Berufsgenossenschaftliche Kliniken – Bergmannsheil
Universitätsklinik der Ruhr-Universität Bochum
Abteilung für Gastroenterologie
Ltd. Arzt: Prof. Dr. B. May

Do cultured human skin explants elaborate coeliac antigen, possibly even Tissue-Transglutaminase ?

vorgelegt von
Claus Reinsberger
aus Paderborn
2001

Dekan: Prof. Dr. med. G. Muhr

Referent: Prof. Dr. med. B. May

Koreferent:

Tag der Mündlichen Prüfung:

To my Parents

Table of contents

1.) *Introduction:*

1.1.) Definition of Coeliac Disease:

Coeliac disease is an inflammatory disorder of the upper small intestine related to gluten ingestion in genetically susceptible individuals. The pathological changes of the bowel mucosa as well as the clinical signs and symptoms recover after institution of a gluten free diet, which proves that the enteropathy is gluten induced [1] [2].

1.2.) History:

Although the disease has been known for more than a century now, the harmful effects of ingested wheat gluten, a group of proteins found in wheat, rye and barley [3], was only discovered in the late 1940s by Dicke [4]. Soon afterwards, with the development of per-oral biopsy-techniques, it became apparent that histological changes were an integral part of the disease process.

1.3.) Pathology:

The characteristic features of the small intestinal mucosa in untreated coeliac disease are loss of villous height, crypt hypertrophy, plasma cell and lymphocyte infiltration in the lamina propria, decrease in the luminal epithelial cell height and infiltration of the epithelium with γ/δ T lymphocytes. These changes occur in three stages: At first only the infiltrative changes can be seen, followed by hyperplastic changes and, finally, there is the phase of destruction [5].

1.4.) Diagnosis:

In 1970 diagnostic criteria were stated by the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) . These were modified in 1990 and say that small-bowel mucosal atrophy with improvement or normalisation on a gluten-free diet and a deterioration of the villous

morphology during intake of a gluten-containing diet prove the occurence of coeliac disease [6]. In adult gastroenterological practice it is unusual to require three biopsies to confirm a diagnosis of coeliac diesease. An original flat biopsy accompanied by a clinical and / or biopsy response to gluten free diet is considered adequate.

1.5.) Prevalence:

The prevalence of coeliac disease was thought to be around 1 in 1000 [1], but the disease is now reported to affect 1 in 200 people [1] [7] with the highest prevalence in the world in the west of Ireland. In many other countries the prevalence of coeliac disease was significantly underrated than shown in previous studies or even unknown. Northern spain for example is reported to have a prevalence of 1:389 [8] and in sweden prevalence among children is even 1:300, although in the seventies childhood coeliac disease was thought to be disappearing [2].

1.6.) Classification:

To explain this increase in prevalence the „iceberg-model" is often chosen with clinical manifested coeliac disease on the top. Beyond this is silent coeliac disease, which means that marked severe damage to the jejunal mucosa is visible, but clinical symptoms are absent. The latent form of coeliac disease follows one step downwards on the iceberg. This term is reserved for individuals with a histologically normal jejunal mucosa. In connection with this the term potential coeliac disease has been introduced to differentiate patients, who had never had a flat jejunal biosy (potential coeliac disease) from patients who had had an abnormal biopsy in the past that recovered on a gluten-free diet. The broadest part on the basis of the iceberg is then built by healthy but genetically susceptible individuals [2] [9]. Regarding this model it is understandable that, according to the actual opinion, coeliac disease is underdiagnosed [10] [11].

1.7.) Genetical aspects:

This genetical component is associated with the HLA Class II extended haplotypes DR3-DQ2 or DR5/7-DQ2 and shows a concordance between monocygotic twins of 70 % [2].

1.8.) Clinical features:

Signs and symptoms of coeliac disease are variable and differ between individuals, so pathognominic clinical features do not excist. In the past the diagnosis was confined to patients with diarrhoea, flatulence, weight loss, malaise, anaemia and / or osteomalacia. Following the introduction of the fibroptic endoscope and the ease of obtaining duodenal biopsies, it was realized that the spectrum of coeliac disease included patients with milder symptoms including non-intestinal features or symptoms caused by malabsorption, such as growth retardation, anaemia, aphtous mouth ulcers, neurological symptoms, infertility, arthritis etc. [1] [12]. Patients even presented with clotting disorder, hypoglycaemia and angina pectoris [13]. Concerning miscarriage it is even recommended to include tests for coeliac disease in the usual routine tests in pregnancy, as up to 50 % of pregnant women with untreated coeliac disease have to expect an unfavourable outcome of pregnancy that could be prevented by a gluten free diet [14] [15]. Prior to the introduction of routine small bowel biopsy it was thought coeliac disease was a disease of childhood and was curable with a period of gluten free diet. It is recognised this was not the tune state of affairs and the typical coeliac is a female in the third or fourth decade [12].

1.9.) Associated diseases:

There are also a few associated autoimmune diseases, mostly belonging to HLA-B8 DR3 phenotype like Insulin Dependant Diabetes Mellitus, Thyroid Disease and Addison's Disease [5]. Furthermore an increased prevalence of Fibrosing Alveolitis, Systemic Lupus Erythematosis and Polyarteriitis has been reported among coeliac disease patients [5]. But the strongest connection exists with Dermatitis Herpetiformis, a condition, where the same

human leucocyte antigens (HLA-B8, HLA-DR3, HLA-DQ2) and the same circulating antibodies (anti-gliadin, anti-endomysial) can be observed as in coeliac disease [12]. A previously reported association between coeliac disease and alopecia areata has to questioned, as a gluten free diet does not have an effect on the course of alopecia areata [16].

