density gradient centrifugation analysis. When recombinant Hsp90 was added to the mixture of rPrP and novobiocin, it bound to novobiocin-induced rPrP aggregates but not to monomeric rPrP, indicating that in the presence of novobiocin, Hsp90 bound to the PrP aggregates can not depolymerize or unfold/refold the PrP aggregates. Either cell fractionation analysis or indirect immunofluorescent microscopical analysis demonstrated that some portions of PrP^C on the membrane was coexist or colocalized with some of membrane-associated Hsp90 in Neuro2a neuroblastoma cells. The findings suggests the followings; novobiocin directly causes misfolding and aggregation of PrP^C; novobiocin-induced PrP^C aggregates are recognized by cell membrane associated Hsp90. Finally, it might be hypothesized that the PrP^C-Hsp90 complex formation triggers the degradation of PrP^C via an unidentified Hsp90-related protein degradation pathway, but it remains to be explored.

CE-46

BOTH RAFT-DEPENDENT AND –INDEPENDENT ENDOCYTIC PATHWAYS MEDIATE PRPC INTERNALIZATION IN POLARIZED EPITHELIAL FRT CELLS

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The cellular prion protein (PrP^C) plays a key role in the pathogenesis of Transmissible Spongiform Encephalopathies. In these prion-related diseases PrP^C undergoes post-translational conversion to the infectious form (PrP^{Sc}). Although PrP^C is known to be located in detergent-resistant membrane domains (DRMs or rafts) in both epithelial and neuronal cells, the mechanism underlying its internalization is still debated, as caveolae and clathrin-dependent processes have been described to be involved in different cell types. By combining morphological and biochemical assays we have investigated the mechanism of PrP^C endocytosis in FRT cells, which lack caveolin-1 and caveolae, and in FRT-cav1 cells which possess caveolin and caveolae. We found that in FRT cells PrP^C is internalized through smooth vesicular invaginations from the plasma membrane. PrP internalization is affected by cholesterol depletion and requires activated Cdc-42, which was previously shown to be involved in raft-mediated internalization of GPI-anchored proteins. In addition, we found that PrP^C endocytosis is also regulated by Eps15-Ap2 binding and dynamin 2, suggesting an involvement of a clathrin-dependent pathway. By using FRT-cav1 cells we also show that, although PrP^C resides in caveolae at steady state, caveolae do not participate to its internalization. In conclusion our data indicate that both a raft-dependent and a raft–independent pathway, but not caveolae, have a mutual non exclusive role in PrP^C internalization.

CE-47

□-SHEET-STRUCTURED PRP90-231 INDUCES SH-SY5Y CELL DEATH THROUGH INTRACELLULAR ACCUMULATION.

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Peptides corresponding to amyloidogenic regions of the prion protein showed, at some extent, PrP^{Sc} physicochemical features and possess neurotoxicity *in vitro*. Recently, we have demonstrated that the recombinant peptide corresponding to the 90-231 region of human prion protein (PrP90-231) triggers SH-SY5Y human neuroblastoma cell death, once refolded into a □-sheet-rich structure. To follow PrP90-231 interaction with cells in relation with its toxicity, we have conjugated PrP90-231 to the fluorescent molecule fluorescein-5-isothiocyanate (FITC). By immunoblotting and MTT reduction test, we have observed that PrP90-231 does not undergo proteolytic cleavage due to the tagging process and does not loose its citotoxicity *in vitro*. Hence we have treated SH-SY5Y cells with subtoxic and toxic concentrations of PrP90-231-FITC (10 nM and 1□M respectively) to analyse, by live cell confocal imaging, the interaction of both peptide concentrations with cells. By using organelle-specific viable trackers, we observed that, after 12-24 hours of treatment at both concentrations, PrP90-231-FITC accumulated in SH-SY5Y cytoplasm and localised into organelles showing endo-lysosomal features. At treatment times longer than 48 hours, cells treated with PrP90-231-FITC 10 nM showed the coalescence of peptide-containing bodies into a perinuclear area without evidencing signs of cell degeneration. Conversely, cells treated with 1□M showed a widespread distribution of large bodies containing PrP90-231-FITC and evidenced features of cell death. Moreover, we have measured, by immunoblotting, PrP immunoreactivity in SH-SY5Y lysates after cell treatment with PrP90-231-FITC 1 □M (exposure times ranging from 30 minutes to 48 hours). We have observed that, after 120 minutes, a 16 KDa immunoreactive band was detected indicating binding/internalisation of full-length peptide. After 24 hours, full-length peptide immunoreactivity decreased and lower molecular bands appeared. In conclusion, our data demonstrate that PrP90-231 is internalised and subjected to partial proteolysis by SH-SY5Y; we hypothesize that cell death process, *in vitro*, is triggered when the amount of internalised peptide saturates cellular proteolytic capacity (Grants by PRIN 2004 to TF).

CE-48

PHENOTYPIC CHARACTERISATION OF MICRODELETIONS WITHIN THE HYDROPHOBIC DOMAIN (AA 114-121) OF THE PRION PROTEIN

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Within the N-terminal part of PrP there is a so-called “toxic peptide”, a highly conserved segment comprised amino acids (aa) 105-125. Within the toxic peptide, extending from aa 112-119 there is a palindrome sequence (AGAAAAGA), also known as the hydrophobic domain or transmembrane region. This segment of PrP showed the highest tendency to form amyloid structures, and altering the sequence could modulate its structure and toxicity. The high amyloidogenic potential of PrP (aa105-125) suggested that this region plays a critical role in the interaction of PrP molecules and their conversion into PrP^{Sc}. It was previously shown that PrP mutants with deletion of codons 114 to 121 (Δ114-121) or 112-119 (Δ112-119), which span most part of the subregion AGAAAAGA, were not convertible into PrP^{Sc} and additionally their overexpression resulted in a dominant-negative effect, i.e. inhibition of endogenous PrP^{Sc} accumulation (Hölscher et al., 1998; Norstrom et al., 2005). Recently we created several additional mutants of PrP^{C}, i.e. Δ114-121, Δ114-115, Δ114-117, Δ114-119, Δ116-119, Δ116-121, Δ118-121, Δ120-121 and investigated their convertibility into PrP^{Sc}. This was done by transfecting scrapie-infected N2a mouse neuroblastoma cells with the deletion mutants carrying a 3F4-tag. All mutants but one mutant, Δ114-115, were resistant to conversion into PrP^{Sc}. To analyse the possible dominant-negative effect of the new mutants we are currently doing co-transfection experiments with 3F4-tagged wild-type PrP combined an untagged PrP-mutant. Loss of 3F4-tagged proteinase K resistant PrP in immunoblots will be indicative of a dominant-negative effect of the mutant to be tested. Our present results indicate that very subtle deletions in the hydrophobic domain of PrP are sufficient to inhibit its conversion into PrP^{Sc}. Data about dominant-negative effects of the mutants will be discussed.

CE-49

ALTERED PROCESSING OF PRION PROTEINS PACKAGED INTO NEURONAL CELL-DERIVED EXOSOMES.

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The mechanism by which PrP^{Sc}, the abnormal isoform of the host-encoded prion protein, PrP^{C}, transfers from the site of peripheral exposure to the lymphoreticular system and subsequently the central nervous system (CNS) is uncertain. In this study we establish that exosomes released from prion infected neuronal and non-neuronal cell lines contain both PrP^{C} and PrP^{Sc}. Exosomes from the infected cell lines were efficient initiators of prion propagation in uninfected recipient cells and produced prion disease when inoculated into mice. Moreover, the exosomal associated prion infectivity was transmittable between heterologous cell types in addition to homologous cells, raising the possibility that PrP^{Sc} containing exosomes can mediate infectivity transfer between tissues *in vivo* and suggesting a relationship between prion spread within the CNS and neuronal exosomes. Further investigation has demonstrated that exosomal derived PrP exhibits distinct biochemical properties compared with total intracellular PrP, suggesting altered processing of particular PrP isoforms in this pathway. These data are important for understanding the normal processing of cellular PrP and the dissemination of infectious prions.

CE-50

CHARACTERISATION OF PRPSC USING ALTERNATIVE PROTEASES TO PROTEINASE K

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PrP^{Sc} is involved in transmission of prion disease. Routine detection of this aberrant form of PrP is based on the different sensitivities of PrP^{C} and PrP^{Sc} to proteinase K (PK). PrP^{C} is completely digested by PK while PrP^{Sc} shows variable resistance to digestion resulting in an N-terminally truncated protein of ~27-30kDa (PrP27-30) Using bioinformatic analysis and literature review proteases were identified that should fully digest PrP^{C} but leave PrP^{Sc} intact, including the N-terminal. Of these proteases trypsin, subtilisin and thermolysin were further investigated. Protease digest concentration gradients were performed on control mouse brains and ME7 infected mouse brains to determine the optimum concentration for complete digestion of PrP^{C} but not PrP^{Sc}. Antibodies SAF83 (SPI-Bio), SAF32 (SPI-Bio) and Ab167(Polyclonal anti-peptide antisera raised against peptide of Hamster PrP (29-54) were used to detect intact PrP^{Sc} after protease digestion. SAF83 recognises an epitope in PrP within the central core of PrP that is accessible in PrP^{C} but becomes misfolded during PrP^{Sc} formation and therefore requires PrP^{Sc} to be denatured for detection. Both SAF32 and Ab167 are N-terminal antibodies, SAF32 detects the octapeptide repeat and Ab167 detects a.a. 29-54. We have shown that trypsin, subtilisin and thermolysin all leave PrP^{Sc} intact after digestion but fully digest PrP^{C}. It should now be possible to characterise intact PrP^{Sc} in its native state using an N-terminal antibody following protease digestion.

CE-51

USING YEAST TO INVESTIGATE THE CYTOPROTECTIVE ACTIVITY OF PRP

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Numerous investigations implicate PrP^C in neuroprotection from toxic or stressful insults. In cultured human neurons and mammalian cell lines, PrP^C abrogates apoptosis induced by Bax, a pro-apoptotic member of the Bcl-2 family. Our results demonstrate that when PrP^C is targeted to the secretory pathway of *Saccharomyces cerevisiae* by the addition of a modified signal peptide, it suppresses Bax-induced cell death. Deletion of the octapeptide repeat region of PrP did not affect Bax rescuing activity, indicating that copper binding to this region is not essential for rescue. However, deletion of a charged region (residues 23-31) encompassing an endocytic targeting motif partially eliminated activity, suggesting that PrP internalization may be required. Cytosolic PrP (23-231) failed to suppress Bax-induced death, indicating that protective activity requires PrP targeting to the secretory pathway. To track Bax localization we constructed an N-terminally GFP-tagged Bax protein. Preliminary results indicate that PrP co-expression enhances survival of GFP-Bax expressing yeast. In the majority of surviving cells, GFP-Bax translocates to mitochondria but the cells do not die. In control experiments, co-expression of GFP-Bax with Bcl-2 results in GFP-Bax localization to both the cytoplasm and mitochondria. These results imply that PrP inhibits Bax activity after translocation to mitochondria, perhaps by preventing oligomerization and formation of cytochrome c-releasing pores. Our research goal is to utilize yeast as a model system to investigate PrP mediated protection from Bax-induced cell death by dissecting the rescue mechanism, identifying other gene products involved, and determining their role in this pathway. The yeast system is advantageous because it offers the possibility of performing genetic screens to identify proteins of interest. Consistency between PrP function in he yeast and mammalian systems will allow us to extrapolate the information learned about the protective function of PrP^C in yeast to the more physiologically relevant mammalian systems.

CE-52

JUNCTIONAL EXPRESSION OF THE PRION PROTEIN PRP C BY BRAIN ENDOTHELIAL CELLS: A ROLE IN TRANS-ENDOTHELIAL MIGRATION OF U937 HUMAN MONOCYTIC CELLS

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Conversion of prion protein (PrP c) to its protease-resistant isoform is involved in the pathogenesis of prion diseases. Although PrP c is highly expressed in neurons and other cell types, its physiological function still remains elusive. Here we intended to evaluate its expression, subcellular localization and putative function in brain endothelial cells, which constitute the blood-brain barrier. We detected its preferred expression at intercellular junctions of freshly isolated brain microvessels and cultured brain endothelial cells of mouse, rat and human origin, co-localized with the adhesion molecule platelet endothelial cell adhesion molecule-1 (PECAM-1); moreover, both PrP c and PECAM-1 were present in raft membrane microdomains. Using mixed cultures of wild type and PrP c -deficient mouse brain endothelial cells, we observed that PrP c accumulation at cell-cell contacts was likely dependent on homophilic interactions between adjacent cells. Moreover, we report that anti-PrP c antibodies unexpectedly inhibited transmigration of U937 human monocytic cells through brain endothelial cells, while not affecting cell adhesion. A similar inhibitory effect was observed with four anti-PrP c antibodies and blocking anti-PECAM-1 antibobies as control. Our results strongly support the conclusion that PrP c is expressed by brain endothelium as a junctional protein which is involved in the trans-endothelial migration of monocytic cells.

CE-53

ANALYSIS OF THE DISTRIBUTION OF CELL-RELEASED BOVINE PRP IN AN EXOSOMAL- AND A SOLUBLE FRACTION.

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Proteolytic, phospholipase-mediated and exosome-mediated release of the prion protein have been reported. Recently, exosomes containing PrP^{Sc} released from prion-infected cells were shown infectious, suggesting exosome release as a means of spreading prions between cells (Fevrier et al, 2004). It is therefore of large interest to investigate the targeting of prion proteins to proteolytic, phospholipase-mediated or exosome-mediated release.
We found the PrP released from transfected cells in a soluble fraction and an exosome bound fraction. The soluble fraction contained proteolytical shed PrP cleaved at the extreme C-terminal end. The exosomal fraction contained the GPI-anchored PrP. A deletion mutant in the C-1 cleavage site affected the C-1 cleavage and a full length PrP was found in the exosomal fraction. Further analysis of the distribution of the PrP in the exosomal fraction and the interaction of the released PrP with various cells are currently being studied.

CE-54

THE ROLE OF PRPC PROTEIN IN NEURAL DIFFERENTIATION OF NSCS INTO NEURONAL AND GLIAL LINE

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The definite role of PrPc protein has not been established yet, however one of PrPc putative roles is its involvement in cell differentiation. The aim of this study was to investigate the possible role of PrPc in differentiation of neural stem cells (NSCs) into neuronal and glial cells, induced in vitro by serum deprivation. We examined the expression pattern of PRNP gene in relation to differentiation at mRNA (RT-PCR) and protein level (immunocytochemistry). We detected the expression of PRNP gene in all steps of differentiation, however, no significant changes in PrP mRNA level were observed. Immunocytochemical analysis demonstrated the presence of PrPc protein during all steps of neuronal cells differentiation which were marked by expression of stage specific proteins: nestin, β-III-tubulin, MAP-2. The expression of PrPc was also detectable in subset of GFAP-positive maturating glial cells. Our results suggest that PrPc expression during differentiation is not restricted to the neuronal cells only, since we revealed PrPc expression in glial cells as well. It may imply PrPc involvement in differentiation of astrocytic and neuronal cells.

(The study was partially supported by KBN grant No. 2P05A 13 29)

Poster Session

DIAGNOSIS

DIA-01

NEUROPATHOLOGICAL AND IMMUNOCHEMICAL FINDINGS OF SUSPECT ATYPICAL SCRAPIE CASES DETECTED IN THE SPANISH SURVEILLANCE PROGRAMME (2003-2005)

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During the last 5 years, scrapie surveillance has been encouraged with the aim of detecting most of the scrapie-affected animals; eradicating the scrapie disease and improving the detection of BSE cases in sheep or goats. As a consequence of the increase in the number of analysis, the number of scrapie cases has also been incremented and atypical cases of scrapie have been described along Europe (E.g. Nor98 cases). The European Food Safety Authority has defined an atypical scrapie case as: samples mild positive to rapid Western Blot (high concentration of PK); clearly positive to modified Western Blot (low concentration of PK) and a low band of <12KDa and finally highly positive in the cerebellum and mildly positive in the brainstem by immunohistochemistry. A retrospective study has been done analyzing suspect atypical scrapie cases diagnosed from 2003 to 2005 by the National Reference centre for TSEs of Zaragoza. Following the EFSA recommendations and the immunochemical protocols developed by Gonzalez et al, the envisaged techniques have been developed: Vacuolar lesional profile; Reactivity of intracellular PrPd to N-terminal antibodies (epitope mapping) and phenotypic characterization by immunohistochemistry with the P4, 6H4, R145 antibodies; Modified Western Blot to determine de glycoform ratio and molecular weights. In addition, the entire PRNP gene coding region of the selected animals has been sequenced in order to visualize the present SNPs.
The results obtained reveal the presence of atypical scrapie cases in our country. In addition, these cases could have been sub estimated because the surveillance applied was based on the analysis of the brain steam by rapid Western Blot**.**

DIA-02

DETECTION OF PRPSC IN PLASMA FROM SHEEP USING A MULTIMER DETECTION SYSTEM - 3D.

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Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases that includes bovine spongiform encephalopathy (BSE), scrapie in sheep and goats, and Creutzfeldt-Jacob disease (CJD) in humans. The putative agent is a host cell surface glycoprotein, the prion protein, that aggregates after misfolding. Reports of transmission of both scrapie and BSE by blood transfusion in sheep have raised concern for the safety of blood and blood products. This concern has heightened after cases of vCJD contracted from blood transfusions were reported.
We have developed a system called Multimer Detection System (MDS), in which a specific epitope-overlapping antibody system is used to differentiate the aggregated PrP^{Sc}/PrP^{C} (multimer) from PrP^{C} (monomer). MDS detected the PrP^{Sc} in various infectious samples - brain homogenate, plasma spiked with brain containing PrP^{Sc} from mouse and plasmas from a diseased hamsters and sheep. The MDS system was improved when we change from a plate to magnetic bead format (MDS-3D). The MDS-3D worked optimally when the plasma sample, buffer, magnetic beads coated with capturing antibody and detection antibody were added simultaneously. This could reduce the assay time by eliminating several incubation steps. Using the MDS-3D system, plasma from diseased sheep could be easily differentiated from normal animals. The MDS-3D system could be applied to human samples and potentially ensure the safety of the blood and blood products.

DIA-03

DIAGNOSTIC VALUE OF CSF BIOMARKERS IN HUMAN PRION DISEASES

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Human transmissible spongiform encephalopathies (TSE), in particular Creutzfeldt-Jakob disease (CJD), are invariably fatal neurodegenerative diseases. Clinical signs and symptoms at early stage may not be easy to distinguish from other diseases by neurological findings. Diagnostic biochemical parameters, including 14-3-3 protein and tau levels in cerebrospinal fluid (CSF), have been used as diagnostic markers but these markers can also be detected in other conditions. We aimed to evaluate CSF levels of 14-3-3 protein, tau, p-tau and Ab42 as diagnostic biomarkers of human TSE in a clinical settting. CSF samples from 47 patients with a definite diagnosis of TSE and 155 non-TSE controls were analyzed. 14-3-3 protein was detected by Western blot while CSF levels of tau, p-tau and Ab42 were determined by commercially available ELISA kits. A positive CSF 14-3-3 protein was detected in 44/47 TSE patients versus 11/109 non-TSE controls (sensibility 0.94; specificity 0.90; positive predicting value 0.80; negative predicting value 0.97). Tau levels and p-tau levels (mean±SE, pg/ml) resulted significantly higher in TSE (respectively 5268.9±890.7 and 37.1±8.5 versus 507.5±41.6 and 19.2±2.0) compared non-TSE subjects ($p<0.01$) while there were no differences in Ab42 levels (340.0±35.6 versus 250.6±79.2, $p=0.99$). The detection of 14-3-3 protein in CSF and the measurement of tau and p-tau levels confirm to be valuable biomarkers in diagnostic work-up of human prion diseases in vitam.

DIA-04

A FURTHER STEP IN THE CHARACTERIZATION OF A NEW GROUP OF MONOCLONAL ANTIBODIES

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In the past few years some new monoclonal antibodies have been raised against both recombinant bovine PrP and the synthetic peptide corresponding to the amino acid sequence of bovine PrP 153-172. They have been characterized by western blot and IHC for their ability to recognize cellular and pathological PrP from different animal species (Barbieri et al., Prion 2003, Munich 8-10 October 2003).
Here we report on the use of such AcM in the detection of PrP from a set of brain samples from wild ruminants including deer, roe-deer, chamois, fallow-deer and ibex.
The AcM directed to the N-terminal part (3G5, 3H1, 4D7), the core region (2B10 and 4E3) and the C-terminal part (4G11) of PrP were able to recognize PrP^C from all the samples.
Among AcM raised against the synthetic peptide, 3E2 recognized PrP from all samples while 3G6 was able to bind no species. These data are consistent with previous results indicating that 3G6 is specific for bovine/human PrP, while 3E2 has higher affinity for sheep and goat PrP with which wild ruminant PrP share a very high degree of amino acid sequence homology (97.2% to 99.6%). Data on the characterization of a new AcM (4E12) able to specifically recognize the mono and unglycosylated form of both cellular and pathological PrP from different animal species including bovine, sheep, goat, hamster and human will also be presented.
AcM were also tested on BASE samples. AcM to the N-terminal part of PrP were unable to detect both BSE and BASE suggesting their epitope is lost after digestion with Pk both in BASE and BSE as expected due to the amino acidic sequence similarity between BSE and BASE PrP (Casalone et al., PNAS, 101, 3065-3070, 2004). AcM to the core of PrP and to C-terminal are able to recognize BASE and the molecular features that distinguish it from BSE such as different glycosylation ratios and lower molecular weight of unglycosylated PrP.

DIA-05

A MULTICENTRE VIRTUAL BANK OF ANIMAL TISSUES AND FLUIDS

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One of the major activities of this Network of Excellence has been the establishment of a virtual bank of animal TSE samples. This objective has been achieved, and is supported by more than twenty institutes representing sixteen countries of NeuroPrion members, and some associate members. The principal goal of this work is to facilitate contact between research groups by publicising the generic content of stores of potentially available TSE material, enabled by centralised data collection software.
The database is located within eDoc on the NeuroPrion website. Researchers can access pdf files that describe the type of bovine, ovine or caprine samples held by each institute, collection protocols, blood stabilisers used and storage media. Email addresses provide links to the primary points of contact at each site, although the publication of such information does not guarantee access to samples, and will be subject to the release conditions operated by each institute**.**

DIA-06

SELDI - ASSISTED IDENTIFICATION OF BIOMARKERS FOR TSE DIAGNOSIS

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Definitive tests for the detection of TSE disease are currently restricted to the presence of the protein PrP^{Sc} found in tissue biopsies. For a pre-clinical diagnostic test to be effective, early markers must be established which ideally can also be found in body fluids such as blood. Our approach to finding new surrogate markers uses differential protein expression profiling and SELDI-MS-TOF technology in brain samples from a well characterised murine scrapie model to establish a panel of markers for scrapie diagnosis. The murine model used in this experiment (intracerebrally injected with ME7scrapie isolate) displays severe pathology in the hippocampus where it was thought that the highest number of disease specific markers would be found. Many potential biomarker profiles were detected by this method, some in the early stages of disease before clinical signs were obvious. The profiles can be used collectively as a panel of markers without formal identification however, by identifying individual proteins we can also establish potential single markers and their role in TSE pathogenesis. We have purified and identified individual proteins using mass spectrometry including SELDI, and western blotting techniques. The identified proteins were then localised in brain sections using immunocytochemical techniques.
Proteins associated with protein folding and oxidative stress have been identified. Other groups have previously highlighted the importance of these pathways in scrapie pathogenesis however the use of these proteins as a panel rather than individual biomarkers will increase the potential of establishing a definitive diagnostic test for TSE disease. Having established and identified markers using this technique in brain tissue we intend to further this work by examining other tissues and blood.

DIA-07

AMPLIFIED IMMUNOHISTOCHEMICAL DETECTION OF PRPSC IN ANIMAL TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES USING STREPTOMYCIN.

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Due to its sensitivity, immunohistochemistry (IHC) of abnormal prion protein (PrPsc) is used to study experimental and natural cases of transmissible spongiform encephalopathies (TSEs) such as Creutzfeldt–Jakob disease in humans or scrapie and bovine spongiform encephalopathy (BSE) in animals. The limits of detection are particularly critical when PrPsc IHC is used for diagnostic purposes. Here, we describe the use of streptomycin sulfate in IHC, providing a novel original and easy way to amplify specifically PrPsc immunohistochemical detection in natural cases of BSE and scrapie, as well as in experimental TSEs in mice models using different PrP antibodies.

DIA-08

RAPID TEST APPLICABILITY IN DETECTION OF CERVID PRP

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Transmissible spongiform encephalopathies (TSE) occur in a number of animal species, (e.g. scrapie in sheep and goats and BSE in cattle). Chronic wasting disease (CWD) has emerged as an important TSE of captive and free-ranging cervids in North America, naturally affecting several species including mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*) and moose (*Alces alces shirasi*). National surveys for TSE in cervids have recently been performed in Europe, the largest of them in Germany (n=6440). As successful surveillance and eradication strategies depend heavily on the tests used, we compared various commercially available rapid tests, originally developed for BSE and Scrapie screening, against prion protein (PrP) in cervids. Positive control samples (confirmed by PrP immunohistochemistry) originated from CWD cases from North America. Three ELISA-based tests, two western blots and one immunochromatographic assay by the companies Bio-Rad and Prionics were evaluated (additional data from other rapid tests will be presented soon). Brain samples from roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus elaphus*) and fallow deer (*Dama dama*) sampled from different geographical regions in Germany were tested without using the proteinase K digestion step in PrP purification. Test procedures, especially those related to dilutions and other modifications to enhance sensitivity, were conducted according to the manufacturers. The western blot systems were used as reference to compare dilutions of PrP. All six tests evaluated were found to be suitable to detect PrP from cervid species tested. However, the analytical sensitivity and specificity demonstrated significant differences between the tests. We suggest the Bio-Rad Western Blot as reference as well as test of the choice for the detection of American cervid PrP giving highest values in quantitative and qualitative sensitivity. The Prionics LIA is best suited for detection of PrP from German cervid species. For future studies on cerivd PrP we recommend modification of the standard protocols, e.g. additional precipitation steps, triggering sensitivity to higer values.