1.10.) Treatment and clinical management:

The essential treatment of coeliac disease is the permanent withdrawl of gluten from the diet that means that all cereal grains known to contain toxic gluten such as wheat, barley and rye are to be excluded from the diet. After a few weeks symptoms will have improved, but mucosal recovery can take months or years. It is furthermore recommended to monitor the progress in a coeliac clinic, to ensure regular consultation with a trained dietician, to add supplements of deficient nutrients, such as iron, folic acid and calcium, to monitor dietary compliance by serological tests and to repeat an intestinal biopsy, if clinical progress is suboptimal [1]. However the possibility remains that patients do not respond to a gluten free diet. Enteropathy-associated T-cell lymphoma, ulcerative jejunitis, an end-stage hypoplastic mucosa and recently discovered mucosal infiltration by leukaemia are causes for a non-responsiveness [17]. The possibility that mucosal abnormalities in coeliac patients on a gluten free diet are related to trace amounts of gluten has been ruled out meanwhile [18]. Whatever reason for the non-responsiveness to a gluten-free diet there may be, those cases require the application of immunorepressive drugs to induce remission.

1.11.) Serology:

Anti-Gliadin-Antibodies:

As the majority of patients only has milder symptoms, the essential investigation which is a small-bowel biopsy had to be improved by other serological screening-techniques. At first an enzyme-linked immunosorbent assay with serum gliadin antibodies was found [19]. This test is now reported to have an inconstant rate of sensitivity and specificity of 70-100% [20]. Problems in this test can be blamed on the fact that false positive gliadin antibodies are frequently found in diseases other than coeliac disease and in healthy individuals [20]. As the antibodies are mainly produced inside the mucosal lining, IgA-type antibodies are predominant [12]. Nevertheless it is recommended to test both IgA -and IgG-Anti-Gliadin-Antibodies, because there is a relatively high prevalence of IgA deficiency among patients with coeliac disease [21].

Saliva:

Furthermore it has been shown that coeliac disease patients produce saliva with higher concentrations of total protein, albumin, IgA and IgG and higher activities of salivary peroxidase and myeloperoxidase than healthy control patients, although there are no differences in the saliva flow rate itself. Following a gluten free diet amounts of amylase, IgA and IgM become lower in coeliac patients than in healthy controls [22]. But nevertheless it is not helpful to rely on salivary IgA- and IgG-Anti-Gliadin-Antibodies concerning diagnosis or follow-up of coeliac diesease patients [23].

Anti-Endomysial Antibodies:

Using an indirect immunofluorescence technique Anti-Endomysial Antibodies (EMA), e.g. antibodies against the endomysial membrane surrounding smooth muscle tissue, specifically elevated in patients with coeliac disease can be detected. Those tests are even more accurate, i.e. more specific and more sensitive [24] than the Anti-Gliadin-ELISA, but 100% sensitivity and 100% negative predictive value can only be reached with a combination of

those two [21]. As with salivary Anti-Gliadin-Antibodies it is not helpful to use salivary Anti-Endomysial-Antibodies as a diagnostic tool [23].

It was proposed that Calreticulin [25] is the main antigen recognized in Anti-Endomysial-Antibody tests, but Dieterich et al. identified the „true" and perhaps sole antigen as being the autoantigen: tissue-transglutaminase (tTG) [26].

Serological Management:

All in all there is still disconcern about the right strategy of the use of the mentioned serological tests: Some authors prefer a two step approach with the determination of Anti-Gliadin Antibodies in the first line to exclude coeliac disease and the confirmatory use of Anti-Endomysial Antobodies in individuals, where a raised level of Anti-Gliadin Antibodies is detected [27] [21]. Other authors regard a one step strategy only with the determination of Anti-Endomysial Antibodies as more economical and more sensitive than the two-step approach [8]. Furthermore it has also been proposed that serological testing might not be enough to detect all coeliac patients and that the true prevalence can only be investigated by a combination of serological and clinical / pathological features [28]. In addition it is also not possible yet to detect latent coeliac disease only by serological tests: Neither differences in the counts of intra-epithelial lymphocytes and $\gamma\delta^+$-T cells, nor serological markers such as Anti-Gliadin-, Anti-reticulin- or Anti-Endomysial-Antibodies are able to differentiate between latent and manifest coeliac disease [29].

1.12.) Pathogenesis:
The pathophysiological mechanism is now thought to be an autoimmune one: tissue transglutaminase is produced and secreted in many different

tissues and organs and is present both intracellularly and extracellularly. It is involved in extracellular matrix assembly, cell adhesion and in wound healing processes [30] and its activities, which are calcium mediated, are selective cross-linking and deamidation of protein-bound glutamine residues [31]. Furthermore tissue transglutaminase is required for activation of transforming growth factor β [32]. The connection of tissue-transglutaminase and coeliac disease was first discovered by Bruce et al. in 1985: They showed its appearance in the human jejunal mucosa and demonstrated its high avidity for gliadin as a substrate. These workers found tissue-transglutaminase activity to be higher in coeliac mucosa than in non-coeliac mucosa [33]. The current theory is that ingested gliadin, which is the alcohol-soluble, prolamin rich reacting fraction of gluten [3] is resorbed through open tight junctions in the epithelial lining of the gut. It has been shown that zonulin, a protein analogue to the Vibrio cholerae derived zonula occludens toxin, induces such a disassembly of tight junctions in the intestinal epithelia and its concentrations in intestinal tissues in the acute phase of coeliac disease is raised [34]. It could therefore well be that zonulin contributes to mechanism of the resorption of gliadin. After passing the epithelial barrier gliadin is deamidated by tissue-transglutaminase [35] that is secreted by mononuclear cells, endothelial cells and fibroblasts of the subepithelial lining of the gut. This deamidation especially of the gliadin-peptides 134-153 unmasks epitopes that allow its binding to antigen-presenting cells via HLA-DQ2. Non-deamidated gliadin and gliadin-/tissue-transglutaminase-complexes are bound in the same way. These antigen-presenting cells are then recognized by CD^{4+}-cells that mediate a Th1- and a Th2- immune-reaction. The Th1-reaction causes a matrix degradation via its main mediator TNF-α, which leads together with other T-cell cytokines to villous atrophy and crypt hyperplasia, the two main histological features of a biopsy. In the Th2-response IL-4 and IL-5 are mainly released and cause a B-cell maturation with the production of IgA antibodies against gladin, tissue-transglutaminase and gliadin-/tissue-transglutaminase-complexes plus the presentation of peptides to B-cells [36] (see table 1). However in recent studies it has been confirmed that tissue-transglutaminase is the predominant antigen for the

production of Anti-Endomysial Antibodies, but at least in some coeliac patients it is not the only one [37] [38]. Furthermore as in various autoimmune diseases a raised expression of heat shock proteins has also been established in coeliac disease. This expression has been shown to correlate with intra-epithelial $\gamma\delta^+$-T cell densities, but not with the expression of mucosal HLA DQ2 positivity [39]. Glutenin, a component of gluten, is said to consist of repetitive peptides that are recognized by T cell epitopes and lead to an increased production of Interferon-γ, so it could well be involved into the disease process [40]. Last but not least recent studies suggest that at least one non HLA gene is also involved into the pathogenesis of coeliac disease [41] [42]. All theses findings indicate that the "true" pathophysiological mechanisms leading to coeliac disease are much more complicating than supposed.