DIA-09

THE SEARCH FOR DIFFERENTIAL GENE EXPRESSION IN CATTLE SPLEEN AND WHITE BLOOD CELLS DURING BSE INFECTION

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Differential gene expression within peripheral tissues of BSE infected cattle could provide the means of identification for early BSE infection. The oral route of infection by the infected agent (PrP^{res}) passes from the gastrointestinal system to the CNS via the lymporeticular system. Little is known of the effect on gene expression of the host organs during this early infection period; although there is some evidence of decreased levels of erythroid differentiation-related factor (EDRF) in the spleen and erythroid cells in the blood of TSE infected mice. Therefore there is a possibility of using differential gene expression in peripheral tissues as an early diagnostic test for BSE infection.
In this project we are investigating the differential gene expression between normal and BSE infected cattle using RNA isolated from spleen and white blood cells. Spleen samples were collected from a DEFRA funded controlled BSE challenge project that was carried out at VLA in Weybridge using Holstein cattle. Blood samples were collected from a BSE challenge set up at Greifswald, Germany using Simmental cattle. Blood samples were collected during the BSE incubation and initially fractionated into PBMs (peripheral blood monocytes). At later time points individual white blood cells were separated into B cells, T cells and macrophages. RNA isolated from the spleen and PBM samples was used to probe bovine specific microarrays, using both dual colour array and the Affymetrix Bovine Genome Array.
Analysis of the microarray experiments produced gene lists of significantly differentially expressed genes. Interestingly there are more down regulated genes during BSE infection compared to up regulated genes against age matched controls. These results require verification by quantitative PCR and classification into groups and families to enable a full understanding of the significance of these findings. It is possible that the outcome of this project could produce a panel of differential genes which may be used as part of an early diagnostic test for BSE infection.

DIA-10

COEXISTENCE OF BRAINSTEM LEWY BODIES, SYNUCLEIN NEURITES AND PRPTSE TYPE 1 IN A PATIENT WITH A 120 BASE PAIR INSERTION IN THE PRNP GENE PRESENTING WITH AN AKINETIC-RIGID SYNDROME.

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We report a 68 years old man with no positive family history for neurological diseases presenting with an akinetic rigid syndrome, resistant to levodopa therapy, who 1 year and 5 months after onset, developed loss of sphincter control and myoclonus. Repeated EEGs did not show CJD characteristic changes, MRIs disclosed only a progressive cortical atrophy, and the 14-3-3 proteins were absent in the CSF. He then developed akinetic mutism, generalized myoclonic jerks, and finally died 20 months after onset. At autopsy, he had diffuse cortical atrophy, more evident in the fronto-temporal areas, cortical and basal ganglia spongiform changes, astrocytic hyperplasia, and synaptic immunostaining for PrP, more prominent in cerebellum. Lewy bodies and neurites were disclosed in the substantia nigra and in the locus coeruleus in spite of no obvious macroscopic discoloration. At the western blot, we found the type 1 PrP^{TSE} (according to the classification of Parchi and Gambetti). *PRNP* analysis revealed valine homozygosity at codon 129, a novel 5 octarepeats-insertion (R2-R2-R2a-R2-R2) between the R3 and R4 wild type sequence, and a point mutation at codon 211 corresponding to a change from glutamate to lysine residue. Cloning analysis is in progress to discriminate whether the insertion mutation segregates in the same allele of the point mutation or not. Occasionally, sCJD may present with an akinetic rigid syndrome mimicking progressive supranuclear palsy syndrome, corticobasal degeneration, or parkinsonism syndromes. Only two cases have been reported with pathological coexistence of idiopathic Parkinson disease and sCJD. However, the genetic complexity of this case is unique and further studies are needed to sort it out the effect of these mutations in the formation and accumulation of PrP^{TSE} and on the coexistence of Parkinson-like lesions.

DIA-11

MAGNETIC RESONANCE FEATURES OF AN ITALIAN CASE OF GERSTMANN-STRÄUSSLER-SCHEINKER DISEASE WITH P102L PRNP MUTATION

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Gerstmann-Sträussler-Scheinker disease (GSS) is a hereditary spongiform encephalopathy most commonly associated to a mutation at codon 102 of the prion protein gene (PRNP). The disease can present with ataxia, spastic paraparesis, extrapyramidal syndrome, dementia or a rapidly progressing neurological syndrome similar to a Creutzfeldt-Jakob disease (CJD). We report a 58 years'old patient with a 2 year history of imbalance and dyslalia and a paternal history of presenile dementia. After 9 month from the onset the neurological examination showed imbalance and slurred speech. The MRI of the brain revealed minimal enlargement of subarachnoid sulci and supratentorial ventricular system without signal alterations. Two years later, the neurological examination revealed a very important gait difficulty with imbalance, dysmetric saccades with fixation instability, diffuse decreased tone, decreased lower limb tendon reflexes, slurred and hecolalic speech and behavioral alterations with apathy and depression. The MRI of the brain showed slight diffuse atrophy and the presence of marked hyperintensity on T2-weigthed and Diffusion-weighted images in the cerebral cortex. The cerebral spinal fluid examination revealed the presence of 14.3.3. protein and an increased Tau protein level. The molecular analysis of the PRNP demonstrated the P102L mutation that confirmed the clinical suspect of Gerstmann-Sträussler-Scheinker disease. The usual imaging findings for GSS are cerebellar and cerebral atrophy and, rarely, a decreased signal changes on T2-weighted images. In one case a high signal change on T2-weighted images in the basal ganglia was reported. The MRI pattern in our case is more suggestive for the sporadic form of CJD whose hallmark is the finding of symmetric changes in the basal ganglia and cerebral cortex. Our findings point to similarities in the MRI findings between CJD and GSS with P102L PRNP mutation, especially when the latter condition evolves to a rapidly progressive neurological disease.

DIA-12

USE OF PROBIT ANALYSIS TO COMPARE RAPID TSE ASSAYS

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Assessment of assay sensitivity should ideally be made using challenging samples which have low concentrations of the analyte of choice and/or are from animals in the very early stages of the disease. The number of TSE positive samples available for assay comparison and evaluation are limited and with the declining incidence of BSE, these samples are likely to become increasing scarce.
The method most widely recommended and used, for sensitivity assessment, is the testing of serial dilutions of numerous positive samples. Serial dilutions have the benefit of producing at least one sample that is close to the assay cut-off but can lead to the production of copious data which are difficult to compare and quantify.
In this study the Enfer TSE Version 2 and Version 3 assays have been assessed by the testing of 140 dilution series, the data produced have been subsequently subjected to Probit analysis. The graphs used for this analysis comprise of plots of the number of samples detected as positive at each dilution against the dilution and allow a simple means of visual comparison. Additionally, an ED50 (median effective dose) has been calculated, this is the theoretical titre at which half of the samples are detected as positive. Data and analysis are also presented where Probit analysis has been used to compare Enfer assays performed manually and by automated processor and also to compare two different types of rapid TSE assays.
Probit analysis has been shown to be a simple method for comparing data from the testing of multiple serial dilutions, providing quantifiable estimates of relative sensitivity (ED50) and enabling comparison of assays with differing outputs such as colorimetric, chemiluminescent and line assays.

DIA-13

DEVELOPMENT OF A BANK OF STANDARDIZED BLOOD SAMPLES FOR THE EVALUATION OF FUTURE PRION DIAGNOSTIC TESTS.

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The risk of interhuman transmission of vCJD via blood is not anymore theoretical with the three cases now attributed to blood transfusion in Great Britain. For the moment, the only protective measures are based on exclusion of donors depending on their background, and on filters for leucodepletion. Screening of blood donors, like it is systematically made for different viral diseases including HIV, would constitute a reinforcement of transfusion safety. Unfortunately, the only tests available and validated in Europe for prion diagnosis are post mortem tests for TSE in ruminants, and are only able to detect PrPres in brain or peripheral organs but not in the blood.

Different tests are currently under development, each of them claiming performances very difficult to estimate in the absence of common standardized samples. Moreover, most of the evaluation trials are performed with blood samples from healthy animals spiked with positive brain, which apparently reflect poorly the properties of the abnormal PrP present in blood. Last, very limited data are generated on blood samples from animals before the onset of clinical stage, to reflect the situation of patients donating blood during the silent incubation phase.

In the context of the NeuroPrion Blooddiag project, we have constituted a library of blood fraction samples from sheep and goats that were experimentally or naturally infected with scrapie versus healthy controls in different research programs. All the animals were referenced and sampled regularly, and blood was fractionated with an adapted protocol, similar to the one used for humans by the French Transfusion Center of Lille (France). Infectivity of the different collected blood fractions (plasma, platelets, red cells and buffy-coat) has been assessed by inoculation of transgenic mice overexpressing ovine PrP gene (tg338). Successful transmissions have already been obtained with several samples and bioassays are still ongoing. With complete information about the animals (genotype, source of contamination, days post-contamination, presence of clinical signs), this unique library comprises now more than 8000 aliquots from the fractions of more than 110 different blood samples. This bank will constitute a powerful tool for the evaluation of the different blood tests currently under development and which already claim for future protection of blood transfusion.

DIA-14

PRION PROTEIN IN MILK AND BLOOD

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The low concentration of the proteinaceous agent in body fluids and its long incubation time complicate epidemiologic analysis and estimation of spreading and thus the risk of human infection. We have developed an adsorption matrix, *PrioTrap*™, which binds with high affinity and specificity to prion protein. The exceptional binding properties of *PrioTrap*™ result from multiple binding motives that recognize different prion protein epitopes, allowing quantitative enrichment of extreme low quantity of prion proteins in biological fluid, like milk or blood. We show that prion protein (PrP^{C}) – the precursor of prions (PrP^{Sc}) – is present in fresh milk from humans, cows, sheep, and goats. The absolute amount of PrP^{C} differs between the species. PrP^{C} is also found in homogenised and pasteurised off-the-shelf milk, and even ultra-high temperature treatment only partially diminishes endogenous PrP^{C} concentration. In view of a recent study showing evidence of prion replication occurring in the mammary gland of scrapie infected sheep suffering from mastitis, the appearance of PrP^{C} in milk implies the possibility that milk of TSE-infected animals serves as a source for PrP^{Sc}. These findings may be relevant for assessing the health risk associated with consumption of milk and milk-derived products. There is also increasing evidence that prions are present in body fluids and that prion infection by blood transmission is possible. As a component of a BSE Test for Live Cattle *PrioTrap*™ is able to diagnose a TSE infection in animals before appearance of clinical symptoms. In this regard we will report on a field study with 1200 post mortem confirmed positive and negative cattle.

DIA-15

CREUTZFELDT-JAKOB DISEASE IS A PROFOUNDLY BEHAVIORAL DISORDER.

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We evaluated the extent and pattern of neuropsychiatric involvement in Creutzfeldt-Jakob Disease. There has been a dearth of formal evaluation of the neuropsychiatric features of sporadic (sCJD) and genetic (gCJD) forms of human prion disease. As a major U.S. referral center for prion disease, we have noted behavioral symptoms to be rather prominent, early features. The neuropsychiatric features of vCJD have been formally evaluated and are profound, yet few, if any, studies have formally evaluated the behavioral features of sCJD and gCJD. The Neuropsychiatric Inventory (NPI; Cummings 1997) is a validated measure for assessing behavioral features in neurodegenerative diseases. Caregivers of 45 CJD subjects (36 sCJD & 9 gCJD), 46 frontotemporal dementia (FTD) subjects, and 199 Alzheimer's disease (AD) subjects were administered the NPI at initial presentation to our center. SPSS was used for statistical analysis; chi-squares (Pearson), one-way ANOVA (Tukey), and Pearson Correlation analysis. Due to multiple comparisons, a cut off of $p < 0.001$ significance was used. The most common behavioral changes in CJD were depression (65%), apathy (65%), and eating/appetite (63%). In contrast, the most common behavioral changes in FTD were apathy (90%), eating/appetite disorder (88%), and disinhibition (83%) and in AD were apathy (49%), depression (43%), and anxiety (38%). CJD subjects had worse delusions, hallucinations and depression than FTD and AD.CJD was similar to FTD in anxiety, aberrant motor behavior, sleep problems, agitation and euphoria.
Behavioral features are a very common in sCJD and gCJD and may be the earliest manifestations of the illness. As a behavioral disorder, CJD lies along a spectrum between AD and FTD, often closer to FTD. Inclusion of neuropsychiatric features in diagnostic criteria needs to be evaluated and may improve diagnostic sensitivity and allow for early diagnosis.

DIA-16

CSF FIDINGS IN A UNITED STATES SPORADIC CJD COHORT

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To determine the CSF profile and diagnostic sensitivity of CSF proteins in a large US sCJD cohort.
As a major prion research center in the US, we have been referred more than 800 potential CJD cases over about the past five years. Data on these patients is stored in a clinical relational database. This database was queried for various CSF findings including cell count (n=132), protein (n=140), IgG index (n=34), oligoclonal bands (n=38), 14-3-3 (n=131), neuron specific enolase (NSE; n=33), total tau (t-tau; n=19) in patients with probable and definite sporadic CJD. T-tau was positive if >1200 pg/ml. NSE was positive if >20 ng/ml. 14-3-3 was measured primarily by western blot (NPDPSC, Cleveland, Ohio). 14-3-3 Diagnosis of probable CJD was based on modifications to WHO 1998 criteria, 2 of the following six sets of symptoms 1. Extrapyramidal/pyramidal 2. Cerebellar 3. Visual 4. Other focal cortical signs (e.g., aphasia, apraxia, neglect) 5. Myoclonus 6 Akinetic mutism AND a positive MRI or EEG. 14-3-3 was not used for diagnosis.
CSF protein level was elevated in 42% of cases (range of 0-123, median 47 & mean 50 mg/dl). WBCs (>6 per HPF) and OCBs were each elevated in 8% of cases, while only 1 subject had an elevated IgG index. The 14-3-3 had a sensitivity of only 50% (47% for definite sCJD and 52% for probable sCJD). T-tau had a sensitivity of 68% (78%for definite and 60% for probable sCJD), while NSE was the most sensitive CSF marker at 73% (84% for definite and 71% for probable sCJD).
Our results are in contrast to other published data that suggest high sensitivity of 14-3-3 and t-tau for sCJD. Interestingly, the only protein marker used in the WHO criteria, 14-3-3, had the lowest diagnostic sensitivity in our study. These proteins should be used with caution. The specificity of these markers is being evaluated in a cohort of non-prion rapidly progressive dementias.

DIA-17

VISUALIZATION OF PRP-AMYLOID IN GERSTMANN-STRÄUSSLER-SCHEINKER (GSS) DISEASE WITH [F-18]FDDNP

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Positron emission tomography was used in conjunction with 2-(1-{6-[(2-[F-18]fluoroethyl)(methyl) amino]-2-naphthyl}ethylidene) malononitrile; [F-18]FDDNP) for visualization of pathology in the living brain of GSS patients. Five subjects with the F198S and P102L mutations in *PRNP* and five controls were scanned. Logan graphical analysis with arterialized venous blood input function was applied for quantitative data analysis. The [F-18]FDDNP distribution volume (DV) parametric images were generated and the region-of-interest (R0I) analysis was performed. *In vivo* results were compared with the known brain pathology pattern. [F-18]FDDNP DV parametric images of GSS subjects show the following results: (1) two symptomatic F198S cases (GSS1 and GSS4) have significantly increased DV values in the basal ganglia and cerebellum (GSS1) and in BG, thalamus and CB (GSS4); neocortical regions have lower DV values compared to those observed in CB, BG, or thalamus; (2) of two pre-symptomatic subjects (GSS2 and GSS5) the older one GSS2 (45 y) has increased BG and thalamus DV values, comparable to the symptomatic subjects and significantly higher than in controls, and cerebellar DV values comparable to controls; the younger pre-symptomatic subject GSS5 (30 y) has no pathology, with DV values within the limits observed in controls for all brain areas; (3) the P102L subject (GSS3) shows qualitatively similar, but milder, pattern of [F-18]FDDNP accumulation in subcortical areas as the two asymptomatic F198S cases. All symptomatic cases show decreased glucose utilization in affected areas. We have demonstrated feasibility of *in vivo* detection of prion pathology using [F-18]FDDNP, a probe with affinity for amyloid-like protein aggregates.

DIA-18

CEREBROSPINAL FLUID PROFILE IN HUNTINGTON DISEASE

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Cerebrospinal fluid (CSF) biomarkers in patients with Huntington disease (HD), Creutzfeldt-Jakob disease (CJD), normal pressure hydrocephalus (NPH), and in healthy controls were evaluated for their potential diagnostic value.
Cerebrospinal fluid was assayed for tau, amyloid 1-42 (A 1-42), neuron-specific enolase (NSE), astrocytic protein S 100b, and cellular prion protein (PrP^C) using commercially available ELISA. Tests were conducted in 15 patients with HD, 17 definite CJD cases with negative 14-3-3 protein (CJD-), 19 definite CJD cases with 14-3-3 positive (CJD+), and in 12 healthy controls.
The CSF levels of tau and A 1-42 were significantly different in HD compared with CJD– (p=0.007 and p=<0.001). There were significant differences of tau (p=<0.001), NSE (p=<0.001) and S 100b (p=<0.001) levels between CJD+ and HD group. No significant difference between HD and healthy controls was found. A significant elevation of NSE level in HD group compared with NPH was detected, as well. In addition, the 14-3-3 positive CJD group exhibited significantly elevated tau (p=<0.001), A 1-42 (p=0.029), NSE (p=<0.001) and S 100b (p=0.001) levels in comparison with 14-3-3 negative CJD group. No significant differences of PrP^C levels between HD and other groups neither differences between CJD+ and CJD- groups were found.
No difference of tau, A 1-42, NSE, S 100b levels between patients with HD and healthy controls was found. Long disease duration, different pathophysiological processes in demential disorders and specific lesion profile in HD may be possible causes of these findings. The mechanism of 14-3-3 elevation and release of other markers into CSF in different stages of CJD is still unknown. However, there is a correlation of CSF marker levels with 14-3-3 positivity/negativity.

DIA-19

DIAGNOSIS OF PRECLINICAL SCRAPIE IN RECTAL BIOPSY SPECIMENS

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The current approach for sheep scrapie surveillance is based on laboratory examinations for disease-associated prion protein (PrP^d) on samples of central nervous system (CNS) collected at post-mortem. Diagnosis of scrapie in live asymptomatic sheep has so far been confined to palatine tonsil or third eyelid biopsies, but these are not practical field procedures. Blood tests are being developed, but their diagnostic efficiency is still to be shown.
We conducted a large survey of immunohistochemical (IHC) detection of PrP^d in rectal mucosa samples collected at post-mortem. Positive results were obtained in 97% of clinical and 86% of preclinical scrapie cases; these rates of PrP^d detection were not affected by factors such as breed, PrP genotype or age of the animals. Furthermore, we assessed the efficacy of rapid tests on the same samples; once optimized, their sensitivity reached 94% of that of IHC.
We have also performed IHC examinations in around 400 rectal mucosa biopsy samples collected from asymptomatic sheep of susceptible PrP genotypes exposed to natural or experimental infection. In natural disease, PrP^d was initially detected approximately half way through the incubation period, and the rate of detection increased in subsequent samplings, as did on tonsil biopsies taken simultaneously. Rectal biopsies also provided positive results animals infected experimentally with either scrapie or BSE agents, with similar efficiency regardless of the route of challenge. Rectal biopsy samples selected from sheep with previous IHC positive results were examined by rapid methods, which provided sensitivity values near 100%.
With training, rectal mucosa biopsies can be performed simply and quickly. Anaesthesia or sedation is not required, and the procedure has no adverse effects on the sheep even when carried out repeatedly. Detection of PrP^d in rectal biopsy samples is a promising approach for diagnosis of clinical and preclinical scrapie in live animals, which can be applied for the screening of sheep flocks; it is also providing good results for the detection of chronic wasting disease in cervids. Samples of rectal mucosa can also be easily taken from animals that are culled as a result of scrapie control measures or that are slaughtered in abattoirs, and can complement scrapie surveillance and monitoring.

DIA-20

TAU DEPOSITS IN VARIANT CREUTZFELDT-JAKOB DISEASE – IMMUNOHISTOCHEMICAL AND BIOCHEMICAL CHARACTERISATION.

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The deposition of hyperphosphorylated tau around PrP plaques in variant CJD (vCJD) has been previously described.[1] The aim of this study is to investigate whether these deposits are unique to vCJD or whether they can found in other forms of prion disease associated with PrP plaques. Twelve patients with vCJD (age 16-62 years, disease duration 6-20 months), 8 patients with sporadic CJD (4 MM1: age 60-75 years, disease duration 2-8 months; 4 MV2A: age 42-71 years, disease duration 7-42 months) and 2 patients with GSS (P102L mutations: age 44-45 years, disease duration 8-89 months) were investigated. Tau immunohistochemistry was performed using 10μm brain sections using AT8 (phosphorylated epitopes 199-205) and pS404 (recognising phospho-tau at serine404). Immunohistochemical analysis of PrP^{res} was undertaken using 6H4. Tau protein was extracted as from the frontal cortex and subjected to SDS-PAGE with immunoblotting with AT8 and pS404.Widespread clusters of tau deposits associated with PrP amyloid deposits were found in the frontal cortex of vCJD patients. These immuno-reactive tau species were seen in fine neuritic processes. Similar deposits were also seen in the molecular and granular layer of the cerebellum. Smaller numbers of tau deposits were also seen in the frontal cortex of MV2A sCJD patients, although these were less clustered in nature. These deposits were also seen in association with PrP plaques. In contrast, there were only a few tau reactive species found in the cerebellum of MV2A sCJD patients. The GSS cases that were examined showed sparse tau immunolabelling. None of the MM1 sCJD cases examined showed any similar tau deposits. Western blotting of the tau extracted from the frontal cortex of vCJD patients detected four pS404 reactive bands (60, 56, 54 and 50kDa). A similar pattern was seen in some of the MV2A sCJD and GSS cases. These results suggest that tau deposition is found in vCJD and may also be found in other prion diseases associated with PrP plaques. The extent of tau immunoreactivity is more widespread in vCJD and may reflect the unique morphology and the large numbers of plaques found in this condition.

1. G.Giaccone et al. Neuroprion 2005

DIA-21

DETECTION OF APTAMER-PROTEN INTERACTIONS USING THICKNESS SHEAR MODE TECHNIQUE

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DNA or RNA aptamers can selectively bind proteins or even low molecular compounds. The selection of the method of detection of aptamer-protein interaction is important in reaching sufficient detection limit as well as for understanding the mechanisms of the protein-nucleic acid interactions. In this work we applied thickness shear mode method (TSM) for detection the interaction between DNA aptamers that selectively bind the thrombin either in heparin or fibrinogen binding sites. We also examined how the binding of thrombin depends on the aptamer structure. We showed, that binding of thrombin to the aptamer immobilized on a surface of the quartz crystal through neutravidin-biotin method is accompanied by decrease of series resonant frequency and by increase of the motional resistance. This suggests that the binding event is accompanied not only by increase of the mass of the sensing layer, but also by increasing of its shearing viscosity. The structure of aptamer significantly affected the sensitivity of the binding. Aptamer of longer oligonucleotide chain providing stabilization of the 3D structure resulted in stronger binding of thrombin in comparison with shorter aptamer without stabilization of the structure. The results obtained can be used in further application of the aptamers as a biosensors for detection e.g. prions.

DIA-22

IN VITRO STUDY OF CURCUMIN LABELING OF PRION PLAQUES AND PRION FIBRILLS

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Amyloid fibril formation and aggregation in the brain is a hallmark of several neurodegenerative diseases for which there is no treatment, yet. Representatives of these diseases are Alzheimer disease and prion diseases. In both neurodegenerative disorder types the diagnosis is typically achieved either late in the manifest stage of the clinical disease, or even postmortem. Amyloid-binding molecules that can serve as imaging agents to quantify amyloid plaque loads in living brain have the potential to determine the extent of in vivo pathology. Several compounds have been developed and may be particularly useful for evaluating antiamyloid-directed therapeutic interventions. Some of the tested compounds that bind and fluorescently label amyloid in vitro like the yellow curry pigment curcumin, may have additional effects. For example, curcumin has potent anti-inflammatory and antioxidant activities and can suppress oxidative damage and inflammation. Low dose curcumin has recently also been shown to effectively disaggregate amyloid beta aggregates and to prevent fibril and oligomer formation (1), revealing its tentative therapeutic potential. Interestingly, it had also been shown to potently inhibit scrapie prion accumulation in scrapie agent-infected neuroblastoma cells (2). The aim of our ongoing work has been to evaluate curcumin for its ability to label prion amyloid plaques in postmortem brain sections of human prion disease victims. Furthermore, we have aimed at determining the binding characteristics of curcumin to recombinant prion protein fibrills in vitro. We employed sequential labeling of 5microm thick tissue sections of paraformaldehyde-fixed paraffin-embedded brain of a variant Creutzfeldt-Jakob disease victim: curcumin labeling was applied first and the labeled sites were photographed. This was followed with immunohistochemistry to prion protein and repeat photographing of the same sites. Fluorimetric binding assay was performed to determine the binding characteristics of curcumin to recombinant prion protein fibrills. The results showed that: (a) curcumin reproducibly and selectively labeled prion plaques in brain tissue sections, and (b) in the fluorimetric assay, curcumin labeled prion protein fibrills (but not the natively folded prion protein) with the binding constant in the submicromolar (Kd=0.3 microM) range. Our results suggest that curcumin may have a considerable diagnostic potential and prompt further studies to better assess its usefulness in prion diseases.