Table 1: Pathogenesis of coeliac disease, modified after Schuppan et al., 1998
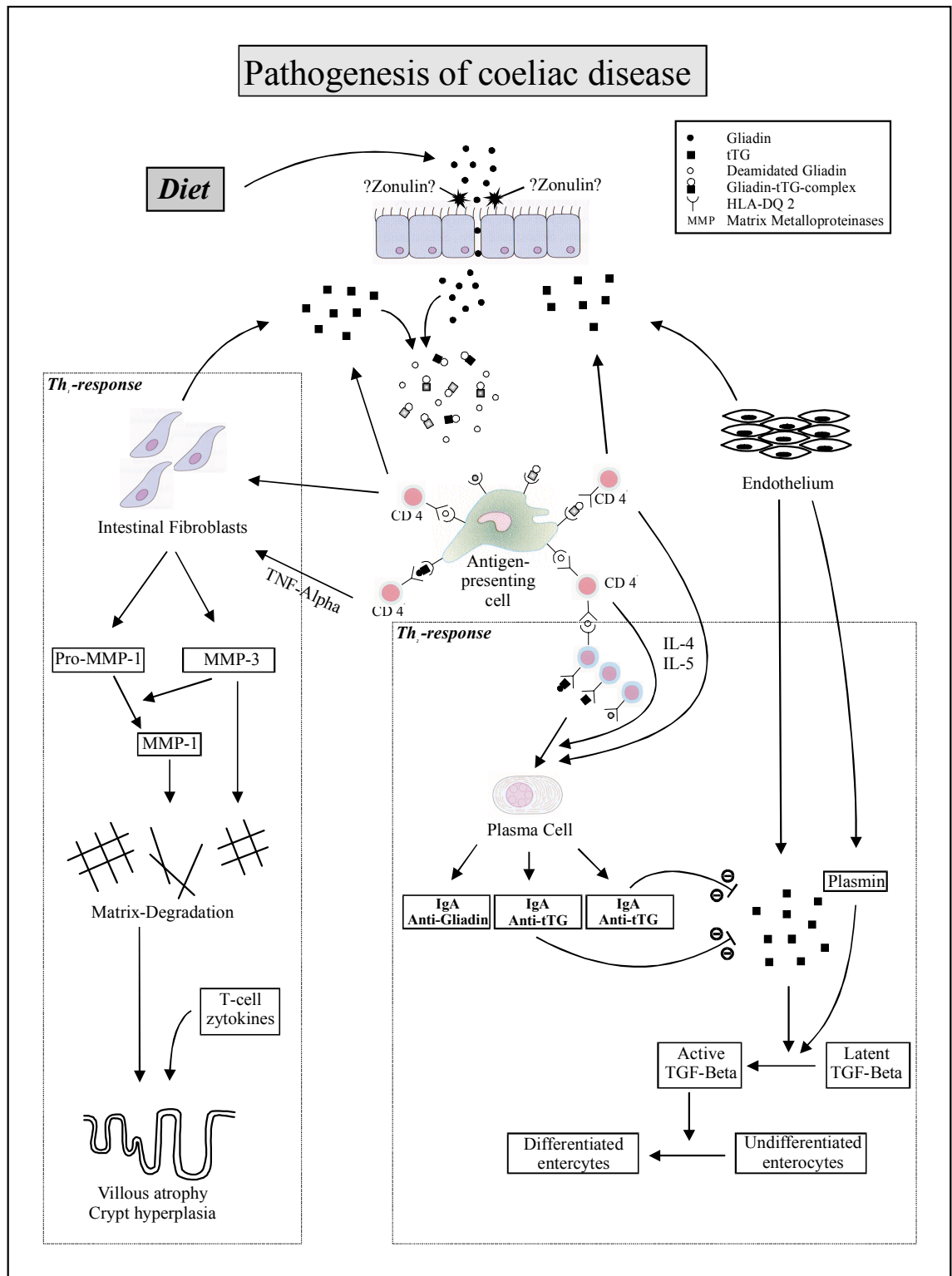
1.13.) Advances in serology techniques:

Having identified tissue-transglutaminase as the main autoantigen it was possible to develop an enzyme-linked immunosorbent assay with tissue-transglutaminase as the substrate. Originally commercially prepared guinea pig hepatic tissue-transglutaminase was used as bound antigen in the early ELISA's. These tests are reported to be highly sensitive and specific [21] [43]. Furthermore they are easy to perform and to evaluate as they are quantitative and not observer-dependant. They also correlate excellently with IgA EMA antibodies obtained by immunosorbent assays [44] [45] [24]. But by comparing these two tests there is a big difference regarding the sources of the antigens: tissue for detecting endomysial antibodies in immunofluorescent assays can be obtained by monkey oesophagus tissue [46], umbilical cord [47] and human umbilical vein endothelial cells (HUVEC) [48], which probably offers the easiest available antigen of these three difficult sources. All three kinds of tissue have been tested and lead to the same results with the same sensitivity and specificity [49].