1 Yang et al. 2005, J biol chem 280, 5892-5901.
2 Caughey et al. 2003, J Virol 77, 5499-5502.

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DIA-23

ACOUSTIC PRION SENSOR

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A thickness shear mode acoustic sensor has been successfully demonstrated as a diagnostic tool to detect infectious prions. The sensor used was a standard AT cut, piezoelectric quartz crystal with gold electrodes. Commercially available, normal recombinant prion protein was immobilized on the gold surface using a thiol linking molecule. The sensor was then contacted by an homogenized brain sample and the resonant frequency of the crystal was recorded at 10 second intervals with the aid of a phase lock oscillator and computer. Scrapie infected brain and normal brain control samples from 21 sheep were tested in a total of 45 trials. No sample pretreatment beyond homogenization was required and the result was available 2 hours after the sample application. Two samples gave false positive results but no false negatives were observed. The test is based on the infection process where a normal recombinant prion protein immobilized on the sensor surface is converted to the misfolded form by an infected sample applied to the sensor.

DIA-24

LABOUR, SPACE AND RESOURCE IMPLICATIONS OF RAPID ASSAY AUTOMATION

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The Enfer TSE Version 3 assay is a 'new' and improved, qualitative immunoassay for the detection of PrP^{Sc} in central nervous tissue of cattle and small ruminants. The automation of sample homogenisation and pipetting that have previously been developed for the Enfer TSE Version 2.0 assay, are directly transferable to the Version 3 assay.
Automation of the ELISA stage of the version 3 assay has now also been achieved with the en4lisa microplate processing instrument. Equipment comparisons for both the automated and manual methods of the Enfer TSE Version 3 assay have been made, demonstrating a reduction in laboratory space required for TSE testing for the en4lisa automated method.
Direct comparisons of labour requirements and laboratory throughput with the Enfer TSE Version 3 manual and automated en4lisa methods, show reductions in resource requirements and increases in maximum output of laboratory areas.

DIA-25

VALUE OF 14-3-3 IN THE DIFFERENTIAL DIAGNOSIS OF CJD

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The clinical diagnosis of Creutzfeldt-Jakob disease (CJD) is basing on a characteristic clinical pattern (rapidly progressive dementia, cerebellar syndrome, visual disturbances, extrapyramidal or pyramidal signs, myoclonus and akinetic mutism) and detection of 14-3-3 in cerebrospinal fluid or periodic sharp wave complexes in EEG. Different studies described 14-3-3 detection in acute diseases such as ischemia, tumours or inflammatory disorders of the brain (Siman 2004, Saiz 1999, Satoh 2003). Whereas some of them are easily to differentiate from CJD, the presentation might overlap. Therefore we investigated the value of 14-3-3 in differential diagnosis of CJD.
In Germany, over 2100 patients were notified to the Surveillance Unit in Göttingen since 1993. In 106 of them, a neuropathologically defined other diagnosis was established. In 90 patients who were tested for 14-3-3 and had a neuropathologically verified diagnosis, 58 (64%) patients had a negative test result for 14-3-3, also during course (in 5 patients). In 32 patients (36%), 14-3-3 was positive.
Of the 32 positive samples, three became negative during course. The diagnoses of these patients were vasculitis and two biopsy-excluded CJD with non-specific gliosis. Six stayed positive during course with the diagnoses of encephalitis (n=3), Alzheimer's disease (n=2) and vascular dementia. In the remaining 23 patients, six (26%) had a hypoxic event prior to the lumbar puncture, four an inflammatory process (17%), and each three (13%) epileptic seizures and cerebral ischemia. These acute processes explain the detection of 14-3-3 as false positive regarding CJD.
This shows a high value of 14-3-3 in differential diagnosis of CJD, when acute processes influencing the test result are excluded.

DIA-26

A RECENTLY DEVELOPED SENSITIVE MICROSCOPY TECHNIQUE APPLIED TO A NEW NEURAL STEM CELL MODEL FOR ASSESSING PRION PROPAGATION.

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Transmissible spongiform encephalopathies (TSEs) are fatal transmissible neurodegenerative diseases observed in various mammal species, including humans. The accumulation of an isoform of the host-encoded prion protein (PrP^{c}) that has become resistant to degradation by proteases is a common feature of TSEs. Although different species possess their own PrP molecule, these are highly conserved and it has become clear that the protease-resistant PrP (PrP^{Sc}) is capable of crossing the species barrier, potentially affecting humans. Diagnostic tests rely on immunochemical techniques (Immunohistochemistry, ELISA, Western blotting) using tissues obtained post-mortem. In the mean time, because of the long incubation period, animal models of infectivity are a constraint and pose ethical problems. Therefore, there is a need for a rapid and reliable method allowing the early detection, identification and characterisation of various strains of resistant PrP in animal tissues. In this project, we are using a Neural Stem Cell (NSC) model developed by our collaborators, which may allow to rapidly assess *in vitro* the infectivity of various strains of PrP^{Sc}. Episcopic Differential Interference Contrast / Epi-Fluorescence (EDIC/EF), a microscopy technique developed in our laboratory, can be used in combination with sensitive fluorescent thiazole dyes able to detect proteins particularly rich in beta-sheets. We are using the NSC model in combination with our detection methods as an alternative to longer and more costly immunochemical protocols. This technique may prove faster, more reliable and more cost effective than current immunochemical methods.

DIA-27

HIGHLY INCREASING OF SENSITIVITY IN SCRAPIE DETECTION BY RAPID TESTS USING PMCA

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PMCA (Protein Misfolding Cyclic Amplification) is a recently described technology for in vitro cycling amplification and aggregation of PrP^{res}. In a similar way to DNA amplification by PCR, PMCA increases the presence of PrP^{res} in a sample with initial minute quantity of PrP^{Sc}.
Previous studies have shown evolution of PMCA from a manual to an automated method that improves reproducibility and efficiency. Also infectivity and PrP^{Sc} detection in blood has been demonstrated in scrapie-afflicted hamsters.
In this work we will show how automated Scrapie PMCA can be linked to UE approved rapid tests to highly increase the sensitivity of them. Also PMCA can be used for PrP^{Sc} amplification obtained from nervous tissue and others as spleen.
In vitro amplified PrP^{res}, show similar characteristics to Scrapie PrP^{Sc} and can be detected by same antibodies that recognizes different epitopes of PrP^{Sc} protein and tests designed for specific capture of PrP^{Sc} show same behaviour using PrP^{res} from PMCA as PrP^{Sc} from scrapie affected sheep tissues.
Results will show that three runs (120 cycles) of automated Scrapie PMCA leads to a 15000 fold increase in sensitivity compared to standard diagnosis methods in nervous tissue.

DIA-28

STYRYLBENZOAZOLE DERIVATIVES FOR IMAGING OF PRION PLAQUES AND TREATMENT OF PRION DISEASES

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Recent prevalence of acquired forms of prion diseases has urged the development of early diagnostic measures as well as therapeutic interventions. We have previously reported that some amyloid imaging compounds, primarily derived from amyloid dyes such as Congo red and thioflavin T, are useful for detection of abnormal PrP plaques and treatment of prion diseases (Ishikawa et al., Journal of General Virology, 1785-90, 2004). To extend our previous findings on the utility of amyloid imaging probes toward a practical step, we have developed and analyzed styrylbenzoazole derivatives with more adequate permeability of blood brain barrier (BBB). The new styrylbenzoazole compounds clearly labeled abnormal PrP plaques in the human brain specimens in a manner irrespective of pathogen strain. A representative compound BF-168 detected abnormal PrP aggregates in the brain of experimental animals when BF-168 was injected intravenously. On the other hand, most of these styrylbenzoazole derivatives inhibited abnormal PrP formation in prion-infected cells with IC50 values in the nanomolar range, indicating one of the most potent classes of inhibitor ever reported. Additionally, BF-168 prolonged the lives of intracerebrally prion-infected mice when the compound was given intravenously at the preclinical stage. The new compounds, however, could not detect synaptic PrP deposition or show pathogen-independent therapeutic efficacy, similar to the amyloid imaging probes we previously reported. The compounds were efficiently BBB-permeable and non-toxic at doses for imaging and treatment in this study; therefore, they are expected as novel lead compounds of therapeutic drugs as well as imaging probes for prion diseases.

DIA-29

SENSITIVE PROXIMITY LIGATION-BASED DETECTION OF BIOMARKERS OF PROTEIN FOLDING DISORDERS

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The development of sensitive and specific techniques for protein detection could provide opportunities for early detection and therapeutic intervention in protein folding disorders, for instance prion and Alzhiemer's diseases. The proximity ligation assay (PLA) is a recently developed method in which specific proteins are analyzed by translating detection signals to DNA sequences. In this method target molecules are recognized by pairs of affinity probes - e.g. antibodies. Proximity probe pairs, used for detection are prepared by attaching DNA strands to the affinity probes. When a pair of such probes bind to a common target molecule, the free ends of the attached oligonucleotides are brought in proximity and can be hybridized to a connector oligonucleotide, allowing the ends to be joined by enzymatic DNA ligation and detected by PCR. We have applied PLA for sensitive identification of peptide oligomers, while excluding detection of protein monomers. Soluble Aβ oligomers, which have been shown to mediate neurotoxicity, may be used as a candidate biomarker for diagnosis of Alzheimer's disease at early stages. The combination of efficient PCR amplification and the use of two or more binding reagents provide very high sensitivity and specificity of detection, surpassing conventional protein detection methods. Furthermore, the proximity ligation technique can be carried out as an *in situ* assay, as in the homogenous assay - requiring very small amount of materials to be tested -, or in a solid-phase format in which the target molecules to be detected are immobilized on a surface using affinity probes, while other materials are washed away. Proximity ligation can, therefore, provide a powerful molecular tool for studying the biology of protein folding disorders and for early diagnosis.

DIA-30

DETECTION OF PRIONS IN BODY FLUIDS – DIAGNOSIS OF CJD

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Transmissible spongiform encephalopathies (TSEs) are fatal disorders of the central nervous system characterised by the progressive accumulation of an abnormal form of the prion protein (PrP). TSEs include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt-Jakob disease (CJD) in humans. It was shown that BSE can be transmitted by the consumption of meat products from BSE infected cattle to humans causing the new variant of CJD (nvCJD) in humans. Among humans nvCJD can be transmitted by blood transfusions and organ transplantations. Due to the slow etiopathology, a general screening of all donors appears obligatory, as the infection risk of a transplant can be unperceived. Thus, our ambition was the development of a detection system for PrP^{Sc} in body fluids.

At present, there are no methods available that allow the detection of PrP^{Sc} in body fluids, because of the extremely low concentrations expected. Therefore, a substantial increase in sensitivity of prion detection systems is necessary. On the basis of a classical ELISA we could establish such a highly sensitive test system by using a new detection technique, the so called Immuno-PCR. The Immuno-PCR combines the specific antigen-antibody reaction with the high amplification rate in PCR. Using Immuno-PCR, sensitivity of the corresponding classical ELISA was increased 10.000 fold resulting in a detection limit of 10pg/mL recombinant prion protein.

By spiking experiments using recombinant prion protein in body fluids it was shown that 10pg/mL recPrP are clearly detectable even when spiked in serum or cerebrospinal fluid. In a further experiment brain homogenate from a BSE-infected macaque and a healthy animal were analyzed in Immuno-PCR. The results demonstrate that PrP^{Sc} from animals which were infected with BSE-positive material can be detected with the presented technique.

Next, the preparation of serum and cerebrospinal fluid was optimized in terms of buffer composition and Proteinase K treatment. Finally, samples from patients with various neurodegenerative diseases were analyzed by the optimized sample preparation combined with the Immuno-PCR. First results on the quantification of PrP^{Sc} in human body fluid samples from patients compared to healthy control individuals will be presented.

DIA-31

COMPARISON OF SEROTONIN LEVELS IN CEREBROSPINAL FLUID AND SERUM BETWEEN SHEEP AFFECTED BY TSE AND HEALTHY SHEEP

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The serotonergic system plays a major role in the regulation of a wide range of biological, psychological and behavioural functions, and its dysfunction is associated with several medical and psychiatric conditions in humans. In addition, serotonin is thought to be involved in the transmission of pain and central pruritus. Experimental studies have demonstrated reduced serotonin levels in brains of rodents infected with transmissible spongiform encephalopathies, and it was hypothesised that the serotonergic system was affected by these diseases, which could also account for the clinical signs. Reduced serotonin concentrations have also been found in blood from scrapie-affected sheep.
Based on these findings, the objective of this pilot study was to investigate if the serotonin concentration in cerebrospinal fluid (CSF) and serum is altered in sheep affected by TSEs compared to healthy controls, which could account for the anxiety and pruritus seen in affected sheep and potentially be used as a surrogate marker.
Serum and CSF was collected at necropsy from 14 sheep with confirmed TSE and clinical signs that included pruritus (6 sheep naturally infected with scrapie, 8 sheep experimentally infected with BSE) and 10 clinically healthy sheep from a TSE-free flock. Serotonin concentration was determined by High Performance Liquid Chromatography. Results between groups were compared with the Mann-Whitney U test.
There was no statistically significant difference in the serotonin concentration in CSF between sheep affected by TSEs and healthy sheep. By contrast, serum serotonin was significantly reduced in TSE-positive sheep ($p=0.03$), and when individual groups were compared, in scrapie-affected sheep compared to healthy controls ($p=0.04$).
CSF serotonin is not a useful marker to explain the clinical signs of TSE in sheep or to distinguish TSE-affected sheep from normal sheep. Although the mean serotonin concentration was significantly reduced in sheep affected by TSEs compared to healthy sheep, there was an overlap in the values across both groups, which makes it unsuitable as a diagnostic test.

DIA-32

MRI IN THE CLASSICAL MM1 AND THE ATYPICAL MV2 SUBTYPE OF SCJD: AN INTER-OBSERVER AGREEMENT STUDY

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The objective of the present study was to establish clinical and radiological features in the atypical MV2 subtype of sCJD compared with the classical MM1 subtype as well as region- and weightening-dependent inter-observer correlation. Clinical features, EEG, 14-3-3 investigation, and MRI hyperintensities of basal ganglia, various cortical regions and thalamus were evaluated in 31 MM1 and 32 MV2 patients. Each MR scan was separately analyzed by two neuroradiologists blinded for *PRNP* genotype/prion protein type.
Myoclonus, akinetic mutism and PSWCs in EEG were significantly more often in MM1 than in MV2 patients. Protein 14-3-3 sensitivity was lower in MV2 (74%) than in MM1 patients (96%). T2-sensitivity for basal ganglia hyperintensities was higher in the MV2 subtype (84% in both observers versus 61% in observer 1 and 42% in observer 2 in MM1 patients). Significant inter-observer agreement was found for basal ganglia and thalamus on T2, FLAIR, PD, and DWI, but for cortical hyperintensities only on DWI. Thalamic changes were significantly more frequent in MV2 patients (86% vs. 12.5% in MM1 patients on DWI).
MRI was the most sensitive investigation in MV2, and 14-3-3 test in MM1 patients. High frequency of thalamic hyperintensities in the MV2 subtype allowed differentiation from MM1 patients. Good inter-observer agreement was found for basal ganglia and thalamus in all weightenings. DWI was the only imaging method allowing reliably reproducible detection of cortical hyperintensities. Since inter-observer correlation for cortical hyperintensities on T2, FLAIR and PD is relatively low, the cortical changes should not be over-interpreted in these sequences.

DIA-33

PRION DETECTION IN BLOOD USING THE EPITOPE PROTECTION ASSAY

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Amorfix is a theranostics company that uses its Epitope Protection technology to develop new diagnostics and therapeutics for diseases involving misfolded proteins. One such disease, vCJD, is of particular concern for the safety of the blood supply as it cannot be detected in blood by current methods, yet it can be transmitted by blood transfusion. In order to detect prions, an assay must distinguish between the normally folded prion protein PrP^{C} and its aggregated disease-causing conformation PrP^{Sc}. Several prion detection assays have used proteolysis to remove PrP^{C}. Since it is unknown what fraction of endogenous prions in blood are protease resistant, we have circumvented this step by developing a chemical means to differentiate between PrP^{C} and PrP^{Sc}, called Epitope Protection. PrP^{C} is selectively modified with short-lived and highly-reactive chemicals which modify selected amino acids in the protein. Such chemical modification efficiently blocks immunological epitopes on PrP^{C} and leaves them unrecognizable to many PrP antibodies. PrP molecules within prion particles are “protected” from chemical modification, and can then be detected by conventional immunoassay after disaggregation of the sample. A remaining challenge is the detection in blood of the very small concentration of PrP^{Sc}, which has been estimated in the low femtogram range. We have developed an ultrasensitive method using a combination of magnetic and fluorescent beads to detect femtogram quantities of PrP. We have tested this methodology by testing blinded panels of vCJD brain and spleen material spiked into plasma, and we can detect vCJD brain homogenate after diluting a 10% homogenate as much as 10^{5}-fold into plasma. Further experiments to validate the assay, such as testing blood from scrapie-infected hamsters at various preclinical time points, as well as testing of blood from human CJD patients, are currently under way.

DIA-34

STANDARDIZED BLOOD SAMPLES OF CREUTZFELDT-JAKOB-INFECTED PRIMATES FOR THE EVALUATION OF FUTURE PRION DIAGNOSTIC TESTS.

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Several sensitive diagnostic tests are under development to detect pre-clinical infection with variant Creutzfeldt-Jakob disease (vCJD) in order to minimize the risk of secondary transfusion-associated infections. Their evaluation will be hindered by the impossibility of creating a library of blood samples from humans incubating vCJD.
We propose a practical alternative using a non-human primate model (Macaca fascicularis) inoculated with several prion strains including vCJD. These animals are currently under study in our laboratory, and are having blood drawn on a regular basis during the pre-clinical and clinical phases of disease. We adapted fractionation procedures from blood transfusion centres to macaque blood to obtain plasma, buffy-coat and red blood cells comparable to those currently processed in humans. Samples are already available from ten primates infected with autopsy verified infections with BSE or vCJD. Fifty animals inoculated with different strains for a variety of research programmes are currently under surveillance and sampled regularly.
This library of standardized blood samples, clearly identified and correlated to the clinical status of the animals, will constitute an important resource for a future validation of screening tests.

DIA-35

SCRAPIE IN SHEEP CARRYING THE LYSINE 171- ALLELE OF THE PRION PROTEIN GENE

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Susceptibility to scrapie in sheep is determined by the infective strain and the host *PrP* genotype, deriving from amino acidic changes encoding five alleles: $A_{136}R_{154}Q_{171}$, VRQ, AHQ, ARH, ARR. In addition to these polymorphisms, a fourth amino acidic change (glutamine to lysine) has been described at codon 171, generating the ARK allele. For this allele no data about scrapie susceptibility are currently available, because of its rarity in sheep population. To investigate the scrapie susceptibility linked to this allele, eight ARK/--- animals coming from a Piedmontese outbreak and 20 ARK/--- sheep from an outbreak in Lombardia Region, were analysed by a SAF immunoblotting, by immunohistochemistry and by two different rapid tests. Three positive ARK/--- sheep, coming from the first scrapie outbreak, were found. Although our results do not permit to estimate the level of susceptibility associated to this allele yet, these findings show that the animals carrying the ARK allele of the PrP gene are not fully resistant to scrapie.

DIA-36

PERFORMANCE OF BSE RAPID TESTS ON AUTOLYTIC BRAIN STEM: THE " IN FIELD" ITALIAN EXPERIENCE

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Routine rapid testing has highlighted some major problems related to in field BSE rapid tests performance in comparison with the EU evaluation. Two of the most significative ones are the number of "initially reactive" samples (samples with positive results at first examination resulting negative under re-testing) and false positive results related to autolytic samples. This aspect is pivotal for Italian BSE active surveillance in risk populations, because the collection of fallen stock is not carried out on a daily basis or because isolated or mountain farms are not easily accessible. Thus a robust test, able to perform on samples in autolytic conditions is needed. To evaluate the robustness of the rapid test systems in use in Italy, we selected 450 autolyzed brain stem samples from bovine older then 24 months. According to the degree of autolysis, assessed and scored using a ranking system from 1 to 3, the samples were divided in 3 categories (low, medium and high autolysis). Samples were dissected in 3 pieces (or in high autolyzed tissue selected from the same tissue mass) randomly assigned to the rapid test systems in use in Italy: Biorad TeSeE, Enfer TSE version 2.0, Abbott and Prionics check LIA. Each aliquot was tested following standard operating procedures (SOPs) prepared according to the manufacturers instructions. The 95% confidence limits for specificity was calculated using the STATA 9 software. Specificity resulted 100% (IC95% 97,57%-100%) for the 3 tests for all categories. Biorad TeSeE identified correctly the samples of all the 3 categories at first examination, Enfer TSE gave 2 initially reactive samples from category 1 (1.3%) and 1 from each of the other two (0.6%), while Prionics check LIA gave one initial reactive sample from the category 1 (0.6%) and could correctly classify the others. Our results show that the 3 tests can be applied even on severe autolytic samples without giving false positive results.

DIA-37

IN VIVO SCRAPIE DIAGNOSIS BY PRPSC DETECTION IN LYMPHOID TISSUE: A COMPARATIVE STUDY USING THIRD-EYELID AND RECTAL MUCOSA BIOPSIES

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Scrapie of sheep and goat is an endemic disease in many countries. The control and eradication of this disease have been hampered by the lack of a practical *ante mortem* diagnostic test.
It is known that PrPsc is detectable in lymphoid tissue of scrapie infected animals even before development of the clinical disease. In most of the susceptible sheep, PrPsc is firstly detectable in the Peyer´s patch and its draining mesenteric lymph node; afterwards, in most of the gut-associated lymphoid tissues and in other lymphoreticular system tissues. *Ante mortem* diagnostic tests based on the detection of PrPsc in palatine tonsil, third-eyelid and rectal mucosa associated lymphoid tissue biopsies have been described. Although the palatine tonsil biopsy is not a practical field procedure, both third-eyelid and rectal mucosa associated lymphoid tissue biopsies have been proposed for scrapie screening analysis in the field.
The objective of this study was to compare the performance of *in vivo* scrapie diagnostic tests using third-eyelid and rectal mucosa associated lymphoid tissue biopsies in field conditions.
Animals belonging to a regularly monitored flock with a high incidence of natural scrapie and genetically susceptible sheep coming from infected flocks detected through the Spanish active surveillance program were included in this study. A sample from rectal mucosa and another one from third-eyelid lymphoid tissue were collected from each animal under local anesthesia. All of them were formaldehyde fixed and routinely processed for histological and immunohistochemical examination.
The results of this field study confirmed that both biopsy samples (third -eyelid and rectal mucosa lymphoid tissue) were suitable for *in vivo* scrapie diagnosis. However, third-eye biopsy caused a higher degree of distress in the animals and therefore it was more difficult to immobilize them for sampling.

DIA-38

SPECIES REACTIVITY OF PRP MOTIF-GRAFTED ANTIBODIES

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In order to further assess the species reactivity of PrP^{Sc}-specific hybrid human IgG monoclonal antibodies containing the 89-112 or the 136-158 polypeptide motif of mouse prion protein (Moroncini G. et al., Proc Natl Acad Sci USA. 2004 101: 10404-9), we performed immunoprecipitation experiments using brain homogenates prepared from normal and prion-infected (BSE, BASE, Scrapie) bovines and sheep. Binding of IgG 89-112 or IgG 136-158 to PrP^{C} in normal bovine or ovine brain was not detected. Conversely, both motif-grafted reagents immunoprecipitated three PrP bands from undigested and pK-digested BSE, BASE and Scrapie brain extracts. Interestingly, the immunoprecipitated PrP bands, as detected by Western Blot, mirrored the canonical WB profile of the three different prion strains assayed, indicating strain-specific binding of these antibodies. Under the same experimental conditions, the parental human IgG antibody did not show any reactivity with PrP^{Sc} nor with PrP 27-30.
In conclusion, the data coming from this study, together with those previously published, identify two independent regions of mouse PrP sequence that possess intrinsic specificity and affinity for epitopes found exclusively on PrP^{Sc} and PrP 27-30 molecules encountered in prion diseases of animals and humans. Grafting such polypeptides into an IgG scaffold imparted upon this antibody the ability to bind to disease-associated PrP isoforms produced by different prion strains (79A, RML, 263K, BSE, BASE, Scrapie) and in different species. The hybrid antibody reagents we have generated may find broad application in the detection of infectious prions in human and animal materials.