As a fourth source of production human fetal lung fibroblasts have been discovered. Those fibroblasts synthesize and secrete antigens that can be purified and bind to antiendomysial antibodies in coeliac disease patient sera [50]. From the ethical point of view it is surely not acceptable to use this source of antigens for routine-tests, but generally fibroblasts can be obtained by several tissues as they belong to the structure of connective tissue in the lamina propria. Fibroblasts monocultures for example are routinely taken from skin biopsies for the purpose of chromosome analyses [51]. It has been shown the media from cultured fibroblasts derived from human skin biopsies contains an antigen, which in Dot Blots reacted with IgA class antibodies in untreated coeliac serum. This antigen could possibly be tissue-transglutaminase. Bruce et al. had suggested tissue-transglutaminase in small intestinal biopsies from untreated coeliacs has different kinetics to non-coeliacs, perhaps inferring there are structural differences between the two molecules. Skin biopsies from coeliac and non-coeliac subjects would allow identification of the antigens produced and if tissue-transglutaminase is

produced it would also allow characterisation and determination, if there are two dictinct molecules. Purification of coeliac tissue-transglutaminase might permit the development of the ideal ELISA to use in screening for coeliac disease and monitoring dietary compliance [52].

Concerning tissue-transglutaminase there is a lack of consensus in the literature, whether this enzyme can be found intra- and extracellularly. Some authors are of the opinion that tissue-transglutaminase is present both intracellularly and extracellularly [30] [53], others dispute an extracellular presence of the enzyme despite its physiological impact on cell adhesion, matrix assembly and wound healing [54]. The original antigen from lung fibroblasts was isolated from the culture medium. In the current study it was decided to investigate not only the medium, but the intracellular compartment of the fibroblasts as well for reactivity with coeliac serum.

Assuming the antigen is tissue-transglutaminase these studies would provide a human coeliac and non-coeliac tissue-transglutaminase for use in an ELISA which should be superior to the guinea pig tissue-transglutaminase ELISA in screening for coeliac disease and monitoring dietary compliance.

As guinea-pig and human tissue-transglutaminase are only 80 % homologous [30], the result of the test with guinea-pig tissue-transglutaminase is impaired [55].

2.)   Aim of the study:

The aim of the study is to characterize antigens in medium with fibroblasts cultured from skin biopsies from coeliac patients and healthy control individuals. In the same way possible antigens from sonicates of the same fibroblasts will be characterised. The antigens that are found in either the medium or the sonicates will be assessed, to determine, if they are tissue-transglutaminase. Therefore it would be expectable that the antigen had either a molecular seize of 80 - 82 kDa [56] [57]. It would also be possible to find sizes of 50 kDa or 78 kDa respectively, as the epidermal isoform of tissue-transglutaminase has in its active form a molecular weight of 50 kDa and as a pro-enzyme a molecular weight of 78 kDa [57]. If an antigen of any other molecular weight is found, it could possibly be the coeliac isoform of tissue-transglutaminase.

The plan was as follows:

1.)   To perform an SDS - Polyacrylamide Gel Electrophoresis to determine, if there were proteins and therefore a possible antigen in the fibroblast culture medium .

2.)   To carry out a western blot-technique using coeliac serum and control serum as first antigen. This should clarify, whether there was/were antigen(s) secreted by the fibroblasts that was/were specific for coeliac disease. Fibroblasts were cultured from coeliac and healthy individuals to see, if there is a specific coeliac form of the antigen(s).

3.)   Using both SDS - Polyacrylamide Gel Electrophoresis and western blotting fibroblast sonicates from the same subjects will be investigated to determine, if there are any intracellular antigens.

3.)   Patients and Methods:

3.1.) Patients

Three Coeliac patients and three healthy individuals were recruited for a skin biopsy. Two of the coeliac patients had a slightly raised gliadin-antibody level, while the other coeliac patient showed a bigger increase. This patient did not show a raised level of anti-endomysial antibodies, but together with one of the fist two patients had a previously flat biopsy.

Furthermore sera from six coeliac patients and four healthy individuals were taken and used as primary antibodies in the western blot. One of the coeliac patients was known to have elevated liver enzymes and a history of high alcohol intake and two were treated patients with no Anti-Endomysial Antibodies. All the coeliacs had elevated anti-gliadin antibodies, anti-endomysial antibodies and a (previously) flat biopsy with different degrees.

All three healthy individuals for the skin biopsy and all four healthy individuals for serum had no coeliac antibodies (Anti-Endomysial. Anti-Gliadin) and could therefore serve as control samples.

Table 2: List of patients

Serum:

No.	Date	Status	Anti-Gliadin AB	Anti-Endomysial AB	Biopsy
1	14/07/99	CD, elevated liver enz., high alcohol intake	376,0	+	previous flat
2	14/07/99	CD, gluten free diet	60,0	+	flat
3	13/08/99	CD, gluten free diet	85,0	weak +	previous flat
4	13/08/99	CD, gluten free diet	36,0	weak +	previous villi
5	14/07/99	silent CD, NIDDM	15,0	weak +	flat
6	14/07/99	CD	318,0	+	flat
7	17/07/99	Control	6,0	-	not done
8	26/07/99	Control	7,0	-	not done
9	12/08/99	Control	4,0		not done
10	12/08/99	Control	3,0		not done

Skin-Biopsy:

No.	Date	Status	Anti-Gliadin AB	Anti-Endomysial AB	Biopsy
A	17/02/99	CD	2,0	not done	villi
B	09/07/99	CD	3,0 (3/2/99)	not done	previous flat
C	09/06/99	CD	65,0	-	previous flat
D	28/04/99	Control	-	-	no biopsy
E	09/07/99	Control	-	-	no biopsy
F	09/07/99	Control	-	-	no biopsy

3.2.) Skin Biopsy

Six skin biopsies were taken (three coeliac and three control) from the middle of the upper arm. After the injection of a local anaesthetic, a round biopsy of 4 mm in diameter was obtained. This was either processed immediately or stored overnight at 4°C.