DIA-39

EVALUATION OF IN FIELD RAPID TESTS PERFORMANCE FOR THE DIAGNOSIS OF OVINE TSE

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Scrapie is known in Italy since 1995 and the number of outbreaks has been dramatically increasing since the introduction of active surveillance in small ruminants in 2002, by means of rapid tests already approved for BSE.
We performed a comparative evaluation among the 2 rapid tests applied in Italy for both sheep and bovine TSE active surveillance (Bio-Rad TeSeE and Enfer TSE kit version 2.0, Abbot) and the IDEXX HerdChek Scrapie Antigen kit and Bio-Rad TeSeE Sheep/Goat, recommended from the EFSA evaluation for classical and atypical Scrapie detection form the obex region, in order to verify their performance in field for the detection of ovine TSE.
The caudal brain stems of Biellese sheep with various genotype belonging to a Piedmont flock with high scrapie prevalence were collected. Samples were subjected to a pre-homogenization protocol and split in aliquots for each test and for the NaPTA western-blot to confirm any positive rapid tests result. IDEXX HerdChek Scrapie Antigen kit and Bio-rad TeSeE sheep and goat could detect additional positive cases in comparison with the two other systems, stressing the importance of applying the most sensitive rapid methods of screening to strengthen TSE active surveillance

DIA-40

PRNP GENE REGULATION DURING PRION INFECTION AND CELL DIFFERENTIATION.

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Prnp is considered as a housekeeping gene and the amount of PrPc during Prion infection is considered as constant during all the disease. However, an overexpression of PrPc is also described during neuronal differentiation via ERK1/2 pathway activation. Using a magnetic cell sorting, based on surface PrP level previously developed in the laboratory, we observed a different PrPc expression between infected cells versus controls. To understand this modification of metabolism of the normal PrP, we focused on the transcriptional regulation of Prnp during prion infection and in different states of cellular differentiation.
We examined ERK1/2 and Notch pathways, already described as involved during prion infection. The studies were performed in different cell lines (GT-1, SN56) infected or not with the Chandler scrapie strain while cAMP and Notch inhibitor treatments were used for differentiation experiments. Notch inhibitor treatments decreased phosphorylated ERK 1/2 and phosphoSTAT in all cell lines and increased the level of PrPres in infected cells. Surprisingly the expression of HES5 (under phosphoSTAT transcriptional regulation) was decreased only in infected cells.
Moreover, the expression of a GFP construct, under the control of different regulatory sequences of the murine prion promoter, appeared in preliminary experiments to react differently to Notch inhibitor treatments depending on the infected status of the cells.
Thus, the metabolic pathways involved during prion infection could lead to variations of PrPc synthesis. Elucidation of the molecular mechanisms involved, and more precisely in Prnp promoter regulation, should offer a new insight to the comprehension of cellular susceptibility to Prion replication.

DIA-41

POTENTIAL COMPLICATION OF BLOOD SCREENING TEST DEVELOPMENT: CASE OF CELLULAR PRION PROTEIN (PRPC) ON RED BLOOD CELLS (RBC)

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Third transmission of vCJD by blood transfusion in UK emphasizes the need for donor screening test for prion diseases. Their only specific marker is pathological form of prion protein (PrPsc), but its detection in blood is significant challenge. Blood contains substantial amount of PrPc both in plasma and blood cells. Conflicting data exist about the quantity of PrPc on RBC. To verify the presence of PrPc on RBC, we used quantitative flow cytometry with widely used monoclonal antibodies FH11, 3F4 and 6H4. We detected binding of 36 (FH11), 80 (3F4) and 260 (6H4) IgG molecules / cell. Low binding of FH11 and 3F4 could be caused by truncation of PrPc. Immunoblot with 6H4 showed that molecular weight (Mw) of RBC PrPc is slightly higher than brain PrPc and deglycosylation provided bands with identical Mw. No band corresponding to truncated PrPc was detected. The unavailability of 3F4 epitope is one of the hallmarks of PrPsc, but RBC PrPc was Proteinase K sensitive. Neither PrPc conformation does not play role in poor 3F4 binding nor glycosylation, because denaturation and deglycosylation of RBC PrPc did not improve its detection with 3F4. This suggests that epitope for 3F4 (MKHM) in RBC PrPc is modified. *In vitro* modification of free amino groups of brain PrPc by NHS-Biotin abolished 3F4 binding. We speculate that 3F4 epitope may be modified by advanced glycation end-products (AGE). AGE modification of brain PrPsc was reported recently. RBC are long living cells providing enough time for PrPc AGE modification. In accord with our hypothesis PrPc on erythroid cells from cord blood was detected equally well with 3F4 and 6H4, suggesting that modification of PrPc occurs after release of RBC into periphery. Methods utilizing FH11 and 3F4 may underestimate quantity of PrPc in RBC. Likewise, tests for presence of PrPsc in blood may encounter difficulties if modification similar to one reported here is present. (GACR 310/04/0419, GACR 310/05/H533, MSM0021620806).

DIA-42

IDENTIFICATION OF METALS ACCUMULATION IN BRAINS OF HEALTHY AND SCRAPIE-AFFECTED SHEEPS BY MAGNETIC RESONANCE IMAGING

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Magnetic resonance imaging (MRI) is widely used to investigate metals accumulation in brain. The aim of this study is to set up a method to carry out MRI on sheep brain, avoiding artefacts due to interface tissue-air, and to investigate metals presence in healthy and scrapie-affected brains to detect possible differences. MRI was performed on four healthy brains preserved and prepared with different protocols. Optimal images, due to absence of morphological artefacts and signal alteration, were obtained dipping whole brains in a plastic container containing 10% formaldehyde solution. To obtain a good quality of imaging several sequences (SE, FSE, GE, and FLAIR) have been performed and images were acquired in the sagittal, coronal and axial planes. Subsequently, we analyzed four brain of healthy and scrapie-affected sheep, carrying out MRI in double blind. Correlation between hyper-intensity and a high abnormal Manganese accumulation, taking into account a possible influence of formalin fixation on Mn concentration in cortex is under investigation.
Imaging showed hyper-intensity in cortex (surface layer) and pineal region for every examined sample; pattern of hyper-intensity is consistent with Manganese accumulation, due to the paramagnetic nature of this element. Use of MRI to discriminate between toxic and physiological concentration of Mn will be evaluated.

DIA-43

DECISION SUPPORT TOOLS IN CLINICAL DIAGNOSIS IN COWS WITH SUSPECT SPONGIFORM ENCEPHALOPATHY

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Reporting of clinically suspected cattle is currently the most common method to detect cases of bovine spongiform encephalopathy (BSE). Improvement of clinical diagnosis and decision making remains crucial. A comparison of clinical patterns, consisting of 25 signs, was made between all 30 BSE cases, confirmed in Belgium before October 2002, and 272 suspected, but subsequently histologically, immunohistochemically and scrapie-associated-fibres negative cases. Seasonality in reporting suspected cases was observed, more cases being reported during wintertime when animals were kept indoor. The median duration of illness was 30 days. The ten most relevant signs of BSE were: kicking in the milking parlour, hypersensitivity to touch and/or sound, head shyness, panic-stricken response, reluctance to enter in the milking parlour, abnormal ear movement or carriage, increased alertness behaviour, reduced milk yield, teeth grinding and temperament change. Ataxia did not appear to be a specific sign of BSE. A classification and regression tree was constructed, using the following four features: age of the animal, year of birth, number of relevant BSE signs noted and number of clinical signs, typical for listeriosis, noted. The model had a sensitivity of 100% and a specificity of 85%. This approach allows the use of an interactive decision support tool (efficacious passive surveillance), based entirely on odds ratios, a statistic independent of disease prevalence.

DIA-44

CHROMATOGRAPHIC METHODS TO PURIFY THE ABNORMAL PRION PROTEIN FOR ANALYSIS

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Technologies used for chemical characterization of the proteins require that the protein of interest has a certain level of purity. Current methods used in prion research are ultracentrifugation, sodium phosphotungstic acid precipitation and immunoprecipitation. Although these methods are useful, there are other methods used in analytical chemistry that are more rapid and efficient. In this study, we describe several different chromatographic approaches to purify the abnormal prion protein. Brain from scrapie infected sheep was prepared by grinding the tissue in liquid nitrogen. The finally ground tissue was then resuspended in 0.15M NaCl and extracted with hexafluoro-2-propanol. A phase separation was induced by the addition of 0.5M Na_2SO_4. The bottom layer which contained the abnormal prion protein was removed and was dried in a vacuum centrifuge. The sample was then resuspended in the appropriate starting mobile phase and centrifuged to remove particulates in preparation for chromatography. Several forms of chromatography were used including normal phase, reverse phase, ion exchange, size exclusion chromatography, hydrophobic interaction chromatography, hydrophilic interaction chromatography and metal ion affinity chromatography. After chromatography, the peak fractions were tested either by Western blot or by fluorescence immunoassay. One of the major problems that occurred was that the some of the solvents required to solubilize the abnormal prion were incompatible with the column matrices, e.g., - 1% formic acid and 50mM hexafluoro-2-propanol are caustic to some columns. To overcome this problem, small disposable cartridges were substituted for the analytical columns when possible. Application of the chromatographic method adapted to these cartridges was used successfully to develop an assay for sheep blood. These purification methods could be used to differentiate between strains of scrapie

DIA-45

FELINE SPONGIFORM ENCEPHALOPATHY: FIRST CONFIRMED CASE REPORTED IN PORTUGAL

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Feline spongiform encephalopathy (FSE), affecting domestic and captive feline species, is a prion disease considered to be related to bovine spongiform encephalopathy (BSE).
Here we report the first case diagnosed in Portugal, highlighting the neuroapthological findings.
In 2004 a 9-year old intact female Siamese cat was referred with chronic progressive behavioural changes, polydipsia, gait abnormalities and episodes of hypersalivation. Clinical signs progressed to tetraparesis and dementia and euthanasia was performed. At necropsy, brain and spinal cord had no significative changes. Tissue samples from brain, cerebellum, brainstem and spinal cord were collected for histopathology and immunohistochemistry for detection of PrP^{res}.
Histology revealed neuropil and neuronal perikarion vacuolation in several areas of the central nervous system together with gliosis and cell rarefaction at the granular layer of the cerebellum. Immunohistochemical detection of PrP^{res} showed a strong and widespread PrP^{res} accumulation as granular and linear deposits as well as associated with some neurons.
These findings are supportive of FSE. To the authors knowledge this is the first confirmed case of FSE reported in Portugal.

DIA-46

IDENTIFICATION OF MOLECULAR BIOMARKERS IN IN BODY FLUIDS OF ME-7 INFECTED MICE.

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The use of PrP^{sc} as a pre-clinical, or general marker for surveillance is limited, due to the fact that it is present in extremely small amounts in accessible tissues, or in body fluids such as cerebrospinal fluid (CSF), blood and urine. Specific detection of these small amounts of the PrP^{sc} conformer are further exacerbated by the presence of a large excess of endogenous PrP. Thus the identification of alternative biomarkers applicable to the development of diagnostic tests or intervention therapies are needed. In this study weekly collections of body fluids were performed on ME-7 infected C57/BL6 mice and age matched controls. The extracted proteins were analyzed by 2-D fluorescence difference gel electrophoresis (2D-DIGE). Differential in gel analysis using DeCyder software (GE Healthcare) was used to identify differentially expressed proteins and gel spots of interest were isolated and identified by tandem mass spectrometry. Currently, 2D-DIGE and Western analysis are being used to characterize the protein profiles of ME-7 and age matched controls throughout disease progression.

DIA-47

DNA APTAMERS BASED BIOSENSORS FOR DETECTION PROTEINS IN BLOOD PLASMA

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DNA aptamers are single stranded nucleic acids with specific binding sites for proteins. They can be immobilized onto a solid support, e.g. by avidin-biotin technology. This aptasensor can be used for detection biologically important proteins, e.g. prions. So far the effectivity of aptamers was demonstrated mostly in buffers but not in a complex biological liquids, e.g. in blood or blood plasma. In this work we examined the sensitivity of detection the protein in a blood plasma using aptamer specific for thrombin. Two basically different methods were used for detection – the method based on electrochemical indicators and the method based on quartz crystal microbalance (QCM). We also examined how the structure of the aptamers affect the protein binding and optimized the method of regeneration of the sensor surface. Finally the sensor was tested in blood plasma of a health donors. We showed, that using the electrochemical indicator – methylene blue – and diffential pulse voltammetry (DPV) or chronocoulometry (CC) it is possible to detect human thrombin wih detection limit of 100 pM. The DPV method was, however more sensitive. The aptamer used selectively binded the heparing binding site of the thrombin. This has been proved in experiments in which the heparin-thrombin complexes were investigated. 2 M NaCl was effectively used for sensor regeneration. After this treatment the sensor can be used at least three times with similar sensitivity. The results obtained can be applied for another aptamer based biosensors, e.g. for detection prions. Detection of thrombin in blood plasma caused certain difficulties. Possibilities of improvement sensor sensitivity in blood plasma is discussed.

DIA-48

LYMPHOID INVOLVEMENT IN PRE-CLINICAL CHRONIC WASTING DISEASE IN ADULT MULE DEER (ODOCOILEUS HEMIONUS)

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The exact dissemination mechanism of the chronic wasting disease (CWD) associated prion, PrP^{CWD}, within natural cervid hosts is not completely understood. Strong evidence suggests that the peripheral nervous system is important in this process. In addition, the tropism of PrP^{CWD} for lymphoid tissue has been extensively described. PrP^{CWD} has been demonstrated in various lymphoid tissues of experimentally and naturally infected mule deer (*Odocoileus hemionus*). However, the extent to which PrP^{CWD} is distributed in lymphoid tissues of pre-clinical free-ranging mule deer has not been reported. We describe six, apparently healthy, male and female mule deer, sampled as part of an ongoing test and cull CWD management program at Rocky Mountain National Park, Colorado, USA. Each deer was found to have immunohistochemical staining (IHC) consistent with CWD in a palatine tonsil biopsy sample. Deer were euthanized and full necropsies performed. Numerous tissues were collected to investigate the distribution of PrP^{CWD} during the pre-clinical stages of disease. Five of the animals were considered to be in the early stages of CWD and one was in a mid disease stage. Lymphoid tissues of the head, neck, thorax, and abdomen including peripheral lymph nodes were IHC positive. Additionally, hemal nodes of the neck, thorax and abdomen were positive. The thymus glands were IHC negative. This report documents the wide distribution of lymphoid tissues where PrP^{CWD} may be found during the early stages of CWD in mule deer. Furthermore, PrP^{CWD} accumulation in hemal nodes suggests the potential for hematogenous dissemination of PrP^{CWD}.

DIA-49

UTILITY OF RECTAL MUCOSAL BIOPSIES FOR DETECTING CHRONIC WASTING DISEASE IN ROCKY MOUNTAIN ELK (CERVUS ELAPHUS NELSON)

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Preclinical diagnostic tests for transmissible spongiform encephalopathies have been described for mule deer (*Odocoileus hemionus*), using biopsy tissues of palatine tonsil; for sheep, using lymphoid tissues from palatine tonsil, third eyelid and rectal mucosa. The utility of rectal mucosal biopsies was investigated in Rocky Mountain elk (*Cervus elaphus nelsoni*), a species for which there is not a live-animal diagnostic test. Postmortem rectal mucosal sections (PRMS) were examined from 308 elk from two herds that were depopulated. The results of the PRMS were compared to immunohistochemical (IHC) staining of the brain stem (vagus nucleus), retropharyngeal lymph nodes and palatine tonsil. Seven elk were found positive in the brain stem and cranial lymphoid tissues, however, only six of these elk were positive in the PRMS. The remaining 301 elk in which PrP^{CWD} was not detected in the brain stem and cranial lymphoid tissues also were free of PrP^{CWD} in the PRMS. Rectal mucosal biopsies (RMB) were taken from 421 live elk from three ranches in which at least two previous cases of CWD had occurred. Three positive non-clinical elk were found in one farm. These elk were killed and CWD was confirmed. PrP^{CWD} also was found around neurons and in non-myelinated nerves within the submucosa. PrP^{CWD} also was found in the connective tissue of the lamina propria of the infected elk. Number of follicles counted in PRMS and biopsies decreased with age of animal. This procedure is easy and rapid to perform and may be suitable as a diagnostic test as part of an integrated management strategy to detect CWD in elk.

DIA-50

ADVANTAGES OF THE MOREDUN RECSPECTM FOR TSE DIAGNOSIS IN RUMINANTS

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Diagnosis of ruminant TSE disease currently relies on detection of abnormal PrP in tissue samples. While largely confined to the CNS in BSE of cattle, abnormal PrP accumulates in tissues of the lymphoreticular system in sheep scrapie and CWD-infected deer. Abnormal PrP can often be detected in LRS tissues before any CNS involvement, and in otherwise asymptomatic animals. A biopsy of LRS tissue in the live animal is therefore a useful test of infection status. Previous studies have focused on biopsies of palatine tonsil and third eyelid; here we report on the development of a biopsy technique for rectoanal mucosa-associated lymphoid tissue (RAMALT). We have designed the Moredun RecSpec^{TM}, a speculum which allows observation and accurate sampling of the rectal mucosa, specifically targeting the lymphoid follicle-rich area between mucosal folds. We employed this method to take sequential biopsies from sheep of the Moredun natural scrapie infected Suffolk flock. Biopsy samples were fixed in buffered formalin and assessed by immunohistochemistry for the number of follicles (valid biopsy if n >=5) and the presence of abnormal PrP. RAMALT biopsies taken using the Moredun RecSpec^{TM} gave better quality biopsy samples (97% biopsies >5 follicles) compared with a parallel palatine tonsil biopsy (75% biopsies >5 follicles) with a significantly greater average number of follicles. Animals do not require sedation and exhibit no signs of distress or subsequent complications allowing sequential biopsies from the same animal. With experience the technique is very rapid (<1min/animal) and the equipment is inexpensive (~ GB£1.50/animal). Sample size is adequate for detection of abnormal PrP in ELISA systems, and we have successfully adapted the technique for use in red deer. RAMALT biopsy by this method can be a useful tool in the detection of preclinical and clinical disease in experimental studies or expanded to flock testing as part of disease control strategy.

DIA-51

MULTIFOCAL “NECROTIZING” LEUKOENCEPHALOPATHY MIMICKING CREUTZFELDT-JAKOB DISEASE

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A multifocal “necrotizing” leukoencephalopathy (MNL) mainly involving the white matter of the pons and characterized by microscopic foci of necrosis have been reported in immunosuppressed and critically-ill patients. A 64-year-old woman with history of chronic respiratory failure of restrictive type was referred to our intensive care unit because of severe hyponatremia (110 mEq/l) and recurrent seizures. At admission, coma without focal neurologic signs was evident. EEG background was in theta range with frequent spike-wave discharges. In spite of adequate correction of sodium levels and systemic antiepileptic treatment, no clinical improvement was observed. CSF analysis disclosed the presence of 14-3-3 protein and very high levels of tau (10.000 pg/ml). A presumptive diagnosis of Creutzfeldt-Jakob disease (CJD) was made. A brain MRI highlighted high signals on DW and T2 scans involving left posterior thalamus and parietal cortex. A second CSF analysis (performed 4 weeks later) confirmed the presence of 14-3-3 protein and greatly raised tau levels (15.000 pg/ml). After about two months the patient died from multiple organ failure. Biochemical examination of frozen brain tissue failed to detect PrPres. Neuropathological examination ruled out a diagnosis of CJD. Multiple microscopic foci of necrosis involving the white matter tracts at the basis of the pons were present on histologic examination. No calcified areas were evident. A presumptive diagnosis of multifocal leukoencephalopathy was made. The case described appears to fulfil the pathologic criteria for MNL in immunosuppressed and critically-ill patients. To our knowledge there are no other reports of MNL mimicking CJD in its clinical, EEG, MRI and CSF aspects.

DIA-52

TIME RESOLVED FLUOROIMMUNOASSAY OF PRPSC IN SHEEP BLOOD.

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Development of a pre-symptomatic blood test for TSEs for human and veterinary diagnostic purposes remains a high priority. This also represents an appreciable challenge given the low levels of the disease biomarker PrP^{Sc} likely to be present in blood. One of the few tests reported as capable of detecting PrP^{Sc} in blood is capillary electrophoresis immunoassay[1] (ICE), however this procedure appears to lack the robustness required for routine application.
A microwell based time resolved fluoroimmunoassay (trFIA), analogous with ICE, has been develop and applied to the analysis of blood following extraction with a solid phase ‘PrP^{Sc} specific’ affinity ligands. These ligands, known as the “Seprion range” are a group of polymeric compounds that have the ability to bind PrP^{Sc}, under selective conditions. Plasma and buffy coat Seprion captured extracts from 30 sheep at the terminal stage of scrapie and 30 control sheep were analysed using the novel trFIA. Highly significant differences were seen between the diseased and control animal groups, for both the plasma and buffy coat samples ($p = <0.0001$). This study provides preliminary proof of principle for the combined Seprion extraction –trFIA procedure, which offers promise as a pre-mortem diagnostic blood test for scrapie.

1. Schmerr, M.J., *et al*. (1999) J. Chromatog. A, 853, 207-214.

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DIA-53

IN SEARCH FOR BLOOD TSE BIOMARKERS USING THE SELDI (SURFACE-ENHANCED LASER DESORPTION/IONIZATION) APPROACH.

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The present work relies on the use of the new SELDI (Surface-Enhanced Laser Desorption/Ionization) proteomic approach to look for TSE related biomarkers. Importantly, the analysis of biological samples and in particular serum is analytically challenging due to the high dynamic concentration range of constituent protein/peptide species. However, SELDI has already demonstrated its ability in proteomic sample analysis leading to the discovery and development of novel multimarker clinical assays for different cancers as well as for neurological disorders.
In the framework of a Neuroprion program (STEM-TSE), we are analyzing different TSE samples including human, ovine, mouse and cattle sera. We will present here the SELDI approach and give examples of the results obtained using human sera. We have indeed selected different peaks which relative level of expression were linked to the presence of a human TSE and/or to a neurodegenerative process. We are now in the process of purifying and identifying the biomarker candidates.

This work is supported by the Neuroprion network of excellence (LOI SELDI-TSE) and the CNRS.

DIA-54

DETECTION OF OVINE SCRAPIE IN RAMALT TISSUE USING AN ELISA

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Earlier studies of recto-anal mucosa-associated lymphoid tissue (RAMALT), obtained from animals at various stages of disease and subjected to immunohistochemistry (IHC), and Western blot (WB) analysis have shown that PrP^{sc} could be detected with a consistency comparable to central nervous system, and other lymphoreticular system tissues. The aim of the present work was to investigate the suitability of using an IDEXX post mortem PrP^{sc} ELISA for the detection of scrapie in rectal biopsy tissue as a potential diagnostic tool for live animal testing. An initial small scale evaluation was conducted with the IDEXX Herdchek Chronic Wasting Disease Antigen Test Kit (CWD-EIA). The test was conducted according to the package insert with ovine RAMALT tissue replacing the cervid retropharyngeal tissue normally evaluated in the test. This preliminary evaluation resulted in a specificity of 100% (n=38) and diagnostic sensitivity of 75% (56/75 positive). Based on this promising data, the decision was made to further optimize the IDEXX CWD-EIA for RAMALT applications. A small panel of IHC positive and negative tissue was made available for optimization purposes. The key steps evaluated were tissue disruption, PrP^{sc} sample capture and PrP detection conditions. Release of PrP^{sc} from the tissue matrix was optimised by the addition of a large ceramic bead to the disruption step and increasing the number of cycles. The addition of digestive enzymes was investigated but was found to have no significant effect on signal strength. An increase in capture time, during the sample incubation step along with the addition of a gentle motion was used to optimize sensitivity. Experiments also demonstrated that there was no significant loss of signal when biopsy sized tissue (approximately 120mg) was used, compared to the standard kit weight of 300mg. Using the optimized protocol, a population of 256 scrapie negative RAMALT samples and 213 scrapie positive RAMALT samples (IHC positive) were evaluated on the IDEXX CWD-EIA Test Kit using the modified protocol. The specificity of the optimized protocol was 100% and sensitivity was 94%. In conclusion, we were able to modify the IDEXX CWD Antigen Test Kit protocol for optimal detection of PrP^{sc} in ovine rectal mucosal tissue.

DIA-55

CNA.42 AND FDC-B1, DIRECTED AGAINST OVINE FOLLICULAR DENDRITIC CELLS?

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Like the variant of Creutzfeldt-Jakob (vCJD) in human and bovine spongiform encephallopathy (BSE) in cattle, natural scrapie in sheep is a transmissible spongiform encephalopathy (TSE) caused by prions (PrPSc). If natural scrapie is endemic in sheep and considered as harmless for human, the BSE species-specific barrier from cattle to genetically susceptible sheep appears to be non-existent. Sheep orally infected by BSE agent developed similar clinical signs than in natural scrapie infection and the similar tissue tropism of the two agents suggest that BSE could potentially be transmitted from sheep to sheep. Following a peripheral exposure, contary to BSE agent, PrPSc is widely distributed in all lymph organs. The presence of PrPSc at early time point in tonsils suggests that tonsillar biopsies would have a role in the diagnosis of preclinical stage of the disease. In lymph organs, follicular dendritic cells (FDCs) seem to be the major site of cellular prion protein expression and the principal sites of infectious agent accumulation. Nevertheless, the ability to recognise specifically the network of FDCs from all secondary ovine lymph organs is limited. In this study, two monoclonal antibodies were tested: CNA.42 directed to human FDCs and FDC-B1, directed to bovine FDCs. Palatine and pharyngeal tonsils were removed from sheep at the age 4 to 6 months, processed for immunocytochemistry and florescence microscopy analysis. This one reveals a clearly positive network inside the germinal center of both tonsils immunostained with FDC-B1. However, the CNA.42 only cross-reacts with paryngeal tonsils follicular dendritic cells. These results permit to postulate that the antigen recognised by CNA.42 is not expressed in the same way by follicular dendritic cells from all lymph organs. Negative staining obtained on ovine tonsils with this antibody must thus be interpreted with full knowledge of the facts. Other lymph organs, as lymph nodes from digestive and respiratory tract, will be studied to highlight if this observation is organ specific or not.

DIA-56

IN-VIVO DIAGNOSIS OF PRION INFECTED MOUSE USING VISIBLE AND INFRARED LIGHT

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To be truly useful, prion diagnostics should identify “suspect” cases during the pathogenesis at as early stage as possible. The currently available methods are quite insensitive when compared with those available for other infectious diseases.
Near Infrared Spectroscopy (NIRS) has proved to be a straight forward method for disease diagnosis based on noninvasive and nondestructive in vivo monitoring of bio fluids and tissue spectral changes. We have examined possible application of NIRS for prion diagnosis.
Scrapie (Chandler and Obihiro strains) infected C57BL/6J mouse brain homogenate was used as the challenge material. Twenty µl of 10% brain homogenates were suspended in 1ml of PBS and intracerebrally injected into the brain of 20 and 4 mice, respectively. Brain homogenate of a normal mouse and PBS, as controls, were injected in 4 mice each. NIR in-vivo spectra of each mouse were acquired in the area of the abdomen and the brain, respectively, at regular time intervals, with portable NIR instrument, Fruit tester 20 (Fantec Ltd., Japan). Spectral data were analyzed by Principal component analysis (PCA) and SIMCA classificator and the difference between groups was expressed as distance between classes. The experiment was carried out until the number of the mice in infected groups became 1 (226 days, 20 spectral measurements at regular time intervals).
It was found that the distance between each class of infected mice and the control group (mice with normal brain homogenate injected) was significant and increasing with the time while the distance between the control and the PBS injected mice was decreasing. Interestingly, periodic ups and downs of the class distances to the control were observed for all groups, over the period of the experiment. In this experiment, the first substantial distance was found at day 50, for Chandler, and at day 90, for Obihiro strain, after the injection.
NIRS proved to be a valuable tool for an early in-vivo diagnosis and for bio – monitoring that could provide new scientific insides in the area of prion disease diagnosis and understanding of its pathogenesis.

DIA-57

PRP^{RES}-LIKE PROTEINS IN SCRAPIE-INFECTED HAMSTER PLASMA SIMILAR WITH THE PRP^{RES} IN BRAIN

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PrPres has hardly been detected in the blood except in leukocytes even during the disease period of animal models, when PrPres is found at high amounts in sensitive tissues. We examined detecting PrPres in scrapie infected hamster plasma (scHaPl) by partial purification and using highly sensitive chemiluminescence immunoblot (IB). Brain homogenate of scrapie-infected hamster (scHaBrh) and scHaPl were obtained at the terminal stage of scrapie (Sc237) infection. The scHaBrh and the scHaPl were digested with PK or PK and PNGase F then purified partially by acidic SDS precipitation. Preparations has analyzed by conventional IB using anti-PrP mAbs and a highly sensitive chemiluminescence reagent (SSWF). Acidic SDS condition was successfully precipitated the PrPres in scHaBrh as well as 3F4-reactive proteins in scHaPl selectively and the SSWF enable the detection of the PrP protein at concentrations as low as in 10^{-9} g brain equivalent. In combination with an image analyzer, treatment of both scHaBrh and scHaPl with PK and PNGase F resulted in the formation of 19-20kDa 3F4-reactive proteins, indicated similar peptide backbones. Acidic SDS precipitation after the PK treatment of the scHaPl discriminated the scHaPl and mock infected (mc) HaPl but between the 3F4 reactive protein species and the protein species of PrPres in scHaBrh showed extreme Mw similarities. Furthermore, 2DE analysis also showed highly similar patterns between scHaBrh and scHaPl on PrPres and 3F4-reactive protein spots, respectively. In conclusion, the 3F4-reactive proteins in scHaPl are expected to be the PrPres in plasma. From these results, a practical screening test for TSE using plasma is expected to develop on the near future.

DIA-58

FEASABILITY BEFORE EVALUATION OF TESEE™ CJD ELISA AND TESEE™ WESTERN-BLOT FOR CREUTZFELDT-JAKOB DISEASE (CJD) DIAGNOSTIC

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Since 1997, we have been using in our lab a sensitive Western-Blot assay with ultracentrifugation step for diagnosis purpose. However, this assay is time consuming and needs some experience for recovering of ultracentrifugation pellet. The Bio-Rad TeSeE CJD ELISA test (TeSeE Elisa) is an improved version of the already existing TeSeE screening test commercialized for the detection of the Transmissible Spongiform Encephalopathies (TSE) in brain or peripheral tissues of animals. The Bio-Rad TeSeE WESTERN-BLOT assay (TeSeE WB) is routinely used for the confirmation of TSE suspected animals. Our goal is to implement rapid and robust tools for a routine CJD diagnostic, at least as sensitive and specific than our current protocol and/or reported protocols.
The aim of this work was to test the ability of TeSeE ELISA and TeSeE WB for detecting the PrP^{res} in CJD patients. For brain detection of PrP^{res}, samples were obtained from 28 patients: 14 non-CJD, 13 CJD and 1 Fatal Familial Insomnia (FFI). For peripheral tissues, 8 patients were selected: 3 vCJD, 2 sCJD, 1 mutation CJD and 2 non-CJD patients. Initial screening was done with the TeSeE Elisa. Then, the confirmation of all suspected cases (repeatedly positive at screening) was done with the TeSeE WB.
The TeSeE Elisa and TeSeE WB were able to detect a significant signal of PrPres in the brain of all patients with 100% specificity and 100% sensitivity. The same results were observed with the two assays when testing 8 spleens and 2 tonsils. The TeSeE WB has detected the typical molecular pattern of the PrP^{res} in brain, tonsil and spleen in the 2 v-CJD patients and has distinguished between PrPres types 1 and 2. The performances (analytical sensitivity, precision) of the two assays were also evaluated during this study.
This feasibility study has demonstrated the adaptability of TeSeE Elisa and TeSeE WB to detect PrPres in human brain, spleen or tonsil. They seem to be a correct alternative to the time consuming method with ultracentrifugation. Furthermore, to consolidate these preliminary results, a complete study will be needed on a larger cohort of patients.

DIA-59

DETECTION OF EARLY MRI SIGNAL ALTERATIONS IN TSE EXPERIMENTAL MODELS

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Transmissible spongiform encephalopathies (TSE) or prion diseases are lethal neurological disorders of animals (e.g. scrapie) and humans (e.g. CJD). Neuropathological features of TSE are spongiform change, neuronal loss, gliosis and accumulation of abnormal isoform of prion protein (PrPTSE). Although according to the WHO criteria, magnetic resonance imaging (MRI) is not a criterion for the diagnosis of CJD, it is recognized as a useful noninvasive diagnostic marker. To date few MR studies were performed on animal TSE models, using conventional MRI techniques. The aim of this study was to investigate the potential role of MRI in early detection of TSE using different animal models.
We infected hamsters intracerebrally (i.c.), intraperitoneally (i.p.) or orally with 263K scrapie strain and mice, i.p., with mouse adapted variant-CJD prions. MRI T2W images (TR/TE = 2500/60-70-90 ms) and diffusion-weighted (DW) images (b = 2136 s/mm2, TR/TE = 2000/50 ms) were carried out at 4.7 T (INOVA SIS 200/183 system, Varian, Palo Alto, USA). The MR signal was detected with a surface coil, volume coil for hamsters or a home-made headphone coil for mice.
MRI analyses showed hyperintensity in T2W images of thalamic nuclei areas in hamster brains (i.e. at 70 days post infection (dpi), i.c.; at 100-130 dpi, i.p.; from 130 up to 150 dpi, oral) and hypointensities in DW images in medulla oblongata of i.p. injected hamsters at 100-130 dpi. In variant-CJD infected mice T2W images revealed hyperintensities also in cortex. In our different TSE animal models we detected MR signal alterations at both pre-symptomatic and symptomatic stages of the disease. In particular: the increase in T2W signal could be detected mainly in orally and i.p. infected animals within a time window starting at about two third of the incubation period; the decrease in DW signal in i.p. scrapie infected hamsters was in general agreement with histopatological findings on disease progression in this model. The direct relationship between MR signal changes and histopatological observations is still to be definitively elucidated.

DIA-60

IDENTIFICATION OF MOLECULAR MARKERS OF BSE PATHOGENESIS: AN INVESTIGATION OF THE IMMUNE SYSTEM OF BSE INFECTED MICE.

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Diagnosis of TSE neuro-degenerative diseases is still based on the detection, at late stages of disease incubation, of the abnormal disease-specific isoform of the host prion protein (PrP^{sc}) accumulated primarily in the central nervous system (CNS) of infected animals. Components of the lymphoreticular system are central to prion pathogenesis and spread to the CNS. Subsequent to infection through either the peripheral or central routes and prior to its appearance in the spinal cord or the CNS, PrP^{sc} usually replicates and accumulates to high levels in the spleen, blood and other lymphoid tissues. In mice PrP^{sc} has been detected in Peyers Patch and spleen within 3 months of BSE infection. This observation gives rise to the possibility of using differential gene expression in peripheral tissues as an early diagnostic test for TSE infection. To support this, work performed at the Roslin Institute identified decreased levels of erythroid differentiation-related factor (EDRF) in the spleen of TSE infected mice and is also reflected in erythroid cells in blood. In the present project gene and protein expression is being investigated in components of the lymphoreticular system to detect genes and/or proteins that are differentially expressed between normal and BSE infected mice (strain 301C, passaged in C57BL *sinc* s^7 s^7) during early infection stages. The BXD12ty mouse model used in this investigation is susceptible to both primary and mouse-adapted BSE infection and displays a short incubation period (263±days). Gene expression across the early stages of infectivity, in comparison to age-matched mock-fed controls have been analysed in the spleen and blood cells using Affymetrix GeneChip® Mouse Genome 430 2.0 Array. A range of genes have been identified that are differentially expressed in infected animals. SELDI analysis was performed on plasma samples and a number of differentially regulated proteins have been identified. Validation is being carried out by qPCR and Western Blot analysis. Confirmation of these results may lead to diagnostic markers of BSE infection.

DIA-61

APPLICATION OF THE SEPRION LIGAND IN PRION DISEASE

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Previously, we have described the development of a PrPSc-specific ligand, Seprion, into a very simple microplate immunoassay which removes the need for sample preparation, including proteinase K. When used as a post-mortem brain assay this Seprion assay has 100% sensitivity and specificity compared to current gold standards (Ref. 1) and has received USDA approval for use in CWD and BSE and EU approval for use in scrapie (including atypical scrapie) and BSE. Recently, we have been investigating the utility of the ligand in an ante-mortem blood screening assay and in a therapeutic compound screening assay. Previously, we have presented the results of a small blind study on whole blood from scrapie-infected and uninfected sheep received frozen from the Veterinary Laboratories Agency (VLA) archive. In this study the assay had a sensitivity of 100% and a specificity of 96%. We have further developed this assay into a format appropriate for use in ante-mortem animal diagnostics and for use by human blood screening services. The results of screening a larger blind panel of sheep plasma samples and a large number of samples from a scrapie infection time course study will be presented. The assay has also been used to screen a large number of compounds for activity *in vitro* in the prevention of amyloid protein aggregation. A number of compounds were identified, some of which were also found to reduce scrapie prion load in infected neuronal cell lines. We conclude that the Seprion ligand is a versatile tool that can be incorporated into: post-mortem brain, lymph node and spleen assays; an ante-mortem plasma assay; and a therapeutic compound screening assay.

References

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DIA-62

DETECTION OF 14-3-3 PROTEINS IN CEREBROSPINAL FLUID IS A LATE DISEASE MARKER IN EXPERIMENTAL SIMIAN VARIANTCJD

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Here, we present data from a study in BSE-infected Cynomolgus monkeys. Cerebrospinal fluid (CSF) samples were collected from infected animals and tested for the presence of 14-3-3 protein isoforms as a biomarker for brain damage to identify the onset of brain damage. An in-house western immunoblot protocol was established and evaluated using 10µl of CSF to detect 14-3-3 protein isoforms: the lower detection limit is around 0.1 ng protein/10µl CSF. CSF samples were collected from Cynomolgus monkeys at regular intervals during the asymptomatic and symptomatic phase of infection. False-positive results were obtained by cell-contaminated CSF samples, whereas false-negative results were seen in samples after repeated freeze-thaw cycles. For the study, freshly obtained CSF samples were carefully tested for cell contaminations, aliquoted and one aliquot was tested on the day of collection. The incubation period (1000 – 1800 days) was defined as the period from the BSE inoculation until the drop in body weight, since the onset of the symptomatic phase of infection was characterized by this drop in body weight rather than by CNS symptoms. The decline in the body weight was followed by behavioural changes and finally by ataxia one to four months later. Monkeys with ataxia were sacrificed and simian vCJD was confirmed at necropsy by the detection of both typical lesion profiles and PrP^{res} in the brain. 14-3-3 protein-positive CSF samples were found exclusively during the phase of behavioural changes and ataxia but not earlier. In conclusion, 14-3-3 proteins are detectable in small amounts of simian CSF samples. However, pathological changes occur earlier than detectable brain damage. Finally, the detection of 14-3-3 proteins in CSF samples is a tool suitable for defining and standardizing the humane endpoint in experimental simian vCJD. The work referenced was performed in partial fulfilment of the study "*BSE in primates*" supported by the EU (QLK1-2002-01096).

Poster Session

THERAPY

THE-01

DNA VACCINE AGAINST PRION DISEASES: A PROMISING APPROACH

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Prion diseases as Creutzfeldt-Jacob are lethal to humans and other animals and are closely associated with the conformational alterations of the ubiquitous prion protein (PrPc). Recently, immuno therapy approaches have focused on the ability of antibodies to prevent propagation of the abnormal form of prion protein (PrPsc). More precisely, it has been decribed that only antibodies recognizing native cell-surface PrPc may interfere with prion pathogenesis. In this context, we are developping a DNA vaccine approach against prion disease. Our objective is to enhance antibody titers against native PrPc compared with that obtained using classical vaccination protocols.
However, protective responses in wild type mice is limited, which is believed to be a consequence of T cell tolerance to the PrPc. To overcome this problem, we have developped a strategy based on modified plasmid vectors encoding a secretory isoform of the human PrPc (hPrP1-229 without the GPI anchor) fused or not with a sequence of the tetanus toxin (829-844), an universal T helper cell epitope. These constructs were compared for their capacity to induce human PrP specific immune response in C57Bl/6 mice. Intramuscular injections of those naked DNA have triggered a specific but low immune response. One hypothesis to explain this result is that the level of human PrPc protein, induced by the way of naked DNA injection, may be too low. Very recently, biodistribution studies of naked DNA vaccines showed that the number of plasmid DNA molecules surviving to transfect cells after intramuscular injection was only a small fraction of the total DNA injected. So, to enhance *in vivo* cells transfection efficiency, we have compared different DNA delivery methods (*in vivo* electroporation, and formulation with polyethylenimine or non-ionoic block copolymers) in terms of plasmid detection, transcrit and protein expression *in situ* (injection site and spleen). Preliminary results indicate promising ways to develop an efficient immunization protocol against prion diseases in wild type mice.

THE-02

ADOPTIVE IMMUNO-CELL THERAPY IN PRION DISEASES :A STUDY OF SOME PREREQUISITES

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Adoptive immuno-cell therapy could circumvent some of the limitations reported with active vaccination or passive antibody transfer against mouse scrapie. These difficulties mainly stem from the strong tolerogenicity of PrP and from the relative inaccessibility of the brain to antibodies. We have presently explored two alternative strategies of adoptive cell therapy. A first one consists in adoptively infusing into wild-type mice, T cells from *Prnp*-/- donors which were immunized against major mouse PrP epitopes. Another one relies on the administration into wild-type recipients, of live activated bone marrow-derived dendritic cells (DCs) loaded with MHC class II-restricted immunogenic peptides of mouse PrP. Regarding the first strategy, we have asked whether adoptively transferred T cells would survive to the various forms of peripheral tolerance, retain their immune reactivity, cooperate with host B cells, and eventually retard scrapie progression without causing adverse autoimmune manifestations. Our results do clearly demonstrate a persistence of the adopted T cells in the wild-type hosts up to 3 months after transfer, with an intact capacity to respond to PrP epitopes and to cooperate with B cells for antibody production. Furthermore, such T cells retard scrapie lymphoinvasion with no apparent clinical or histological signs of autoimmunity. With respect to the loaded-DC strategy, we have looked at the capacity of such cells to activate a helper T and B lymphocyte repertoire specific of PrP. Loaded DCs elicit a reproducible antibody response to native, membrane-bound PrP, even in wild-type mice, presumably harbouring a strongly repressed B cell repertoire. These results provide encouraging answers to some major prerequisites for adoptive cell therapy: 1) infused anti-PrP T cells are not ineluctably tolerized in a PrP-positive environment, 2) a persisting B cell repertoire to native PrP can be activated, notably by peptide-loaded DCs in wild-type recipients and 3) adoptive cell therapy appears to be active against prion propagation in scrapie-infected mice.

THE-03

MONO AND BI-FUNCTIONAL ANTIBODIES AS THERAPEUTICS FOR PRION DISEASES

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Prions are the infectious proteinaceous agents of Transmissible Spongiform Encephalopathies (TSEs) or prion diseases. According to the *protein-only* theory, prions propagate by recruiting and converting the cellular prion protein (PrP^{C}) to the disease causing isoform (PrP^{Sc}or scrapie). No effective therapies exist at present for these fatal diseases.
Although several reports have shown that both antibodies and Fab fragments against PrP^{C} are able to cure scrapie-infected cells, their effectiveness in prion disease therapy in vivo has not yet been evaluated because passage of antibodies to the brain through the blood brain barrier (BBB) is prevented. In order to overcome this problem, monoclonal antibodies against transcytotic receptors present at the BBB have been used to deliver different therapeutic molecules into the brain by exploiting their transcytotic pathway. Therefore, in order to test the potential of the anti-prion antibody D18 in passive immunotherapy as a therapeutic intervention in TSEs, we have produced both monofunctional (scFv) and bifunctional (diabody) versions of the anti-PrP D18 Fab.
Our studies suggest that D18 scFv might be directly used in TSE therapy in vivo because its dimensions could allow a faster and more efficient spreading of the molecule in the brain after i.c. injection and even a passage into the brain after nasal administration.
Addionally, we have also produced a small chimeric antibody (or diabody) containing both anti-PrP and anti-transferrin receptor specificities that should be able to cross the BBB via this transcytotic pathway.
We have shown that some of these molecules are able to bind recombinant PrP and PrP^{C} from mouse brain homogenate and reduce PrP^{Sc} levels in scrapie-infected GT1 cells. We are currently evaluating their potential therapeutic effect after intracerebral and peripheral administration in prion-infected mice.

THE-04

EVALUATION OF ANTI-PRION DRUG POTENCY IN PRIMARY NERVE CELL CULTURES INFECTED BY PRIONS FROM VARIOUS SPECIES

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Transmissible spongiform encephalopathies (TSE) arise as a consequence of the infection by prions of the central nervous system and are incurable. Neuronal loss and gliosis, associated with the accumulation of misfolded PrP protein (PrP^{Sc}) are hallmarks of prion diseases, however the mechanisms underlying these disorders remain unclear. We recently described a cellular model consisting of cerebellar granule cells derived from transgenic mice expressing ovine PrP. Upon exposure to low infectious doses of natural sheep scrapie agent, such cultures were found to accumulate *de novo* PrP^{Sc} and infectivity, as assessed by protease digestion and mouse bioassay. Both neurons and astrocytes were found to sustain prion propagation, and a late neuronal death was observed in infected cultures [(1)]. In an attempt to generalize these observations, we found that it is also feasible to propagate mouse, hamster and human prions in cultures derived from transgenic mouse lines expressing the cognate PrP^{c}.
Primary cell cultures are widely used to search for molecules with protecting activity in neurodegenerative or neuro-infectious diseases as well as to assess their neurotoxicity. Thus we sought to evaluate the activity of Congo red, chlorpromazine and a polyene antibiotics, three compounds known to cure prion-infected cell lines, in these newly developed cell systems. We observed that the anti-prion activity in such cultures is species- or strain-dependent, and is in good agreement with results reported *in vivo* in rodent models. Our findings suggest that prion-infected primary neuronal cultures may be relevant tools for anti-prion drug appraisal, including human TSE agents.
[(1)] Cronier S., Laude H., Peyrin J.M. (2004) Proc Natl Acad Sci U S A. 101, 12271-6.

THE-05

DEVELOPMENT OF A CELL THERAPY STRATEGY FOR THE TREATMENT OF PRION DISEASES

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Once a patient becomes infected with a prion disease, or transmissible spongiform encephalopathy (TSE), the progression of infection is inexorably fatal. Although the potential for late stage therapies using chemical molecules seems limited, cell therapy strategies which have been shown to be effective in other neurodegenerative conditions might ameliorate TSE induced neuropathology. By taking advantage of prion "resistant" polymorphisms Q171R and E219K that naturally exist in sheep and humans, respectively, we have evaluated a cell therapy strategy combined with a gene therapy approach. In order to orchestrate a brain repair with prion resistant cells, our specific objective is to genetically modify embryonic stem cells (ES), by introducing “dominant negative” PrP mutants, before their transplantation in scrapie infected mouse brain.

According to a strategy employed by Dr. A. Aguzzi, we have differentiated embryonic stem cells (ES) *in vitro*. When placed in a “neural” differentiation medium, the ES cells formed embryoid bodies (EBs) enriched in neural precursors that are collected for transplantation. We have then set up optimised conditions for gene delivery in these cells using lentiviral systems. We succeeded to transduce the EBs with the lentivirus carrying the dominant negative PrP mutants. We have previously shown that these lentivirus were able to inhibit prion replication *ex vivo*. To test the feasibility of this graft approach, C57bl/6 mice have been intra-cerebrally inoculated with the Me7 prion strain and transduced EBs have then been injected into different area of the mice brain using a stereotaxic frame.

We are now assessing the effect of the transplantation on the development of a prion disease. Although we do not expect, for this first pilot study, an increase of the incubation period, we hypothesize an inhibition of the prion replication through the dominant negative properties of the mutated PrPs in the grafted area, as well as a brain repair around the transplanted area.

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THE-06

TARGETING THE UNTRANSLATED REGIONS (UTRS) OF THE PRNP GENE

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Although the underlying mechanism for the conversion of normal cellular prion protein (PrP^{C}) into the pathological isoform (PrP^{Sc}) is not fully understood, current strategies for therapy focus on either inhibiting this process or abolishing the formation of PrP^{Sc}. $PrP^{0/0}$ knockout mice lacking the PRNP gene cannot produce the infectious agent, are protected against the disease and show only a subtle phenotype. This indicates suppressing the expression of PrP^{C} may be a reasonable approach to prevent accumulation of PrP^{Sc}. We are currently investigating the possibility of specifically down-regulating PrP^{C} expression at the translational-level. Using a luciferase-reporter assay with the gene for firefly luciferase under the translational control of the 5’UTR of the human PRNP gene we are monitoring the effect of several compounds on PrP5’UTR-mediated translation in stably transfected SH-SY5Y cells in a 96-well format. Enhanced green fluorescent protein (EGFP) acts as a specific internal control reporting cell viability and transcription levels. A second construct exploiting the 5’UTR of the Alzheimer amyloid precursor protein (APP) gene counterscreens for non-specific hits on PrP5’UTRs. An Iron-responsive Element (IRE) Type II has been found in the APP5’UTR to regulate its translation (Rogers et al., 2002). Compared to APP5’UTR, preliminary data for PrP5’UTR show similar effects on iron treatment in the luciferase assay, suggesting its RNA secondary structure as a possible target for translational regulation (Bandyopadhya et al., 2006). Additional studies indicate a specific regulation of PrP5’UTR-conferred translation. We intend to further confirm and characterise this process by inserting small deletions into the 5’UTR and mapping regions essential for regulation. Compounds identified in a high-throughput screen with FDA-approved drugs, which specifically down-regulate translation mediated by PrP5’UTR, will be tested for their effect on expression of PrP^{C} and propagation of PrP^{Sc} in scrapie-infected N_2a cells.

Rogers et al. (2002). *J Biol Chem* 277, 45518-45528
Bandyopadhya et al. (2006). *J Biomol Screen.* In press

THE-07

EFFECTIVENESS OF AN ORALLY ADMINISTERED ANTI-PRION CHEMICAL

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We reported previously that some amyloid imaging probes or amyloidophilic chemicals developed for diagnosis of Alzheimer's disease are effective as anti-prion chemicals when administered intravenously (J Gen Virol. 2004;85:1785-1790). We have developed and tested new amyloidophilic chemicals with better brain-permeability. We have concluded that relatively longer retention in the brain than that of the chemicals might be also important to improve their effectiveness as anti-prion chemicals (J Neurochem. 2006; in press). Here, we report the anti-prion effectiveness of an orally administered amyloidophilic chemical that was developed originally as a drug candidate for Alzheimer's disease and which possesses satisfactory permeability and relatively longer retention in the brain than the imaging chemicals.
This chemical, called compB, inhibits PrPres formation in the cells that are persistently infected with RML prion at a subnanomolar dose, but it is not effective in cells that are infected with Fukuoka-1 prion or 22L prion. When administered orally in a form of the mixture with powder feed from the inoculation to the disease terminal, compB prolonged the incubation times of mice who had been inoculated intracerebrally with RML. Its effectiveness was dependent on the compB dose. The highest dose (300 mg/kg/day) prolonged the incubation period by 2.5 times that of the control. The compB effectiveness was also observed in infected mice when it started from different time points after inoculation, although the better effectiveness depended on the earlier start of administration. Oral compB was also beneficial for mice that had been inoculated intracerebrally with Fukuoka-1 or 22L. The effectiveness in these mice, however, was not so prominent as that in the mice with RML. The mice with 263K prion showed no significantly different incubation times using oral compB.
Improvement of the pharmacokinetic parameters of compB seems to be necessary for better efficacy. Furthermore, we must elucidate the mechanism of the prion strain-dependent effectiveness and cope with it. Nevertheless, the findings of this study encourage us to pursue chemotherapy for prion diseases.