3.3.) Cultivation of Fibroblasts

a) All the work was done sterile under a laminar-flow. Components for medium were aliquotted out in volumes that were enough to allow for errors in measurement. Volumes of the additives to the Hams F10 media were aliquotted as follows:

   0,35 ml Ultroser G
   0,5 ml Penicillin/Streptomycin
   0,4 ml Amphotericin B
   0,2 ml L-glutamine

Tissue from the skin biospy was spliced into 25 small pieces and equally distributed among 5 different sterile Falcon flasks. Then 5 ml of medium was added prepared as follows:

   20 ml   Hams F10 + HEPES medium
   4,0 ml   Foetal calf serum
   0,32 ml   Ultroser G
   0,4 ml   Penicillin/Streptomycin
   0,2 ml   Amphotericin B
   0,1 ml   L-glutamine

The Falcon flasks were then incubated at 37°C for seven days.

b)   On day seven growth was assessed under an inverted microscope and noted. If significant growth had occurred **subculturing** took place and medium was replaced and stored. Then each flask was rinsed with 2,5 -

3,0 ml of PBS, after which 1 ml of Trypsin/EDTA was added and the flasks were incubated for 15 min. The flasks were gently tapped to ensure that the slices were loosened and separated from one another.The flasks were again checked under an inverted microscope. 9,0 ml of the complete medium were now added to the cell suspension in Trypsin/EDTA. After further gentle shaking of the flasks, 5,0 ml of the medium containing cells (equally to 50 % of the cells) were removed and added to a fresh sterile Falcon flask with the result that 5,0 ml of the cell suspension was now in each of the flasks.

On the next day medium was changed when the cells have settled and attached to the flask again. Old medium was disposed and 5,0 ml of fresh medium was added to ensure the removal of the Trypsin/EDTA.

c)   If, after the incubation period, only small colonies or overgrowing of small colonies developed, **reseeding** of the flasks was necessary to allow redistribution of an overgrowing colony over a large area and subsequent exponential growth. Therefore medium was removed and the flasks were once washed with 2,5-3,0 ml of sterile PBS. 1,0 ml of Trypsin/EDTA was added to the cells and left for 15 min to allow to detach. After that cells were gently tapped to ensure that they were loosened and the flasks were checked again under an inverted microscope. 5,0 ml of the complete medium was then added and the cells were left to settle overnight

On the next day the old medium with Trypsin/EDTA was removed and replaced by 5,0 ml of fresh medium.

These proceedings were repeated over and over again until in the end there were pure fibroblasts of each individual. For Sonicating and SDS Polyacrylamide Gel Electrophoresis with the subsequent Western Blot only samples from 7 days old flasks were chosen that showed a homogenous picture with neither overgrown nor small colonies to guarantee comparable amounts of cells.

d)   2 seven days old flasks of each individual were taken and medium was emptied out and replaced by 1 ml of Trypsin/EDTA. After 15 min of incubation the flasks were examined using an inverted microscope to determine if the cells were no longer stuck to the plastic of the flask. Aliquots of the Trypsin/EDTA-cell suspensions were taken into Eppendorf-pipettes. Those were spun in a centrifuge at 1500 Rpm for 10 min. The Trypsin/EDTA suspension was pipetted out, but the pellet remained inside the Eppendorf-pipette. The pellets were resuspended with PBS (1X), one Eppendorf-pipette with 0,5 ml the other with 1,0 ml. After that both suspensions were pipetted together, to ensure that cells from both flasks are now in one Eppendorf-pipette. They were spun again at 1500 Rpm for 10 min and resuspended in 1,0 ml PBS (1X). The cellular suspensions were next sonicated in a MSE-Soniprep 150, Ultrasonic Disintegrator, Loughborough, Leicestershire for 10 sec at mid power. After 10 seconds the samples were checked under the inverted microscope to ensure the disruption of the cells. The samples were finally stored in the freezer until SDS Polyacrylamide Gel Electrophoresis and Western Blot were carried out.

3.4.) SDS Polyacrylamide Gel Electrophoresis

Denaturating polyacrylamide gel electrophoresis was carried out on a 10 % polyacrylamide seperating gel using a 3,5 % stacking gel to concentrate the applied protein sample.

The following stock solutions were prepared using HPLC grade water:

(a)   30% Acrylamide-Bis:

   29,2 g Acrylamide, 0,8 g N.N'-methylene-bis-acrylamide dissolved in 50 ml, made up to a final volume of 100 ml.

   This solution can be stored in the dark at 4°C for up to 30 days.

(b)   1,5 M Tris/HCL, pH 8,8:

        18,15 g Tris base, adjusting pH to 8,8 with 1 N HCl, made up to 100 ml distilled water and stored at 4°C.

(c)   0,5 M Tris/HCl, pH 6,8:

        6,0 g Tris base, adjusting pH to 6,8 with 1 N HCl, made up to 100 ml distilled water and stored at 4°C.

(d)   SDS reducing sample buffer (10X):

5,0 ml	-	2,5 M Tris/HCl, pH 6,8
4,0 ml	-	β-mercaptoethanol
0,2 ml	-	0,5 % (w/v) bromophenol blue
0,8 ml	-	88 % (v/v) glycerol
1,6 g	-	SDS

        This solution was made up the day before use.

(e)   SDS (5X) electrode running buffer:

9,0 mg	-	Tris base
43,2 g	-	glycine
3,0 g	-	SDS

        dissolved in 600 ml distilled water and pH adjusted to 8,3 with 1 N HCl. If precipitation occurs then the buffer can be heated to 37°C before use. Prior to electrophoresis, 60 ml of this stock solution was diluted with 240 ml of water.

(f)   Coomassie blue stain:

        0,1% Coomassie blue R-250 in fixative: 40% methanol and 10% acetic acid

(g)   10% ammonium persulphate:

        prepared fresh daily

A 10% separating gel was obtained by mixing the above solutions in the following proportions:

(a)	30% Acrylamide-Bis	13,35 ml
(b)	1,5M Tris/HCl, pH 8,8	10,00 ml
	Distilled water	16,43 ml

This mixture was degassed for at least 15 min before adding 200 µl 10% Ammoniumpersulfate (APS) (g) and 20 µl TEMED. It was then swirled gently to mix and transferred to a small beaker with a good lip before pouring between plates (all volumes given above are sufficient for four gels). The gel was poured to approximately 1,5 cm from the top and the gel was then over layered with distilled water to exclude oxygen which prevents solidification of gel. After polymerisation excess water was poured off and dried with filter paper prior to pouring the stacking gel.

A 3,5% stacking gel was obtained by mixing the above solutions in the following proportions:

(a)	30% Acrylamide-Bis	2,33 ml
(c)	0,5M Tris/HCl, pH 6,8	5,00 ml
	Distilled water	12,55 ml

This mixture was degassed for at least 15 min and 100 µl 10% APS (g) and 20 µl TEMED added. It was swirled gently to mix and poured as already described above. The combs for moulding the wells were inserted and the gel was allowed to polymerise.

15 µl crude extract of protein samples were incubated with 10 µl SDS reducing sample buffer (10X) (d) for 5 minutes at 95°C prior to loading. 10 µl of denaturated sample were loaded into each well. The standards, Low Molecular Weight Calibration Kit for SDS Electrophoresis, Amersham Pharmacia, Biotech, were phosphorylase b (94 kDa), albumin (67 kDa),

ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20,1 kDa) and α-lactalbumin (14,4 kDa).

Electrophoresis was carried out using a *Mighty SmallIII*[TM] (SE 250) mini gel protein system from Hofer Scientific. The current setting was a constant 30 mA per running plate. Western Blot was performed directly after the SDS-PAGE.

3.5.) Western Blot

The following solutions were used as follows using HPLC grade water and stored at 4°C:

(a)   Tris buffered saline (TBS), pH 7,5:

            20 mM Tris, 500 mM NaCl, adjusting to pH 7,5 with 1 N HCl

(b)   0,1 % Tween 20:

            0,1 ml Tween 20 + 100 ml TBS buffer (a)

(c)   Blocking solution:

            5 % low fat milk (LFM) in TBS buffer (a)

(d)   Primary antibody:

            coeliac or control sera, diluted 1/100 with blocking solution (c)

(e)   Secondary antibody:

            Alkaline phosphatase conjugated to Anti-Human IgA (α-chain specific), diluted 1/30.000 with TBS buffer (a)

(f)   Alkaline phosphatase buffer, pH 9,5:

            0,1 M Tris, 0,1 M NaCl, 10 mM MgCl$_2$

adjusting to pH 9,5 with 1 N HCl

(g) Alkaline phosphatase substrates:

SIGMA *FAST*[TM] BCIP/NBT buffered substrate tablets (5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium), 1 tablet diluted in 15 ml of water

(h) Transfer buffer, pH 8,8:

25 mM Tris (a), 192 mM Glycine, 20 % (v/v) Methanol

Adjustment of pH was not carried out using acid or base. The pH should be between 8,1 - 8,4 depending on the quality of the Tris, Glycine and Methanol. The Methanol was analytical reagent grade, as metallic contaminants in low grade methanol will plate on the electrodes. If the pH does not fall within the range of 8,1 - 8,4 no buffer acid or base should be added, the buffer must be remade with better quality components

Prior to transfer, the Bio-ice cooling unit was filled with distilled water and frozen overnight. The nitrocellulose membranes (one per gel) were marked by cutting one corner in order to facilitate proper orientation of the resulting bands and stored together with filter paper (four per gel) and fibre pads (two per gel) in transfer buffer (h) overnight.

Gels were equilibriated in transfer buffer (h) for 30 min prior to blotting, facilitating the removal of electrophoresis buffer salts and detergents which would adversely affect the transfer process.

Procedure:

After the electrophoresis the lanes with the standards were first divided from the rest of the gel. They were not to be blotted, but to be stained with Coomassie Blue R-250 immediately. All the preparations for the blot and the blot itself were carried out in the cold room at 4°C. The Mini-blot electrode was placed in the buffer chamber and the tank was half filled with transfer

buffer (h). A small stir bar was placed on the bottom of the unit and the unit was placed on a magnetic stirrer. When the system was running the movement of the stir bar generates air bubbles indicating that the current was running. Wearing surgical gloves, the gel and the membranes were sandwiched between four sheets of filter paper (two sheets on each side) which was in turn between the two filter pads. Care had to be taken to ensure no air bubbles were trapped between the gel and the membrane. Once the cassette had been assembled it was placed into the half filled buffer chamber. There was enough room in the buffer chamber for two cassettes which means that two gels can be blotted at the same time. If there was no second gel to be blotted the second cassette was loaded empty into the buffer chamber. After the loading, the Bio-ice cooling unit was placed in the buffer chamber, next to the electrode. The buffer chamber was then filled to the top with transfer buffer (h). Once connected to the power pack the transfer was carried out at a constant voltage of 100 V for approximately one hour.

After the blot, gels were stained with Coomassie Blue R-250 for approximately 1 hour and destained in aqueous 40 % Methanol and 10 % acetic acid to make sure that the blot was successful and no protein was left in the gels. Standards were stained in the same way. The gels were subsequently stored in 2 % acetic acid solution.

The development of protein bands on the nitrocellulose membrane was carried out as follows. All procedures were carried out at room temperature. Each membrane was placed in one box which was placed on a rock/roller. This box was used during the whole incubation periods:

1 h	-	in blocking solution (c)
3 h	-	in primary antibody in blocking solution (d)
5 x 5 min	-	washing in TWEEN 20 in TBS buffer (0,1 %) (b)
5 min	-	in TBS buffer (a)
1 h	-	in secondary antibody in TBS buffer (e)
5 x 5 min	-	washing in TWEEN 20 in TBS buffer (0,1 %) (b)
5 min	-	in TBS buffer (a)

2 x 5 min   -   in alkaline-phosphatase buffer (f)

Finally the membranes were incubated with alkaline phosphatase substrates (g) in the dark until a reaction developed (approximately 5-15 min). The reaction was stopped by washing a number of times with distilled water. All the membranes were then stored in distilled water.

4.)   Results:

4.1.) General Comments:

The 10% separating gel is only able to separate between proteins of 12 to 90 kDa of size, so it was recognized that the three clear bands belong to the three standard proteins with the smallest size. In every gel/membrane three smaller blurred bands were to be seen in the high molecular weight area. So the three bands in the lanes of the low molecular weight markers were α-lactalbumin (14,4 kDa), trypsin inhibitor (20,1 kDa) and carbonic anhydrase (30,0 kDa).

Blotting was always successful as there was never any protein left on the gel after the blot.