THE-08

AN ANTIPRION COCKTAIL AGAINST CREUTZFELDT-JAKOB DISEASE

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Creutzfeldt-Jakob disease and other human or animal prion diseases have lacked an efficient pharmacotherapy so far. Although highly effective in the ScN2a cell model of prion disease, heterocyclic compound quinacrine has shown ambigous or insufficient power in *in vivo* experiments.
Here, we present data on improved pharmacological options for prion diseases, particularly for patients with Creutzfeldt-Jakob disease. Combination therapies have been successful clinical strategies in infectious diseases like tuberculosis or AIDS. An antiprion cocktail of several blood-brain barrier-permeable pharmaceuticals already in clinical use was tested for antiprion activity *in vitro* and *in vivo*.
In the ScN2a cell model, the tricyclic antidepressants or iminodibenzyl derivatives like clomipramine showed a clear structure-activity relationship for antiprion effects that were additive to those of quinacrine. We found that the mechanism of action of these heterocyclic compounds can sufficiently be explained with a redistribution of cholesterol from the plasma membrane into intracellular compartments, thereby destabilizing conversion-mandatory lipid rafts. Adding another drug alterning cellular lipid metabolism, HMG-CoA reductase inhibitor simvastatin to quinacrine and clomipramine proved to be a highly efficient cocktail with synergistic antiprion effects.
When such a cocktail was administered orally to mice of the fast-replicating tg20 mouse strain that had been inoculated with RML prion several weeks before, a significant delay of incubation time was observed compared to mock treated controls. We thus could significantly improve a pharmacotherapeutical regimen for treating prion disease and propose such a combination therapy for treating patients with Creutzfeldt-Jakob disease.

THE-09

QUINACRINE BINDING TO PROTEINS: MECHANISM, IMPLICATIONS FOR ANTI-PRION THERAPY AND APPLICATION AS IN VITRO SCREENING ASSAY FOR PRP BINDING

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Tricyclic aromatic (TCA) compounds have been proposed to be candidates for treatment of TSE. Direct interaction with PrP^C has been suggested as mechanism of drug action. We here show by means of NMR-spectroscopy that binding of TCA compounds occurs with millimolar *k*D to motifs consisting of two neighboring aromatic residues. Binding is independent of the secondary structure of the double-aromatic residue motif and independent of the side chains attached to the tricyclic aromatic compound and is not specific to PrP. Moreover, biologically inactive 9-aminoacridine (9-aa) binds with similar *k*D than anti-prion active quinacrine ruling out direct interaction of TCA´s with PrP^C as drug active mechanism. However, binding of 9-aa to PrP^C can be used as reporter for binding of other proteins to PrP^C by measuring changes in T1-NMR relaxation times of 9-aa upon PrP-protein complex formation. This assay is applied to probe the binding of two proteins reported to bind to PrP^C under NMR conditions.

THE-10

SIMVASTATIN-TREATMENT PROLONGS SURVIVAL TIMES IN A MURINE PRION MODEL

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Prion diseases are fatal and at present there are neither cures nor palliative therapies known/available, which delay disease onset or progression. Cholesterol-lowering drugs have been reported to inhibit prion replication in infected cell-cultures and to modulate inflammatory reactions. We aimed to determine whether simvastatin-treatment could delay disease onset in a murine prion model. Groups of mice were intracerebrally infected with two doses of scrapie strain 139A. Simvastatin treatment commenced 100 days post infection. The treatment did not affect deposition of misfolded prion protein PrP^{res}. However, expression of marker proteins for glia activation like major histocompatibility class II and galectin-3 was found to be affected. Analysis of brain cholesterol synthesis and metabolism revealed a mild reduction in cholesterol precursor levels, whereas levels of cholesterol and cholesterol metabolites were unchanged. Simvastatin treatment significantly delayed disease progression and prolonged survival times in established prion infection of the CNS ($p \leq 0.0003$). The results suggest that modulation of glial responses and the therapeutic benefit observed in our murine prion model of simvastatin is not due to the cholesterol lowering effect of this drug.

THE-11

NEW HEPARAN SULFATE MIMETICS FOR THE TREATMENT OF PRION DISEASES : A FIRST STRUCTURE-ACTIVITY IN VITRO STUDY

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Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative disorders with long incubation periods such as Creutzfeldt-Jakob disease in man. TSEs are characterized by the accumulation in the brain of an abnormal isoform (PrPres) of the host encoded prion protein (PrPc), which is partially resistant to proteolysis. PrPres, is the only specific marker of the infection, and the inhibition of its accumulation is often used to evaluate the efficacy of therapeutic drugs. There are currently no effective therapies for TSEs. Thus, the development of new therapeutics in human TSEs is of crucial importance. One class of molecules that has shown limited but significant efficiency in the treatment of TSEs is sulfated polyanions such as Pentosan polysulfate and Dextran sulfate 500. However their use is limited by their toxicity. Heparan sulfate bind PrP and play an active role in the PrP catabolic pathway. For this reasons, we thought that new heparan sulfate mimetics (HMs) initially developed for their ability to stimulate tissue repair, would represent good candidates for the development of a PrP-targeted therapeutic against prion diseases. Here, we report the first structure-activity study concerning the relationship between the antiprion activity of HMs and their degree of sulfation, their molecular size, and the influence of different hydrophobic cores. In order to do that, we tested in a cellular model chronically infected with Chandler scrapie strain (ScGT1-7), the ability of a battery of molecules on their capacity to inhibit PrPres formation. Our data show that optimal anti-prion activity of sulfated polysaccharides can be reached with medially sulfated molecules. This activity can readily be enhanced by introducing hydrophobic moieties (phenylalanine derivatives and ethylhexylamine) and by reducing molecular size. In conclusion, this novel generation of HMs with low molecular weight and hydrophobic functionalities open new insights for prion therapeutics.

THE-12

EARLY SUCCESSES IN THE DEVELOPMENT OF A PRPSC VACCINE USING A MOUSE MODEL SYSTEM

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The United States National Wildlife Research Center (NWRC) develops effective wildlife damage management methods through research to understand human and wildlife conflicts. As part of our mission, the NWRC has a very active Chronic Wasting Disease Project that has several ongoing research areas that aim to understand and prevent the spread of the endemic prion disease of cervids, Chronic Wasting Disease (CWD), in North America. Building on the success of the NWRC's vaccine based immuno-contraceptive efforts, we are determining the feasibility of developing a vaccine to reduce or prevent the spread of CWD in domestic and wild populations of cervids. Using a unique adjuvant technology developed at the NWRC, we tested five prion peptides coupled to a carrier protein to identify possible CWD vaccine candidates. Following vaccine treatments which produced antibodies to the individual peptides as measured by ELISA in CL57BL/10 mice, all mice were challenged with the Rocky Mountain Laboratory mouse scrapie strain via an intraperitoneal route. The progression of the disease was monitored by western-blot analysis and IHC. All five peptides tested have increased average days until death relative to the challenge controls. Two peptides increased survival very significantly ($p = 0.0005$). The unique nature of the adjuvant and the use of a relatively new carrier protein may have provided a basis for these promising initial results. While vaccinate groups still succumb to the disease, results suggest that an immunological approach to controlling CWD or other prion diseases is plausible. Our future directions are to continue work in a mouse model system to improve vaccine constructs and formulations in addition to moving into more relevant animals such as *Ovis aries* and/or cervids.

THE-13

ACTIVE IMMUNIZATION IN A HAMSTER MODEL OF SCRAPIE

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Vaccination has been shown to be effective in mouse models for neurodegenerative conditions characterized by protein misfolding, such as Alzheimer's disease (AD) and Transmissible Spongiform Encephalopathies (TSEs). Many different immunogens and strategies of intervention have been proposed for the immunotherapy of prion diseases, based on the necessity of agent replication in lymphoid tissue prior to neuroinvasion. Here we report the use of two synthetic oligopeptides (PrP 119-146 and PrP 142-179) corresponding to the central part of hamster (Mesocricetus auratus) prion protein, as a vaccine candidate in hamster scrapie model. Immunization with these oligopeptides, in particular PrP119-146, prolonged survival time (>23 days) in animals challenged intraperitoneally with 263K strain of scrapie agent. Peptide specific seroconversion and antibody titres were measured by ELISA; brain lesions and the content of glial fibrillary acid protein (GFAP) were analysed by histopathology and immunohistochemistry. The amount of prion protein (PrPres) both in spleen and brain was evaluated by immunohistochemistry and western blotting. Moreover, mRNA expression for GFAP and pro-inflammatory cytokines (TNF-□, IL-1□) were evaluated by RT-Real Time PCR. Immunized animals showed a specific, but of low titre, antibody response. Increased survival after challenge was associated with reduction of cerebral lesions, PrP deposition (both in brain and spleen), GFAP and cytokines expression. Therefore, our results indicate that, even if associated with a modest humoral response, vaccination can slow down PrPres deposition and decrease neuroinflammation, allowing to develope a targeted strategy to prevent or reduce the pathology due to prion infections.

THE-14

EXPERIMENTAL TREATMENTS FOR HUMAN PRION DISEASES

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Human prion diseases, also known as transmissible spongiform encephalopathies (TSE), are caused by accumulation of the abnormal isoform of the prion protein in the central nervous system. At present there is no proven specific or effective treatment available for any forms of TSE. Some oral agents, such as quinacrine or flupirtine, have shown promising results in vitro and are currently being investigated in clinical trials.

Pentosan polysulphate (PPS), a polysulphonated polysaccharide, has been shown to significantly prolong the incubation period when administered to the cerebral ventricles in preclinical trials with rodent TSE models. Cerebroventricular administration of PPS has been carried out in patients with different forms of TSE and was shown to be well tolerated in doses up to 220 µg/kg/day. Currently, a total of 22 patients have received PPS for periods of time ranging from 2 to 39 months. Proof of efficacy has been difficult because of the lack of specific and objective criteria for measurement of response to treatment. However, preliminary clinical experience demonstrated extended survival in some patients receiving long-term PPS. A young man with vCJD is surviving for a total of 5 years after initial symptoms, and for 39 months after start of PPS infusion. Other vCJD patients have also shown overall survival times well above the mean values expected with their disease.

Further prospective investigation of the cerebroventricular administration of PPS is essential to clarify the observed trends and to ascertain possible clinical benefits in patients with TSE.

THE-15

MHC CLASS-I RESTRICTED PEPTIDES FROM PRION PROTEIN CAN INDUCE SPECIFIC CYTOTOXIC T CELLS IN TOLERANT MICE.

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It is fundamental to improve the methods of prevention and therapy of Transmissible Spongiform Encephalopathies (TSE) for which no efficient therapy is available today. The disease is associated with conformational changes of a normal host protein, PrPc, which is converted into a protease resistant form, PrPSc. Whereas antibodies against native PrPc were shown experimentally to efficiently control PrPSc accumulation and pathogenesis, the possibility that cytotoxic T lymphocytes (CTL) directed against PrP-derived peptides would clear PrPSc-producing cells has never been explored.
The objective of this work was to generate CD8+ CTL against PrP by engineering potent immunogens to overcome tolerance in C57BL/6 mice. Immunization strategies will include the use of 1) PrP low affinity peptides that have been modified to increase their binding to MHC class I molecules, 2) the elimination of $CD4^{+}CD25^{+}$ regulatory T (Tr) cells to raise or improve the generation of T cells specific for PrP and 3) recombinant adenoviruses (Ads) vectors expressing homologous PrP genes or minigenes encoding MHC class I-binding peptides. In a first step, we immunized C57BL/6 mice with modified PrP nonamer peptides designed to bind with high affinity to H-2Db molecules and measured the frequency of IFN secreting specific T cells in response to the modified but also to the natural peptides. The cytotoxic potential of splenocytes from immunized mice was evaluated by their capacity to lyse modified and natural peptide-loaded targets in *in vitro* and *in vivo* assays. Among ten H-2Db-restricted PrP peptides, only few were able to induce CTL efficient in vitro and in vivo. The removal of CD4+CD25+ Tr cells by pretreatement of recipient with cyclophosphamide before immunization significantly increased the frequency of IFN secreting T cells specific for the natural and modified peptides .Experiments using immunization with recombinant Ads or plasmids expressing the entire PrP molecule or minigenes encoding the relevant peptides are in progress.
Further work will be performed to verify that some of the peptides that induce efficient CTL, are naturally processed and presented on the cell surface of PrP-expressing mice, and to test the protective capacity of these effectors on PrPSc accumulation and prion disease progression.

THE-16

VACCINATION APPROACHES AGAINST PRION INFECTIONS

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Prion diseases are neurodegenerative infectious disorders for which no prophylactic regimens are known. In order to induce antibodies/auto-antibodies directed against surface-located PrP^{c}, we used a covalently linked dimer of mouse prion protein expressed recombinantly in *E. coli*. Employing dimeric PrP as an immunogen in combination with adjuvant CpG, we were able to effectively overcome auto-tolerance against murine PrP in PrP wild-type mice without inducing obvious side effects. Treatment of prion-infected mouse cells with polyclonal anti-PrP antibodies generated in rabbit or auto-antibodies produced in mice significantly inhibited the endogenous PrP^{Sc} synthesis. In addition, we found immune responses against different epitopes when comparing antibodies induced in rabbits and PrP wild-type mice. Only in the auto-antibody situation in mice an immune reaction against a region of PrP is found that was reported to be involved in the PrP^{Sc} conversion process. Our data clearly show that we also induce a Th1-type T-cell response in this auto-immunization situation. Studies on improved antigen application and the efficacy of tolerance breakers like anti-OX40 antibody are ongoing. Our data point to the possibility of developing an active immunoprophylaxis against prion diseases.

THE-17

MODIFICATIONS OF PRP METABOLISM IN VITRO AFTER PENTOSAN POLYSULFATE TREATMENT VERSUS A NEW HEPARAN MIMETIC.

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Numerous compounds have already been evaluated *in vitro* for therapeutic purposes in prion diseases, with disappointing results *in vivo*. Sulfated polyanions, including polysulfate (PPS) and heparan mimetics, number among the more effective tested drugs. In this study, we focused precisely on the PrP metabolism after short treatments and on the anti-prion mechanism of a new Heparan mimetic (CR36) versus PPS.
CR36 was much more efficient than PPS to reduce PrPres accumulation in the chronically infected Sc GT1 cell line with a long lasting anti-prion activity. As assessed by FACS analysis, both compounds reduced PrP expression at the cell surface in several cell lines, with no change in *Prnp* gene expression. This effect appeared to be specific of PrP-GPI and endogenous heparan sulfate proteoglycans were necessary for the PPS and CR36 effect. By using a strategy of complementation of PrP deficient cell with engineered PrP-GPI, we observed a extremely fast endocytosis post PrPc-GPI incorporation into the plasma membrane for both compounds with moderate kinetic variations between CR36 and PPS. After cycloheximide pre-treatment of murine PrPc-GPI overexpressing CHO cells, an increase of the kinetic of PrP endocytosis was observed with CR36 versus PPS treatment.
Immunocytochemistry experiments confirmed the decrease of PrP cell surface expression but also showed a different surface localization pattern of PrP after treatment with CR36 versus PPS.
Thus, these two sulfated polyanions seem to decrease the PrPres precursor at the cell surface by different molecular mechanisms which need further investigations which could lead to original therapeutic approaches.

THE-18

SPECIFIC ANTI-PRION ANTIBODIES LEAD TO NEURONAL APOPTOSIS

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The mechanism(s) of the prion-mediated neuronal degeneration are not fully understood. Previous studies have shown that neurones that lack PrP^{C} expression are unable to sustain accumulation and deposition of PrP^{Sc}. Furthermore, lack of PrP^{C} fails to trigger any neurotoxic effects that are normally seen with PrP^{Sc}, indicating that PrP^{Sc} is not the only factor responsible for neuropathological changes to take place. PrP^{C} may also play an important role in the control of neuronal survival as has been demonstrated by complexation of PrP^{C} to anti-prion antibodies that led to neuronal apoptosis.
Our report shows that treatment of neuronal cells with anti-prion antibodies led to a dramatic over-expression of cytosol apolipoprotein E (apoE), release of cytochrome *c* from mitochondrial compartment and ultrastructural changes consistent with apoptosis. Our current data demonstrate that fine selection of antibodies to be used in prion therapeutic context is possible and even be encouraged although caution should be exerted when considering prion-mediated immunotherapy approaches.

THE-19

A SYSTEMATIC REVIEW OF PRION THERAPEUTICS IN EXPERIMENTAL MODELS OF PRION PROPAGATION

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Over the past forty years, a large number of putative treatments for prion diseases have been studied both in humans and in experimental models. The arrival of variant CJD in the UK in the 1990s has intensified the search for effective therapeutic agents, using an increasing number of animal, cellular and in vitro models of prion propagation. To date, however, there has been little systematic analysis of the resulting data. We present here the first comprehensive, systematic review of the data on experimental approaches to prion therapeutics from the published literature. Articles detailing the effects of treatments in models of prion propagation were identified by systematic literature searches, data tabulated according to experimental type, and a summary of results by therapeutic agent type created[1]. The diversity of the experimental data precludes formal meta-analyses as routinely performed with systematic reviews of clinical trials; nevertheless the availability of all data together allows some qualitative analysis, which can inform both experimental and clinical research and which is timely at the advent of clinical trials in human prion diseases. Here we show selected examples of the tabulated data from in vivo and in vitro approaches to date.

1 Trevitt C and Collinge J. A systematic review of prion therapeutics in experimental models. 2006. Brain (in press).

THE-20

LRP-DOWNREGULATION USING SIRNA DELIVERY VIA LENTIVIRAL VECTOR GENE TRANSFERT

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Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases, which include Scrapie in sheep, BSE in cattle and CJD in humans. Prions, the causative agents of TSEs, are known to interact with the cellular prion protein (PrP^{c}) by inducing conformational changes. The 37 kDa/ 67 kDa laminin receptor (LRP/ LR) acts as the cell surface receptor for the cellular PrP (1) and the infectious prion protein (2,3). It has been shown that LRP/ LR is essential for PrP^{Sc} propagation in neuronal cells (4). The accumulation of PrP^{Sc} in scrapie-infected neuronal cells ($N2aSc^{+}$) has been prevented by transfection with small interfering (si) RNAs specific for the LRP mRNA (4). These results demonstrate the necessity of the laminin receptor for the PrP^{Sc} propagation in cultured cells. Vector-based application of siRNAs circumvents the transient effect of downregulation of gene expression and allows persistent suppression and therefore analysis of loss-of-function-phenotypes that develop over longer periods of time. Transduction of recombinant HIV-based lentiviral vectors expressing siRNAs directed against defined regions of the LRP mRNA resulted in reduction of both PrPres and LRP levels in scrapie-infected neuronal cells. To further enlighten the role of LRP/LR in prion diseases, injection of recombinant LRP-specific RNA interference (RNAi) lentiviral particles into mice using an intracerebral (i.c.) or intraperitoneal (i.p.) route was performed. Western blot analysis of the cortical brain area of mice intracerebrally injected with lentiviral vectors expressing siRNAs directed against the LRP mRNA showed a downregulation of the 67kDa LR. Subsequent prion inoculation in these mice will prove whether the knockdown of LRP/LR by RNAi might prolong the onset of TSE or even prevent prion disease.

(1) Gauczynski et al. (2001) EMBO J. 20, 5863-5875. (2) Morel et al. (2005) Am. J. Path., 167, 1033-1042 (2005). (3) Gauczynski et al., (2006) J. infect. Dis. in press. (4) Leucht et al. (2003) EMBO rep 4, 290-295.

THE-21

A TRANS-DOMINANT NEGATIVE 37KDA/67KDA LAMININ RECEPTOR MUTANT AS A POTENTIAL THERAPEUTIC TOOL FOR THE TREATMENT OF TSES

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Prion diseases are a group of rare, fatal neurodegenerative diseases that affect both animals and humans. TSEs are usually rapidly progressive and clinical symptoms comprise dementia and loss of movement coordination, common hallmarks of TSEs is the accumulation of an abnormal isoform (PrP^{Sc}) of the host-encoded prion protein (PrP^{c}) in the brains of affected individual.
The discovery that the 37kDa/67kDa laminin receptor (LRP/LR) acts as the cell surface receptor for the cellular (PrP^{c}) (1) and infectious prion protein (PrP^{Sc}) (2,3) opened a new perspective for the development of an anti-prion therapy. *In vitro* studies using an N-terminally truncated LRP mutant, representing the extracellular domain of LRP/LR (LRP102-295::FLAG), revealed a reduced binding of (i) recombinant cellular PrP to mouse neuroblastoma cells, (ii) infectious moPrP 27-30 to BHK21 cells and (iii) interfered with the PrP^{Sc} propagation in chronically scrapie-infected mouse neuroblastoma cells (4). A cell free binding assay demonstrated the direct binding of the LRP102-295::FLAG mutant to both PrP^{c} and PrP^{Sc} (4). The secreted LRP102-295::FLAG mutant may act in a trans-dominant negative manner as a decoy by trapping PrP molecules (4). In order to test the therapeutic potential of the LRP mutant *in vivo* transgenic animals were generated expressing LRP102-295::FLAG ectopically in the brain. Transgenic animals showed no phenotype and transgene expression was detected in cortical and cerebellar brain regions. An intracerebral prion inoculation of these mice will prove, if the expression of the LRP102-295::FLAG mutant will impair the PrP^{Sc} accumulation in the brain and delay or prevent a manifestation of a prion disease. Thus, the LRP mutant might represent an alternative therapeutic tool for the treatment of TSEs.
(1) Gauczynski et al. (2001) EMBO J. 20, 5863-5875. (2) Morel et al. (2005) Am. J. Path., 167, 1033-1042 (2005). (3) Gauczynski et al., (2006) J. infect. Dis. in press. (4) Vana & Weiss (2006) J. Mol. Biol. 358, 57-66.

THE-22

INHIBITION OF PRPSC FORMATION BY SYNTHETIC O-SULFATED GLYCOPYRANOSIDE AND THEIR POLYMERS

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Sulfated glycosaminoglycans (GAGs) and sulfated glycans inhibit formation of the abnormal isoform of prion protein (PrP^{Sc}) in prion-infected cells and prolong the incubation time of scrapie-infected animals. Sulfation of GAGs is not tightly regulated and possible sites of sulfation are randomly modified, which complicates elucidation of the fundamental structures of GAGs that mediate the inhibition of PrP^{Sc} formation. To address the structure-activity relationship of GAGs in the inhibition of PrP^{Sc} formation, we screened the ability of various regioselectively *O*-sulfated glycopyranosides to inhibit PrP^{Sc} formation in prion-infected cells. Among the glycopyranosides and their polymers examined, monomeric 4-sulfo-*N*-acetyl-glucosamine (4SGN), and two glycopolymers, poly-4SGN and poly-6-sulfo-*N*-acetyl-glucosamine (poly-6SGN), inhibited PrP^{Sc} formation with 50% effective doses below 20 μg/ml, and their inhibitory effect became more evident with consecutive treatments. Structural comparisons suggested that a combination of an *N*-acetyl group at *C*-2 and an *O*-sulfate group at either *O*-4 or *O*-6 on glucopyranoside might be involved in the inhibition of PrP^{Sc} formation. Furthermore, polymeric but not monomeric 6SGN inhibited PrP^{Sc} formation, suggesting the importance of a polyvalent configuration in its effect. These results indicate that the synthetic sulfated glycosides are useful not only for the analysis of structure-activity relationship of GAGs but also for the development of therapeutics for prion diseases.

THE-23

PASSIVE IMMUNOTRANSFER OF ANTIBODIES DIRECTED AGAINST THE PRION PROTEIN RECEPTOR LRP/LR AS A THERAPEUTIC STRATEGY IN TSE THERAPY

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Recently we identified the 37/67 kDa laminin receptor (LRP/LR) acting as the cell surface receptor for the cellular Prion protein (PrP^{c}) (1) and the infectious Prion protein (PrP^{Sc}) (2). We prooved that polyclonal antibodies were able to abolish PrP^{Sc} propagation in scrapie infected neuroblastoma cells (3), demonstrating that the disruption of the LRP-PrP interaction is a relevant strategy in therapies against TSEs.
We injected the anti-LRP antibody W3 intraperitoneally into scrapie infected mice. Spleen analysis with respect to the PrP^{Sc} content will reveal whether this antibody is able to reduce PrP^{Sc} propagation. We did not observe any side effects after W3 application and monitor the survival times of the mice. Since a polyclonal antibody format is inappropriate for passive immunotransfer into humans, we developed single chain antibodies directed against the LRP/LR employing a phage display technique. Two scFvs termed N3 and S18 have been selected and characterized by epitope mapping, western blotting and FACS analysis. Both scFvs were able to recognize specifically the denatured form of LRP as well as the native form on the cell surface of BHK cells overexpressing LRP. The ability of these antibodies to interfere with the LRP-PrP interaction was proven by pull-down assays (4). In addition, a therapeutic effect of the scFvs on scrapie infected mice was investigated by passive immunotransfer. Although a significant reduction of peripheral PrP^{Sc} propagation in spleen of scrapie infected mice was observed, incubation times were not significantly prolonged. To circumvent the problem of fast renal antibody clearance, we are developing liposomes encapsulating the scFv and antibody secreting myotubes as alternative delivery systems.
(1) Gauczynski et al. (2001) EMBO J. 20, 5863-5875 (2) Gauczynski et al. (2006) J. infect. Diseases, in press (3) Leucht et al. (2003) EMBO rep 4, 290-295. (4) Rey et al., submitted.