4.2.) SDS Polyacrylamide Gel Electrophoresis

Performing SDS - Polacrylamide Gel Electrophoresis with medium of two coeliac and two control patients and staining the gel with Coomassie-blue several bands were visible (see fig.1). There are obvious bands in each of the four samples, but are of different intensities. The biggest band could be seen in the area of around 20 kDa. It appeared to be so big that it covered the whole area from around 25 kDa to 33 kDa. This band was very broad and thick in comparison to the other ones. It appeared biggest in one of the coeliac patients, where the clearest bands were visible and in one control.
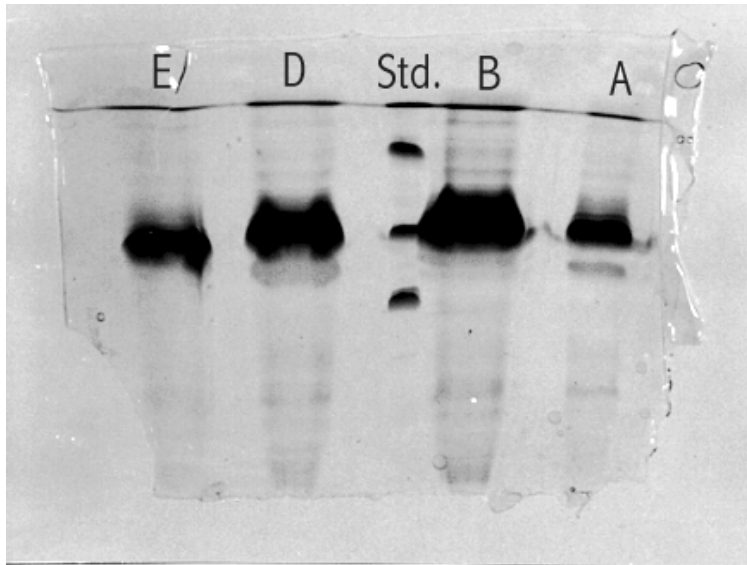
Figure 1:　　　　**SDS Polyacrylamide Gel Electrophoresis**

　　　　　　E:　　　Medium of skin Biopsy Sample of patient E (control)

　　　　　　D:　　　Medium of skin Biopsy Sample of patient D (control)

　　　　　　Std:　　Sample with standard molecular weight markers (α-lactalbumin: 14,4 kDa, trypsin inhibitor: 20,1kDa, carbonic anhydrase: 30,0 kDa)

　　　　　　B:　　　Medium of skin biopsy sample of patient B (coeliac patient)

　　　　　　A:　　　Medium of skin biopsy sample of patient A (coeliac patient)

4.3.) Medium

Concerning the medium samples the results were as follows: The media of three coeliac patients (A,B,C) and the media of three healthy controls (D,E,F) were tested with two coeliac sera (1,2). The number and density of bands developed on the membranes depended on which of the two coeliac sera were used. Utilizing the serum of patient 1 as primary antibody in the western blot several bands were visible. The broadest one was in the relatively middle to low molecular weight area. Comparing the result with the low molecular weight standards this band can be classified as around 40 kDa (note four standards are present, particularly the four with the lowest molecular weights, see figure 2). It was slightly more intense in medium of patients A and C, but it developed in medium of every patient (see figure 2).

Furthermore another large band developed in the low molecular weight area of about 30 kDa. It is a little bit more blurred than the 40 kDa band. It was visible in every patient as well. Besides these two bands several vague bands occured in the high molecular weight area. They were quite weak and difficult to differentiate. They appeared in medium of every patient.
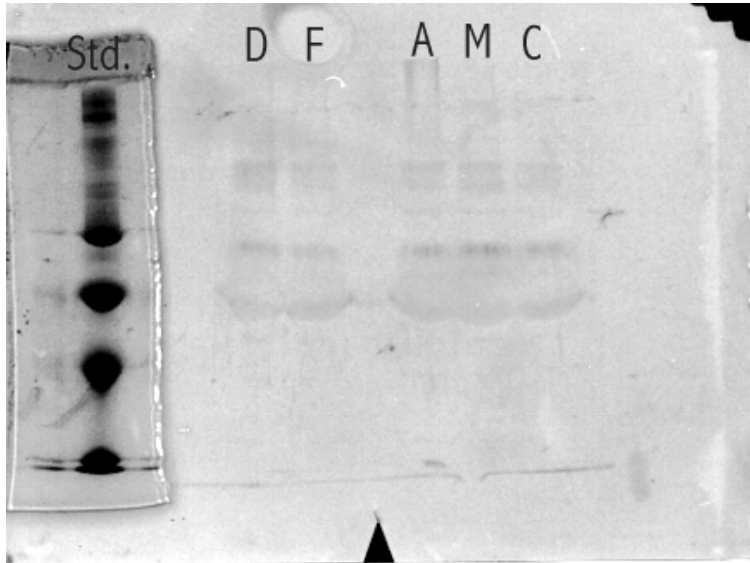

Figure 2:   **Western Blot of serum of patient 6 (coeliac patient)**

Std.:   Sample with standard molecular weight markers (four standards are present: α-lactalbumin: 14,4 kDa, trypsin inhibitor: 20,1kDa, carbonic anhydrase: 30,0 kDa, ovalbumin : 43,0 kDa)

D: Medium of skin biopsy sample of patient D (control)

F: Medium of skin biopsy sample of patient F (control)

A: Medium of skin biopsy sample of patient A (coeliac patient)

M: Medium without any skin biopsy sample

C: Medium of skin biopsy sample of patinet C (coeliac patient)