THE-24

RECOMBINANT AAV ENCODING FOR SINGLE CHAIN ANTIBODIES DIRECTED AGAINST THE PRION PROTEIN RECEPTOR LRP/LR AS A GENE THERAPEUIC APPROACH IN TSE THERAPY

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The 37kDa/67kDa laminin receptor (LRP/LR) acts as the cell surface receptor for the cellular prion protein (1) and the infectious prion protein (PrP^{Sc}) (2). The necessity of the laminin receptor for prion propagation has been shown by downregulation of LRP/LR using siRNAs and antisense RNAs on scrapie infected neuroblastoma cells (N2a) (3). In addition, a LRP/LR specific polyclonal antibody (W3) was able to abolish PrP^{Sc} propagation in scrapie infected neuroblastoma cells (3), demonstrating that the disruption of the LRP-PrP interaction is a relevant strategy to treat prion diseases. Since single chain antibodies provide alternative tools for therapeutic approaches, which are used e.g. in clinical trials for cancer treatment, we developed single chain antibodies directed against LRP/LR. Selection by phage display resulted in two scFvs termed N3 and S18 (4). Both scFvs are able to recognize specifically the denatured form of LRP as well as the native form on the cell surface of N2a cells. Since passive immunotransfer is not sufficient for permanent scFv delivery, we developed recombinant AAV encoding for scFv. We proved expression of the scFvs in the brain of mice after intracerebral AAV injection and treated scrapie infected mice intracerebrally with rAAV expressing scFvs by a stereotactic device. Although spleen analysis revealed a significant reduction of the PrP^{Sc} levels, the survival times were not significantly prolonged. In addition, we are improving the single chain antibodies by chain shuffeling to improve the dissociation constant to the antigen, which might result in a more efficient in vivo effects.
(1) Gauczynski et al. (2001) EMBO J. 20, 5863-5875. (2)Gauczynski et al. (2006) J. infect. Dis. In press (3) Leucht et al. (2003) EMBO rep 4, 290-295. (4) Rey et al., submitted.

Poster Session

PRION SAFETY

SA-01

DECONTAMINATION OF INSTRUMENTS USED IN 'HIGH RISK' SURGERY: DEVELOPMENTS OF RF GAS-PLASMA METHODS FOR DECONTAMINATION MONITORED BY SENSITIVE PROTEIN MEASUREMENTS CARRIED OUT DIRECTLY ON INSTRUMENT SURFACES.

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The resistance of the TSE infective agent to conventional decontamination procedures is well documented and achieving satisfactory levels of protein decontamination from medical instruments to eliminate the possibility of iatrogenic transfer is a significant challenge. A year ago we demonstrated the effectiveness of the use of RF gas-plasma as a method of removing residual contamination from reprocessed surgical instruments and of removing TSE infectivity from experimentally contaminated stainless steel spheres (Baxter *et al, J. Gen. Virol.,* 2005, 86, 2393). In parallel, as part of a survey commissioned by the UK Department of Health, a number of trays of sterile reprocessed surgical instruments, chosen at random from the Sterile Services Departments (SSDs) of several NHS Hospital Trusts in England and Wales, were analysed for contamination. Of 120 instruments examined, >99% were shown to harbor protein bioburdens in the range 10-1200 □g per instrument (Baxter *et al, J. Hosp. Infect.,* 2006, in the press).
We have recently developed a range of novel fluororescent labeling reagents in which the fluorescence is suppressed by incorporation of a labile quenching group. Reaction with a protein eliminates the quenching moiety to give a covalently modified protein with 10^3-10^4 fold increases in fluorescence. In contrast to conventional methods of monitoring contamination on surfaces, which require attempted removal of the soil and subsequent derivatisation prior to measurement, these can be used for direct derivatisation of proteins on the surface and the increase in fluorescence measured directly using a surface scanning spectrofluorimeter. This technique enables us to monitor contamination on surgical instruments with a detection level of ~10 attamoles/mm^2. (*ca* 10^6 protein molecules/mm^2) – a sensitivity previously only achievable in solution. Here we describe the use of this approach for directly monitoring the effectiveness of current hospital cleaning protocols and of RF gas-plasma protocols in the decontamination of 'high risk' neurosurgical instruments.

SA-03

PRIONZYME; A NEW ENZYMATIC APPROACH TO THE DECONTAMINATION OF SURGICAL INSTRUMENTS AND CONTAMINATED ANIMAL WASTE PRODUCTS.

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The emergence of variant CJD (vCJD) has raised concerns that there may be significant numbers of people incubating the disease at a pre-clinical or sub-clinical level. Since (v)CJD is transmissible by surgery, transplant and probably by blood transfusion, and the agent(s) responsible is largely unaffected by standard sterilisation procedures, there are important implications for public health. Working with Genencor International we have developed a novel enzyme-based process for prion inactivation. After screening a wide range of proteases, a genetically engineered subtilisin variant was identified, which under alkaline conditions, eliminated BSE-301v *in vitro* and reduced infectivity by more than 7-logs in a VM mouse model. This enzyme, termed Prionzyme, has recently been independently certified for use for prion inactivation by an EU notified body. The studies have been extended to look at a model of BSE inactivation in complex biological samples (meat-and-bone meal to mimic contamination of animal waste products). These studies also show very significant reduction in infectious titres suggesting that a protease based approach is suitable for treating potentially TSE-contaminated waste material. The results will be discussed with respect to the development and implementation of novel methods for inactivating TSE agents, focusing on the sterilization of surgical instruments. The studies will be related to other ongoing work on prion inactivation within the TSE Research group at the HPA.

SA-04

DECONTAMINATION PROCEDURES FOR PRIONS: STANDARDIZATION OF IN VITRO AND IN VIVO MODELS FOR EVALUATION OF EFFICIENCY

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Prions are unique infectious agents which have been shown to be transmitted iatrogenically, both experimentally and clinically, through contaminated surfaces and could constitute a major public health concern with regard to the large tissue distribution of the vCJD agent in humans. There is currently no standard model to evaluate the safe decontamination of surfaces, including reusable medical devices, meat processing and pharmaceutical production surfaces.
We previously evaluated the efficiency of different decontamination procedures on prion-contaminated surfaces, including reference methods (bleach, sodium hydroxyde and autoclaving) and new procedures. Some of them, including new friendly detergent formulations and vaporized hydrogen peroxide, showed high efficiency against prions and are reputed to be compatible with the different surfaces to be treated. This initial study was performed by using steel surfaces contaminated with a scrapie strain adapted to hamster (263K), largely used in decontamination because of its resistance.
We extended here our studies to other surfaces (plastic, glass), showing that these procedures are also efficient on them. In parallel, similar studies were performed with a BSE-related strain, adapted to mice (6PB1). As for the previous model, disease was transmitted through contaminated wires after implantation into the brains and we established a relation between the infectivity titre, the transmission rate and the incubation period. The results obtained with BSE were similar to those observed with the 263K model reinforcing the relevance of the later for decontamination studies.
The developed *in vivo* scrapie method is proposed as a first standard to evaluate existing and developing prion decontamination technologies. It is clear that further investigations will continue to identify practical methods for evaluating surface prion decontamination.

SA-05

DISINFECTION OF VARIOUS PRION STRAINS BY ACIDIC SODIUM DODECYL SULFATE

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Prions are resistant to standard disinfection and sterilization procedures, resulting in well documented cases of iatrogenic prion transmission from surgical instruments. With the increase in surgical and dental procedures in the aging population, a growing number of pre-symptomatic Creutzfeldt-Jakob disease (CJD) patients are undergoing procedures and no special precautions are being taken with the instruments before reuse. We investigated the inactivation of prions by incubation with sodium dodecyl sulfate (SDS) at various pH values. As judged by sensitivity to proteolytic digestion, PrP^{Sc} was denatured at room temperature by SDS at pH values ≤4.5 or ≥10. When various concentrations of SDS and acetic acid were tested, the duration and temperature of exposure acted synergistically to inactivate both hamster Sc237 prions and human CJD prions. Using highly sensitive bioassays in transgenic mice, we found that CJD prions were over 100,000 times more resistant to inactivation than Sc237 prions, demonstrating that inactivation procedures must be validated against the strain of interest in a specifically susceptible host. As a model for surgical instrument disinfection, the inactivation of prions bound to stainless steel wires was evaluated by direct implantation into the brains of transgenic mice. Some procedures that significantly reduced prion titers in brain homogenates had a limited effect on prions bound to the surface of stainless steel wires. We have extended our studies to BSE prions, bioassayed in transgenic mice expressing bovine PrP, and to the mouse-adapted BSE strain, 301V, bioassayed in transgenic mice overexpressing the B polymorphic allele of mouse PrP. Our findings, which have been directly validated against the prion strains in the respective susceptible hosts, form the basis for a noncorrosive system suitable for inactivating prions on stainless steel surfaces.

SA-06

HIGH PRESSURE TREATMENT AS A TOOL TO INACTIVATE TSE AGENTS AND TO STUDY PRION PROTEIN FOLDING AND AGGREGATION

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High hydrostatic pressure is a mild processing technology with a promising potential in food pasteurization and sterilization by minimizing undesirable alterations such as vitamin losses and changes in taste and color. In addition, high pressure is a thermodynamic parameter providing useful information about the free-energy landscape of proteins. High pressure treatments of proteins can reveal conformations that are not obtainable by other physical variables like temperature, since pressure favors structural transitions accompanied with smaller volumes. Here, both the potential use of high pressure to inactivate infectious TSE agents and the application of this thermodynamic parameter for the investigation of prion protein aggregation and folding will be discussed.
Our results show that high pressures (6-8 kbar) combined with temperatures (60-80 °C) below sterilization conditions are able to induce a remarkable lose in the proteinase K resistance and infectivity of prions which can be attributed to changes in the structure of the prion protein. However, discrepancies under non-physiological buffer conditions, and between isolated and native prions, point out that pressure effects hardly depend on prion conformation and aggregation and that fractions exist which are especially high pressure resistant. Any treatment leading to more aggregated and dehydrated prions result in an increased pressure resistance. In general, our results confirm that the biggest amount of prions in conditions close to native are pressure sensitive and loose infectivity soon during pressurization.

SA-07

IINVESTIGATING THE EFFICIENCY OF VALIDATED WASHER-DISINFECTOR CYCLES ON THE REMOVAL OF PRIONS FROM SURGICAL STAINLESS STEEL SURFACES

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A key feature of the prion agent involved in the initiation and propagation of transmissible spongiform encephalopathies (TSE's) is its ability to withstand chemical and enzymatic degradation. This poses a serious concern for health services with respect to the decontamination of surgical instruments and the removal of prions: these have been shown to be transmissible via neurosurgery and contaminated neurosurgical instruments such as EEG depth electrodes. Although several methods of prion decontamination have been shown to be successful, due to their aggressive nature, none are applicable to surgical instruments. Furthermore, a validated cleaning cycle which can guarantee their complete removal from surgical instruments does not currently exist. This study simulated the conditions of validated cycles and cleaning products currently used within sterile service departments: a HAMO™ 100 prion inactivating detergent cycle with cleaning time of 15 minutes and the newly recommended 7 minutes, as well as Enzycare®II and Instru-Klenz® cleaning cycles, on surgical grade 316L stainless steel wires inoculated with a 10% mouse brain homogenate infected with ME7 Scrapie. For each cycle the pre-treatment was altered using either a Klenzyme® soak, a PRE-Klenz® spray or no treatment, as was the length of drying time of the wires following inoculation. The degree of prion removal was assessed, in addition to general organic contamination, in terms of percentage area coverage of the wires using a rapid episcopic differential interference contrast microscopy technique and a novel and sensitive fluorescent staining protocol. The efficiency of prion removal varied, depending on the pre-treatment and agents used for each cleaning cycle, as well as the effect of prior tissue drying time. The results have been shown to correlate with on going *in vivo* studies.

SA-08

NOVEL MODELS TO ASSESS THE IATROGENIC TRANSMISSION RISKS FOR VCJD IN DENTISTRY USING BSE-301V IN VM MICE.

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The potential for transmission of vCJD by dentistry has been suggested to be very low based on current risk assessments, and there is no evidence to suggest that people have been infected through this route. To provide further underpinning for such assessments, this study will help to identify the risk of iatrogenic transmission between patients during certain invasive dental procedures. Groups of VM mice (n=10) were inoculated with BSE-301V by scoring infected orthodontic dental files along their gum line and leaving in the gingival margin tissue for 5 minutes. The 100 mice were then culled bimonthly (2 up to 20 months) to assess the speed and route of BSE-301V infection in the VM mouse, and to provide evidence for relative infectivity of different tissues. 10 tissues including brain, spleen and various oral tissues have been harvested. These tissues will then be processed and re-inoculated intra-cranially into VM (n=6) mice to assess the infectivity of these tissues. To date this study has completed the primary infections and collected the range of tissues from the time course, which are now being processed for re-inoculated. This study will be complemented by assessing the infectivity of oral vCJD tissues in additional ongoing studies. These human tissues will be injected intra-cranially into RIII mice and scored after a fixed time-point. These results will be discussed with respect to our understanding of instrument decontamination and the potential transmission of (v)CJD via general surgery.

SA-09

EVALUATION OF THE MICROSCOPIC METHOD FOR THE DETECTION OF ANIMAL DERIVED CONSTITUENTS IN FEEDINGSTUFFS

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The BSE (Bovine Spongiform Encephalopathy) epidemic in Europe has heighten the need to adopt strict control measures to avoid the risk of spreading of the disease through meat and bone meal (MBM) based animal feedingstuffs.
The aim of this study was to evaluate the reproducibility and the accuracy of the microscopic examination (Directive 2003/126/EC), as official method to evaluate the presence of MBM and to identify the animal classes, by a ring trial.
CReAA (National Reference Centre for surveillance and monitoring of animal feed) provided nine European labs with a set of 35 samples prepared using a commercial feedingstuff for calves as common matrix: positive samples were made by adding a 0.1% concentration of animal MBM meal (either mammal or chicken or fish).
The reproducibility of the microscopic method was very good: *k-overall* was 0.83 (95%CI: 0.77 – 0.88). With regard to accuracy considering the results in terms of presence/absence of MBM the sensitivity was equal to 100% (95%CI: 95.98 – 100.00) and the specificity was 94.67% (95%CI: 90.87 – 97.21) for all the raters together. In terms of kind of constituent (eg. mammal, poultry, fish) the specificity was 97.13% (95%CI:94.43–98.75) for fish, 98.61% (95%CI:96.48–99.62) for mammal and 98.61%(95%CI:96.48–99.62) for chicken. Sensitivity showed a high variability among the animal classes: fish 100% (95%CI: 90.26-100), mammal 48.15% (95%CI:26.67–68.05) and chicken 44.44% (95%CI:25.48-64.67).
The microscopic method show high level of accuracy and reproducibility with some limits in discriminating between chicken and mammal contamination.
Acknowledgments to the other European laboratories.

SA-10

DUAL STAINING OF PRION AMYLOID AND GENERAL PROTEINACEOUS CONTAMINATION ON SURGICAL STEEL SURFACES ENHANCES PRION-POSITIVE SIGNAL DIFFERENTIATION.

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Transmissible spongiform encephalopathies are a group of rare, transmissible, and fatal neurodegenerative diseases associated with the protein agent (PrP^{sc}). As such, the sensitive and rapid detection of prion PrP^{sc} amyloid on the surface of suspect surgical instruments is of great importance and may even allow remedial action to be taken by sterile service departments (SSDs) prior to any further operative intervention and possible iatrogenic transmission. However, conventional PrP^{sc} detection methodologies tend to rely on the inefficient and unreliable removal of suspect material from a surface using swabs or wipes prior to antibody analysis. Here we show how the combination of an advanced light microscope technique, Episcopic Differential Interference Contrast / Epi-Fluorescence (EDIC/EF) microscopy, and the combination of fluorescent dyes (Sypro Ruby and thiazole derivatives) can be used to detect, *in-situ*, sub-micron (attomole) levels of both general and PrP^{sc} protein contamination in brain and spleen sections, smears, and homogenate on surgical stainless steel surfaces and surgical instruments. This technique can be used to verify that surgical instruments are substantially free from prion protein soiling and hence reduce the risk of iatrogenic transmission. This technique was used to screen the contamination of instrument sets obtained from health service trusts and also assess decontamination protocols for PrP^{sc} infected brain material dried on stainless steel surfaces. The results indicate that drying times in excess of 10 minutes and different detergent or enzymatic cleaning chemistries significantly alter the ability to remove PrP^{sc} from the surfaces. This supports the requirement to prevent instrument drying before processing in SSDs, irrespective of the ultimate cleaning chemistry.

SA-11

STAINLESS STEEL COMPOSITION AS A DETERMINING FACTOR FOR PRION BINDING

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Prions are uniquely resistant to conventional sterilization procedures and inactivation by various chemical treatments. This resistance of prions to inactivation has led to several cases of prion transmission following inadequate sterilization of injected materials both in animals and humans. Prion-contaminated electrodes have been implied in spreading prion infections and, and since prions may accumulate in lymphoid tissues for long periods of times, surgical instruments used in operations on non-symptomatic prion-infected individuals are at a potential risk for transmission of diseases. This has led to the awareness that instruments and materials used in dentistry, surgery and slaughtering may be a source of prion spread. In the present study we investigate whether metals and/or stainless steel of different composition differ in their prion binding capacity. We here report that stainless steel powders of different composition, exposed for 24 hrs to scrapie (RML-strain)-infected GT1-1 cell lysates, show a marked difference in their binding of PrP^{Sc} as determined by Western blotting. We also observed that the difference in prion binding capacity of the steel powders was at least partly due to their difference in nickel content. Thus, stainless steel devoid of nickel may be considered for use in instruments that are at risk of being exposed to prions.
This study was supported by grants from US Army DAMD17-03-102288 and by EC LSHB-CT-2006-019090

SA-12

VALIDATION OF A FT-NIR MICROSCOPY METHOD FOR THE SCREENING OF BONE PARTICLES IN THE SEDIMENT FRACTION OF FEEDING STUFF

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The aim of the present study is to validate as screening method the FT-NIR microscopy technique for the analysis of animal meals in feeds.
A commercial feed has been spiked at three different concentration levels, 0.05%, 0.1% and 0.3%, Each sample, at the three different concentration, has been analyzed in ten replicates. The sedimentation fraction has been separated from the samples following the same preparation procedure of the official method (Directive 2003/126 EC), and then analyzed with the FT-NIR mycroscopy. The NIR spectra have been collected in the range between 6000-4000 cm^{-1}, with a resolution of 8 cm^{-1} , the data have been further elaborated by using the mapping technique. Following the same procedure,35 samples of the proficiency testing organized in Italy by C.Re.A.A in 2005, have been tested, and the results obtained compared with those of partecipantes using the official method.
The following parameters ha been defined:

- Specificity, selectivity
- Stability/ruggedness
- Detection limit
- Precision

It has also been demostrated that the false compliant rate is <0.5% (ß error), as it is demanded in the case of screening method.
A linear correlation has been found between the results obtained at the three different concentration levels.
This method should be used in the laboratory for feed control as screening method; in the case of a suspected non-compliant result , this should be confirmed by the official method.

SA-13

COPPER AND HYDROGEN PEROXIDE ACT IN SYNERGY FOR PRION DECONTAMINATION

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Over the past five years, a growing interest has emerged in the role of metal ions in transmissible spongiform encephalophathies (TSEs). The amino terminus of PrPC contains indeed a series of octapeptide repeats, which are among the most conserved regions of mammalian PrPs, and have been implicated in the binding of divalent metal ions, particularly copper [1]. Whether this binding is of structural or functional significance is still elusive. Copper is an essential redox transition element able to induce ion-mediated damage to proteins. This process has been known since 1894 as the Fenton reaction and consists of reduction of Cu2+ by an electron donor and generation of the hydroxyl radical through the reduction of hydrogen peroxide (H2O2) by the reduced metal. We demonstrated previously that PrPC undergoes a site-specific cleavage of the octapeptide repeat region on exposure to Cu2+ and H2O2 [2]. In view of this, we investigated the effect on PrPSc and demonstrated the interest for a novel copper – hydrogen peroxide formulation for prion decontamination. Here, we show that a formulation of copper metal ions in combination with hydrogen peroxide has a dramatic reduction effect on PrPSc present in prion infected brain homogenates including samples from human CJDs. Animal assays confirmed the reduction effect on prion infectivity which put forward the major interest of this novel copper – hydrogen peroxide formulation for prion decontamination [3].
[1] Lehmann S. Metal ions and prion diseases. *Curr Opin Chem Biol* 2002; 6:187-92.
[2] McMahon HE, Mange A, Nishida N, Creminon C, Casanova D and Lehmann S. Cleavage of the amino-terminus of the prion protein by reactive oxygen species. *J. Biol. Chem.* 2001; 276:2286-91.
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SA-14

POTENTIAL OF ENZYMATIC DIGESTION FOR DECONTAMINATING INFECTIOUS PRIONS

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Infectious prions (PrPres) are problematic for decontamination efforts because they are resistant to standard disinfection methods. Decontamination methods currently recommended (i.e., bleach, autoclaving, NaOH) can be damaging to materials, caustic to personnel, and are not possible in many situations. Improved and more versatile disinfection methods are needed. Enzymatic digestion is a potential means to decontaminate prions. Though PrPres is protease resistant, there are proteases with different specificities and conditions for activity. We used a scrapie mouse model to evaluate the effects of two proteases with digesting activity that eliminated in vitro (ELISA and Western blot) detectability of chronic wasting disease prions. At 34 weeks after inoculation, no mice inoculated with untreated prion-positive brain were alive while 81% and 48% of mice inoculated with brain treated with the proteases were alive. No mice inoculated with bleach-treated or autoclaved prion-positive brain died. Results suggest that enzymatic digestion with either of the enzymes can decrease infectivity. Suboptimal conditions may account for these treatments not being as effective as standard bleach and autoclaving decontamination, but the protection afforded provides support for the concept of successful enzymatic decontamination. Further evaluation of digestion under improved conditions should yield more successful decontamination that will allow for more practical treatment of waste, instruments, and surfaces; make environmental decontamination feasible; and provide a method for treating some organic materials. These improvements would greatly facilitate management of prion diseases in a variety of settings.

SA-15

DECONTAMINATION OF THERMOLABILE INSTRUMENTS FROM PATHOLOGICAL PRIONS

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Pathological prion proteins are known infectious agent for transmissible TSE, the PrPsc have been found to be distributed all over the lymphatic tissues, and in the blood as well. We have reported on a pathological prion infectivity model using hamsters and prion protein contaminated steel wires to demonstrate transmission and decontamination of infectious prion proteins. We showed that the hamster adapted pathological prion proteins could bind to the steel wire in dried form and cause disease after the contaminated wires are implanted or inserted only for a very short time. When the contaminated wires were treated with different cleaning, disinfection and/or sterilization procedures before implantation, infectivity was reduced which was manifested directly by a prolonged surviving time of the test animals. This model is very useful as a prion bioassay to validate reprocessing procedures for surgical instruments.

Because the pathological prion proteins are highly resistant to most of the routine hospital sterilization procedures, reusable surgical instruments need to be regarded as a potential source of transmission. Delicate surgical instruments such as endoscopes need gentle sterilization procedures, but prion proteins are known to be very resistant toward convenient sterilization processes. Most of the processes described as being effective against pathological prion protein are a combination of high-alkaline detergents and steam sterilization.

In searching of low temperature sterilization processes, we also tested combinations of different cleaning disinfectants/detergents followed by a low temperature sterilization process, since a thorough cleaning of the instrument before sterilization is a critical step in decontamination.

After several long-time bioassay studies, we found that washing with alkaline detergent followed by sterilizing in a hydrogen peroxide gas plasma sterilizer (Sterrad) to be the most effective low-temperature, non-autoclaving reprocessing procedure against infectious prion proteins. All the animals have survived until the end of the test (18 months) without showing any clinical signs.

SA-16

CELLULAR ASSAY FOR IN VITRO TSE TITRATION : LATEST IMPROVEMENTS AND POSSIBLE VALIDATION AS AN ALTERNATIVE TO BIOASSAY.

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Titration experiments of transmissible spongiform encephalopthies (TSE) agents, which are required for the validation of manufacturing processes of plasma derived products, involve either rapid immunochemical PrP-res detection, mostly by Western Blot (WB), or time-consuming and expensive infectivity protocols, that consist of intra-cerebral inoculation of laboratory rodents, which is the only validated method for the titration of infectivity.
The LFB has developed an alternative *in vitro* infectivity titration assay, based on the infection of TSE permissive cells. This assay has shown several characteristics that make it an interesting alternative to the reference titration methods for measuring TSE infectivity : it is highly sensitive (~ 80 times more than WB, and comparable to bioassay), specific and reproducible. It is fast (less than 8 weeks), less expensive than bioassay and was shown to be suitable for the validation of manufacturing processes.
Experiments are ongoing in order to increase the sensitivity of the assay and/or to reduce its duration.
Work is also in progress regarding the validation of the assay for regulatory recognition (comparison to bioassay experiments and analytical validation).
Latest developments of the tissue culture infectivity assay will be presented, and recognition by regulatory authorities will be discussed.

Workshop
of the
NEUROPRION CERVID GROUP

CHRONIC WASTING DISEASE (CWD): CURRENT KNOWLEDGE AND EUROPEAN PERSPECTIVE-2006

Tuesday 3rd October 2006, 11.00 - 18.30
Sala Berlino (Berlino Room), Lingotto Congress Centre - Via Nizza 280 - Torino
To register: http://www.newteam.it/PRION2006/

Introduction:
Transmissible spongiform encephalopathies in cervids: past, present and future.
11.00-11.10. Dr Dolores Gavier-Widén
National Veterinary Institute (SVA), Uppsala, Sweden

SESSION I: Chronic Wasting Disease in North America: Current knowledge
Chairpersons: Sylvie Benestad / Aru Balachandran

11.10-12.00. Overview of CWD: Review of the epidemiology, surveillance and control. Ongoing research projects
Dr Mike Miller
Wildlife Health Program, Colorado Division of Wildlife
Wildlife Research Center, Fort Collins, Colorado, USA

12.00-12.50. Pathological lesions associated with Chronic Wasting Disease
Dr Terry Spraker
Colorado State University Diagnostic Laboratories
Fort Collins, Colorado, USA

13.00-14.00 Lunch

14.00-14.20. Clinical signs and antemortem diagnosis of chronic wasting disease
Dr Lisa Wolfe
Colorado Division of Wildlife
Wildlife Research Center, Fort Collins, USA

14.20-14.40. Species specific influences of genotype on CWD susceptibility
Dr Katherine O'Rourke
Animal Disease Research Unit,
Agricultural Research Service, USDA, USA

14.40-15.10. CWD: Review of the diagnostics for CWD. Current situation and ongoing projects in Canada.
Dr Aru Balachandran
National Reference Laboratory for Scrapie and CWD
Animal Diseases Research Institute
Canadian Food Inspection Agency, Ottawa, Ontario, Canada

SESSION II: CWD- Studies and testing in Europe
Chairpersons: Dolores Gavier-Widén / Terry Spraker

15.10-15.30. Discrimination between CWD, BSE and scrapie by molecular profiling
Dr Mick Stack
Veterinary Laboratory Agency, Weybridge, UK.

15.30-15.50. Efficient transmission and characterization of CWD in bank voles
Dr Umberto Agrimi
Istituto Superiore di Sanitá, Rome, Italy

15.50-16.20: break

16.20-16.40. Phenotypes of PrP^{CWD} accumulation in cervids: the sheep experience
Dr Lorenzo González
Veterinary Laboratories Agency, VLA, Lasswade, UK

16.40-17.00. Rapid and discriminatory diagnosis of TSEs in lymph nodes
Dr Jan Langeveld
CIDC, Lelystad, Netherlands

17.00-17.20. The EU-wide survey for Chronic Wasting Disease in European cervids: design of the survey, objectives and implementation
Dr Koen Van Dyck and Dr Sinéad Diederich
TSE Section, DG SANCO, EC, Brussels, Belgien

17.20-17.40. Application of a TSE surveillance program in Germany. Design of the program, difficulties and practicalities. Recommendations to the rest of Europe. Comparison of different prion testing systems. Final results.
Dr Kai Frölich and Tina Blasche
Institute for Zoo-and Wildlife Research, Berlin.

17.40-18.00. TSE surveillance of deer in the UK
Dr Paul Webb
Veterinary Laboratory Agency, Weybridge, UK.

18.00-18.20. Results of the European proficiency testing for the diagnosis of CWD
Dr Sylvie Benestad
National Veterinary Institute, Oslo, Norway
Dr Dolores Gavier-Widén
National Veterinary Institute, Uppsala, Sweden

18.20-18.25. Genetic variability and selection of the Cervus elaphus prion (PrP) gene
Matteo Perucchini
Institute for Animal Health, Edinburgh, Scotland

ORGANIZERS:

- NEUROPRION-Cervids Group
- European Wildlife Disease Association
- CERMAS (Centro di Referenza Nazionale Malattie Animali Selvatici) CERMAS belongs to Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta
- CEA (Centro di Referenza Nazionale per le Encefalopatie Animali) Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta – Turin

Coordinator: Dolores Gavier-Widen, Department of Wildlife Diseases, National Veterinary Institute (SVA), S-75189 Uppsala, Sweden, **E-mail:** dolores@sva.se

OVERVIEW OF CHRONIC WASTING DISEASE: EPIDEMIOLOGY, SURVEILLANCE & CONTROL

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Chronic wasting disease (CWD), a contagious prion disease of several cervid species, has emerged from obscurity to become what some consider one of the most important wildlife health problems on the North American continent. Although the long-term implications and relative importance of CWD remain to be determined, the detection and apparent extent of CWD in both free-ranging and captive cervid populations already has had appreciable impacts on both wildlife management and the captive cervid industry. The 1990s and early 2000s were largely a period of discovery with respect to CWD: we began to appreciate the extent and potential severity of CWD epidemics in free-ranging and captive populations, we developed improved diagnostic and surveillance tools, and we gained insights into its epidemiology and potential host range. Ongoing and recently completed studies have provided further insights into epidemic trends, transmission dynamics, spatial epidemiology, potential population impacts, surveillance strategies, and the apparent ineffectiveness of traditional control approaches. Armed with existing tools and knowledge, animal health and wildlife management professionals are now challenged to craft practical but effective strategies for detection, control, and prevention, recognizing that knowledge and tools will continue to improve with better understanding of CWD and other prion diseases in coming years.

PATHOLOGICAL LESIONS ASSOCIATED WITH CHRONIC WASTING DISEASE

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The pathological lesions of chronic wasting disease (CWD) are similar to other TSE's. Gross lesions are primarily observed in terminal stage of CWD. Gross lesions include emaciation, generalized serous atrophy of adipose tissues, and mild atrophy of lymphoid tissues. Bronchopneumonia is commonly found, especially in elk. In captive deer it is common to find the rumen partially filled with water. Abomasal ulcers are occasionally seen in mule deer, but have not been seen in elk. Immunohistochemistry staining is first observed in lymphoid tissues of the head. Internal and peripheral lymphoid tissues accumulate PrP^{CWD} early in the disease course, but appear to follow the cranial lymphoid tissues. Nerves of the intestinal mucosa and myenteric plexuses begin to accumulate PrP^{CWD} early. A minimal amount of PrP^{CWD} can be seen in the vagus nerve adjacent to the trachea and in the spinal cord. PrP^{CWD} first accumulates in the vagus nucleus of the brain stem the spreads through out the rest of the brain. The cerebellum accumulates PrP^{CWD} last. The earliest histological lesions can be found in the vagus nucleus and are characterized by mild astrocytic hypertrophy and proliferation followed by spongiform degeneration of the neuropil. Vacuolization of the neuronal perikarya, neuronal denrites and axons follow. Neuronal degeneration and dropout appears to be the last change to occur. Spongiform degeneration follows the distribution of PrP^{CWD}. To date, areas of spongiform degeneration free of PrP^{CWD} demonstrated by IHC have not been found. There is no evidence of edema, suppurative or non-suppurative encephalitis in these areas of spongiform degeneration.

CLINICAL SIGNS & ANTEMORTEM DIAGNOSIS OF CHRONIC WASTING DISEASE

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Chronic wasting disease (CWD) presents a diagnostic challenge for those working with captive and free-ranging cervids because clinical signs are subtle and nonspecific throughout much of the disease course and antemortem diagnostic tools are limited. The image of a drooling, stumbling, emaciated deer or wapiti has been represented and repeated often as “the” presentation for clinical CWD over the years; however, both the clinical course and clinical presentation of CWD in deer and wapiti can vary widely, just as with many other diseases. Although the most recognizable signs of end-stage CWD are behavioral alterations and loss of body condition, infected animals can show a number of other signs during the disease course that vary in presentation and duration among individuals and are inconsistently seen. Clinical diagnosis is further complicated by the relatively late occurrence of signs in the overall disease course and the common occurrence of other intercurrent health problems or interceding causes of death. Clinical signs seen in CWD cases are nonspecific, and can include behavioral changes, ataxia, head tremor, hyperexcitability, hyperesthesia, piloerection, intermittent tremors (primarily in wapiti), dilated, spastic, or flaccid esophagus, sialorrhea, odontoprisis, dysphagia and swallowing difficulties, loss of body condition, polydypsia, and polyuria; pruritus, sometimes seen in scrapie, has never been reported in CWD cases. Signs may be most obvious during or after handling, anesthesia, or other stressful situations, but tend to become more consistent and recognizable as disease progresses. Because there are many potential differential diagnoses, CWD should be included as a differential in postmortem evaluations of adult North American cervids or closely related species when poor body condition or neurological signs are apparent. CWD presently can be diagnosed in live cervids by demonstrating PrPCWD via immunohistochemistry (IHC) of lymphoid tissue from biopsies of tonsil or rectal mucosa. With sufficient lymphoid follicle counts, CWD cases are likely detectable by biopsy IHC within the first 6–9 months of infection, well before signs first appear.

SPECIES SPECIFIC INFLUENCE OF GENOTYPE ON CWD SUSCEPTIBILITY

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Chronic wasting disease in the United States has been reported in 4 cervid species, including mule deer (Odocoileus hemionus), white tailed deer (Odocoileus virginianus), Rocky Mountain elk (Cervus elaphus), and Shira’s moose (Alces alces). Pathogenesis and genetic predisposition vary among the species. After adjustment for the polymorphisms associated with the nonfunctional Prnp pseudogene, the Prnp gene in white tailed deer is more variable than in the other species. A coding change at codon 96 (glycine to serine) is associated with predisposition to natural disease although the alternative allele is not protective. The relationship between codon 96 genotype and CWD is reported in two farmed populations and one large sample set of free-ranging white tailed deer. The genetic variability in white tailed deer can be used to examine disease transmission patterns. Deer DNA samples (n=195) for which there was accompanying sex, age, CWD pathology, Prnp functional and pseudogene sequence information were analyzed for parentage and kinship using 29 nuclear microsatellite loci. Genetic differentiation, genetic distance, population structure analyses, parentage likelihoods and exclusion probabilities, and kinship coefficients were used to identify dyads related by first order and kin groups containing at least 3 individuals. Dyads were partitioned using likelihood parentage and relationship indices, and chi square analyses were used to test significance (alpha 0.05) of frequency of dyads of CWD-positive individuals compared to that expected by chance. The study confirmed a matrilineal kin group-based social structure, even for deer in confinement, and supported the hypothesis of adult-to-adult transmission reported in studies of free ranging deer. The use of microsatellite or other kinship markers may be useful in novel foci of infection for which behavioral and movement data are not available. The Prnp gene in Rocky Mountain elk is remarkably conserved among farmed and free ranging animals. Homozygosity for the allele encoding leucine at codon 132 is rare in CWD-positive animals and high-dose oral infection results in infection with a prolonged incubation period. In addition to the use of genetics in pathogenesis and transmission studies, additional sequence analysis of other species and populations within and outside the US should be undertaken to confirm the presence of the epitopes used in monoclonal antibody-based diagnostics.

DIAGNOSIS OF CWD: CURRENT SITUATION IN CANADA AND ONGOING RESEARCH PROJECTS

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3. Colorado State University, Ft. Collins, CO, USA 4. VLA, Weybridge, Addlestone, Surrey,UK

Chronic wasting disease (CWD) of cervids is one of the naturally occurring animal transmissible spongiform encephalopathies (TSEs) in Canada. Since 1996, a total of 245 cases have been reported in captive elk and white-tailed deer in Saskatchewan (SK) and Alberta and in mule deer in Toronto zoo. In wild deer populations, CWD was first detected in 2000 with 103 confirmed cases to date in five focal areas in SK. During the past hunting season, 14 CWD-infected deer were detected for the first time in a location in Alberta near the SK border. The CFIA implemented a national program to eradicate CWD in 2000 and made it a Reportable Disease under the *Health of Animals Act* in 2001. Diagnosis and confirmation of CWD was made by several methods, including clinical signs, microscopic lesions of spongiform encephalopathy, immunoassays (immunohistochemistry-IHC, ELISA and Western blotting -WB) and electron microscopy.
Histopathology, limited to the detection of spongiform lesions in brain tissues, is inadequate as a confirmatory test. IHC assay for PrP^{CWD} deposition in brain is an approved diagnostic test for CWD and is used extensively in diagnosis and prevalence surveys. PrP^{CWD} also accumulates in certain lymphoid tissues of deer well before neuroinvasion and the appearance of clinical signs and IHC has been proposed as a preclinical test. WB is an alternative method often more sensitive and easy to set up, hence increasingly used in reference laboratories. It also has the potential for the differential diagnosis of CWD from other animal TSEs. The development of high throughput ELISA- based rapid diagnostic tests has greatly enhanced the capacity for CWD surveillance. Since epidemiological investigations rely heavily on confirmatory tests, the standardization of methods across diagnostic and reference laboratories is critical. Susceptibility of different species of cervids and ruminants to CWD, tissue tropism and distribution of CWD prion and genetic determinants of pathogenesis are among the high priority research projects underway. The preliminary results of these studies will be presented and the implications discussed.

DISCRIMINATION BETWEEN CWD, BSE AND SCRAPIE BY MOLECULAR PROFILING

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If CWD in cervids was discovered in Europe one of the first questions would be whether there could have been contact with the agents of BSE or scrapie. Techniques which can discriminate between experimental BSE in sheep, and natural scrapie cases are now available to examine any positive cases which may arise from extended surveillance. Studies using a modified version of the Prionics®-Check WESTERN blot have shown differences in the electrophoretic mobility of the PrP^{res} protein bands, in particular the molecular weight of the unglycosylated protein band, which is higher for scrapie samples than for sheep experimentally infected with BSE. Discrimination is also possible by parallel testing using two monoclonal antibodies (mAbs). MAb 6H4, is raised in mice to human PrP (amino acid sequence 144-152) and detects PrP^{res} in cattle and sheep; the second, is mAb P4, (ovine PrP sequence 89-104), which is more selective for scrapie PrP^{res}. The basis for discrimination is the location of the N-terminal cleavage site for proteinase K digestion, whereby a short amino acid sequence remains intact in sheep scrapie samples, but not in the BSE samples. When the molecular profiles for a limited number of CWD samples were examined a different molecular weight profile to scrapie and BSE was obtained with the unglycosylated band molecular weights being higher than both, irrespective of whether they were derived from elk, mule deer or white-tail deer.. Application of a wider range of discriminatory tests which utilise this parallel testing may indicate whether CWD in cervids is a single "strain", independent from BSE and scrapie.

EFFICIENT TRANSMISSION AND CHARACTERIZATION OF CHRONIC WASTING DISEASE IN BANK VOLES

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Chronic Wasting Disease (CWD) is a transmissible spongiform encephalopathy (TSE) of free-ranging and captive cervids. It represents the only TSE affecting wild animals populations under natural conditions. Its geographic distribution in United States and Canada is currently expanding, posing new challenges to animal and human health. Although the transmission of CWD to humans or domestic animal species has not been proven, the availability of tools for characterising CWD strains and comparing them with those of other animal and human TSEs, would represent a significant improvement in protecting human and animal health.

The bank vole *(Clethrionomys glareolus)* is a rodent species which proved to be very susceptible to a number of animal and human TSEs. The vole PrP gene is polymorphic at codon 109 codifying either Methionine or Isoleucine. On this basis, two lines of voles named Cg109MM and Cg109II, have been obtained. The vole model was used for transmitting two cases of elk CWD and for comparing their transmission characteristics with those of other human and animal TSEs. CWD transmitted to bank voles with survival times of 185-190 d.p.i. in Cg109II and 260-280 d.p.i. in Cg109MM. Subsequent passages in the two vole's lines were accompanied by reduction of survival times up to 35±3 d.p.i. in Cg109II and 60±5 d.p.i. in Cg109MM.

Preliminary analysis of survival times, lesion profiles, PrP^{Sc} molecular characteristics and PrP^{Sc} deposition pattern, showed some differences among the two CWD isolates, suggesting that different CWD strains could be involved. The comparison of CWD isolates adapted to Cg109MM with several TSE isolates previously adapted to the same vole's line, shows substantial differences between CWD and other sheep, bovine and human TSEs.

These findings show that voles are susceptible to CWD and can be used for biological strain typing. The susceptibility of bank voles to different human and animal TSEs makes it as a valuable animal model in which the biological characteristics of isolates of different TSEs from various species can be compared. Furthermore, voles provide a model with very short survival times for adapted CWD, which could be of interest for studying the biological properties of CWD strains in laboratory animals.

PHENOTYPES OF PRPCWD ACCUMULATION IN CERVIDS: THE SHEEP EXPERIENCE

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Chronic wasting disease (CWD) is a naturally occurring prion disease of cervids in North America. It was first recognized almost 40 years ago but its origin and relationship to other transmissible spongiform encephalopathies is unclear. Despite the fact that it affects several species of free ranging and farmed deer and elk, little is known about strain variability of the aetiological agent.

For several years we have been using two immunohistochemical (IHC) approaches to study and characterize the phenotype of disease-associated PrP (PrP^{d}) accumulation in sheep TSEs. One of them, the so-called epitope mapping, is based on the use of a panel of antibodies against different amino-acid sequences of PrP. This method has allowed us to distinguish between experimental sheep BSE, natural scrapie and a experimental source of sheep scrapie (CH1641). The other approach, called PrP^{d} profiling, is based on the identification and scoring of different morphological and cell associated types of PrP^{d} that accumulate in the brain of clinically affected animals. The IHC phenotypes defined in this way appear to correlate mainly with the source of infection or prion strain and, in some cases, also with the PrP genotype, while other factors such as the route of infection, breed and dose do not have any effect.

We believe that these approaches may be useful for the definition of IHC phenotypes in CWD and further assessment of the factors that can influence such phenotypes. We have performed some preliminary examinations on a few CWD cases in American and Canadian elk which indicate the presence of several types of intra- and extra-cellular PrP^{d} deposition in those brains. It would appear that the American cases can be differentiated from the Canadian ones on the basis of the relative proportion of those types, i.e., on their PrP^{d} profiles. Moreover, those profiles are very different from that of a single UK red deer that developed clinical disease after intra-cerebral challenge with BSE. This animal showed a different epitope mapping pattern compared to all of the CWD cases examined. Although these are very preliminary observations they would point towards a lack of relationship between BSE and CWD and also perhaps towards the existence of more than one strain of CWD agent.

RAPID AND DISCRIMINATORY DIAGNOSIS OF TSES IN LYMPH NODES OF CWD INFECTED WILD LIFE ANIMALS

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Live and early screening in deer and elk for CWD would offer a tool for the determination of the TSEs status of wild life. Using our recently published method* for detection of scrapie and BSE in retropahryngeal lymph nodes (RLN) we applied the methods on some lymph nodes from white tail deer, mule deer and elk. Ovine scrapie and BSE infected RLNs were used for comparison. Using antibodies SAF32, 12B2, L42, and F99 at respectively 0.5, 0.2, 0.2 and 2 µg/ml in Western blots and 8 mg tissue equivalents per lane (except elk RLN, 0.4 mg TE), the method was capable to detect the positive animals. The molecular aspects of the deer and elk PrP^{res} samples appeared to differ from those in scrapie and BSE infected sheep RLN. Also, the antibody reactivities confirm that the CWD samples can be considered different from BSE. Methods and other molecular aspects will be discussed within the limits of the experiments.
The present data indicate that WB of suitable lymph nodes can be a promising and sensitive tool for applying surveillance of live wild game for CWD because of its speed and sensitivity.

*Langeveld JPM, Jacobs JG, Erkens JHF, Bossers A, van Zijderveld FG, van Keulen LJM. 2006. Rapid and discriminatory diagnosis of scrapie and BSE in retro-pharyngeal lymph nodes of sheep. BMC Veterinary Research 2:19.

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THE EUROPEAN UNION (EU)-WIDE SURVEY FOR CHRONIC WASTING DISEASE (CWD) IN EUROPEAN CERVIDS: DESIGN OF THE SURVEY, OBJECTIVES AND IMPLEMENTATION

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CWD has never been detected in a cervid in the EU. Nevertheless recognising cervids in Europe could potentially have been exposed to CWD, through imports, as well as to other TSEs, the European Commission (EC) sought the advice of the European Food Safety Authority (EFSA) experts and consulted Member States’ experts on the design of a suitable survey for Europe; North American experts were also consulted. Practical considerations were taken into account in discussing the scope, diagnostics and time frame for a potential survey. These elements were elaborated further in discussions with the Member States. Regulatory process required a change to TSE Regulation 999/2001 to allow the survey to take place; the legal basis to launch the survey should be in place in the latter half of 2006, allowing a start during the 2006 hunting season. The survey will be conducted over 2 hunting seasons, finishing in early 2008. The goal of the survey is to detect CWD if it is present in red or white-tailed deer in the EU. Member States with sufficiently large wild (red and white-tailed) or farmed (red) deer populations to allow them to achieve meaningful sample sizes will in particular be targeted. Other Member States will participate through collection of samples from particularly high-risk animals (clinically sick, fallen and road-killed deer). Member States will take into consideration geographical and epidemiological risk factors when selecting deer for sampling; targeted deer will be over 18 months; in wild deer, males will be particularly targeted. Rapid testing of hindbrain samples, as agreed by the Community Reference Laboratory (CRL) for TSEs, will be used. Follow-up on any non-negative result will be supervised by the CRL. Member States will be required to submit reports of their deer testing annually, and the EC will assess those data and take further action where appropriate.

SURVEILLANCE OF TSE IN CERVIDS FROM GERMANY AND ANALYSIS OF RAPID TESTS FOR DETECTION OF CERVID PRP

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ABSTRACT

Transmissible spongiform encephalopathies (TSE) occur in a number of animal species. Chronic wasting disease (CWD) has emerged as an important TSE of captive and free-ranging cervids in North America, naturally affecting several species including mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*) and moose (*Alces alces shirasi*). We performed the recently largest active survey on TSE in German cervids and investigated 4250 roe deer (*Capreolus capreolus*), 1416 red deer (*Cervus elaphus elaphus*) and 1390 fallow deer (*Dama dama*) between 2002 and 2005. All cervids (n=7056) were tested negative for TSE. This survey provides no evidence for the existence of prion diseases in free-living German cervids.

As survey and possible eradication strategies rely on the tests performed, we compared various commercially available rapid TSE tests for their analytical sensitivity against PrP in cervids. Positive control samples (confirmed by PrP immunohistochemistry) originated from CWD cases of North America. Additionally, we tested brain (n=6) and lymph node (n=6) samples from roe deer, red deer and fallow deer within Germany, without applying the Protease K digestion step. Three ELISA-based tests (Bio-Rad TeSeE, TeSeE sheep/goat, Prionics-Check LIA), two Western blot tests (Bio-Rad TeSeE Western Blot and Prionics–Check WESTERN) and one immunochromatographic assay (Prionics-Check PrioSTRIP) were evaluated. To enhance sensitivity, the tests were modified in consultation with manufacturers. Western blot systems were used as reference to compare dilutions of PrP. All six tests were suitable to detect PrP of cervids from all species tested. However, analytical sensitivity demonstrated significant differences between the tests. So we achieved lowest PrPres quantitative sensitivity for the Prionics-Check LIA and figured the BioRad TeSeE Western blot to be the most sensitive test for detection of PrPres in cervids.

TSE SURVEILLANCE OF DEER IN THE UK

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Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) that affects deer and elk. It has been widely reported in the USA and in Canada, but there have been no known natural cases of CWD confirmed in the UK or elsewhere in the European Union (EU). Prior to the proposed EU CWD surveillance regulations, the UK embarked upon a limited surveillance programme of wild cervids. Material was supplied via the UK deer tuberculosis surveillance programme or from animals culled from within maintained herds in National parklands. These samples were assessed using the Bio-Rad TeSeE enzyme-linked immunosorbant assay (ELISA) or by PrP immunohistochemistry using the UK statutory diagnostic protocol[*] using mouse monoclonal antibodies F89 and F99 used as a cocktail.

A summary of the data generated so far will be presented, along with a discussion of the challenges we encountered in setting up appropriate surveillance and quality assurance in the absence of indigenous disease.[1]

[1] Copies of statutory diagnostic techniques are available on the Community Reference Laboratory (CRL) for TSEs website at http://www.defra.gov.uk/corporate/vla/science-tse-rl-intro.htm

THE 2006- EUROPEAN PROFICIENCY TESTING FOR THE DIAGNOSIS OF CHRONIC WASTING DISEASE

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Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy that affects several species of cervids. CWD has never been reported in Europe. However in most European countries only a very limited number of animals have been tested. Therefore, it is not known if the disease occurs in Europe and if the European cervids, either farmed or free-ranging species, are susceptible to CWD or to other prion diseases. A survey for CWD for the whole EU, aiming to start in the Autumn of 2006, is currently under planning. The cervids group, a working group of Neuroprion, has organized a proficiency testing with the following objectives:

1. To provide CWD material to laboratories that will be diagnosing the disease.
2. To determine if the screening and confirmatory methods available for TSEs at these laboratories are appropriate to detect CWD.
3. To obtain an estimate of the variability of results between/among the laboratories and to identify potential difficulties.
4. To offer a training exercise, including discussions on interpretation of results.

Frozen and paraffin-embedded samples of brain and lymphoid tissues of elk and deer with CWD were kindly provided by THE CFIA, Canada (Dr Balachandran). The following laboratories participated of one or more of the ring tests: NVI (Oslo, Norway), SVA (Uppsala, Sweden), VLA (Weybridge, UK), FVB.IZW (Berlin, Germany), CEA (Turin, Italy), UNIZAR (Zaragoza, Spain), LNIV (Lisbon, Portugal) and CVRL (Celbridge, Ireland). The tests applied were: rapid tests, western immunoblot and immunohistochemistry. The work is still in progress (by August 2006) and the preliminary results will be presented.