Using the serum of patient 2 only one band developed. In comparison to the bands that were seen with medium of patient 1 this band was very weak and could be assigned to the area of around 30 kDa. Again it appeared in every patient equally (see figure 3). Interestingly the same band appeared, when the media of the same patients were tested with control serum (patient 7). It was in the same range of molecular weight, but its intensity was even a little bit stronger than the one observed with patient 1.
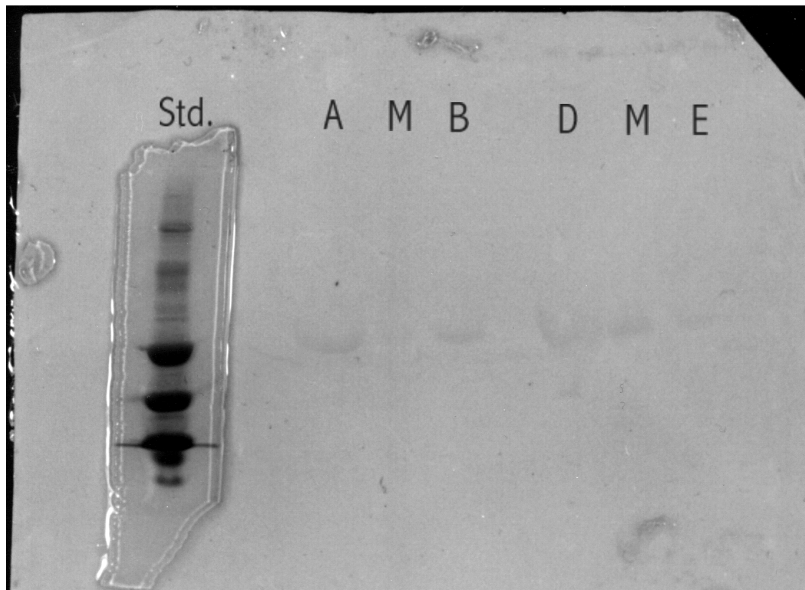
Figure 3: Western Blot of serum of patient 2 (coeliac patient)

Std.:   Sample with standard molecular weight markers (α-lactalbumin:

        14,4 kDa, trypsin inhibitor: 20,1kDa, carbonic anhydrase:

        30,0 kDa)

A: Medium of skin biopsy sample of patient A (coeliac patient)

M: Medium without any skin biopsy sample

B: Medium of skin biopsy sample of patient B (coeliac patient)

D: Medium of skin biopsy sample of patient D (control)

E: Medium of skin biopsy sample of patinet E (control)

Most interestingly the same result was seen, when the „empty" medium was tested, e.g. medium in which fibroblasts had not been cultured. Exactly the same bands with the same intensities developed as had previously, when the same sera were used. This was regardless of whether this was coeliac or control serum (see figures 2 and 3).

4.4.) Fibroblast-Sonicates

Concerning the blots of the fibroblast-sonicates it is important to differentiate between those that were incubated with coeliac, treated coeliac and healthy

control serum. Membranes treated with coeliac sera (patients 1,2,5 and 6) were very different. When testing serum of patient 5 no visible reaction was seen on the membrane. Using serum of patient 2 one broad band in the very high molecular weight area appeared (see figure 4). This reaction could only be seen in fibroblast-samples taken from coeliac patients: patients A and C showed the development of this band, whereas this reaction was not detectable in control fibroblast samples of D and F. This band was also not as clearly shaped as the usual other bands, so the test with this serum was repeated. This time no band at all developed, not even in coeliac fibroblast-serum.
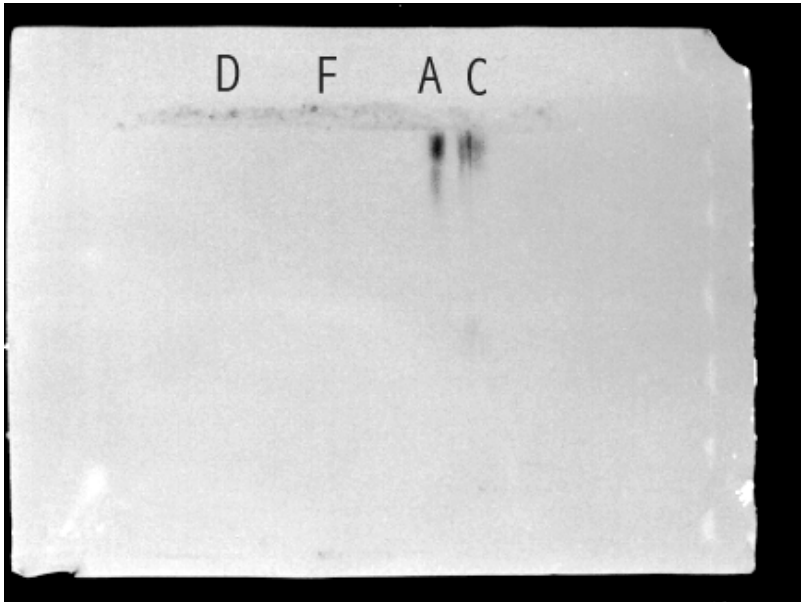

Figure 4: Western Blot of serum of patient 2 (coeliac patient)

D: Fibroblast-sonicate of skin biopsy sample of patient D (control)

F: Fibroblast-sonicate of skin biopsy sample of patient F (control)

A: Fibroblast-sonicate of skin biopsy sample of patient A (coeliac patient)

C: Fibroblast-sonicate of skin biopsy sample of patinet C (coeliac patient)

Also, when using serum of patient one as primary antibody in the blot, only coeliac fibroblast samples showed a reaction (see figure 5): In patients A and C a ladder-like picture became evident. This ladder consisted of around 10 steps, which means that there were about 10 distinct single bands. They were all in the area between 15 and 20 - 25 kDa and developed equally in

both coeliac patients. The strongest band developed on the top of the ladder at around 20 - 25 kDa. There was no reaction and therefore no band in the control fibroblast-samples. Additionally to the clearly shaped bands one reaction occured in the area between 20 and 30 kDa. This reaction appeared between two lanes (A and C) and could not be reproduced and is an artefact. Otherwise the same picture occurred after a repetition of this test.

Figure 5: : Western Blot of serum of patient 1 (coeliac patient)

Std.: Sample with standard molecular weight markers (α-lactalbumin: 14,4 kDa, trypsin inhibitor: 20,1kDa, carbonic anhydrase: 30,0 kDa)

D: Fibroblast-sonicate of skin biopsy sample of patient D (control)

F: Fibroblast-sonicate of skin biopsy sample of patient F (control)

A: Fibroblast-sonicate of skin biopsy sample of patient A (coeliac patient)

C: Fibroblast-sonicate of skin biopsy sample of patinet C (coeliac patient)

Only one band developed after serum of patient 6 was used in the western blot. This band was not as intense as the ones observed with other sera and was in the area of about 20 - 25 kDa (see figure 6) . It could be identified in coeliac and in control fibroblast-samples, but it was slightly more intense in coeliac samples (A and C). No other bands or reactions occured.
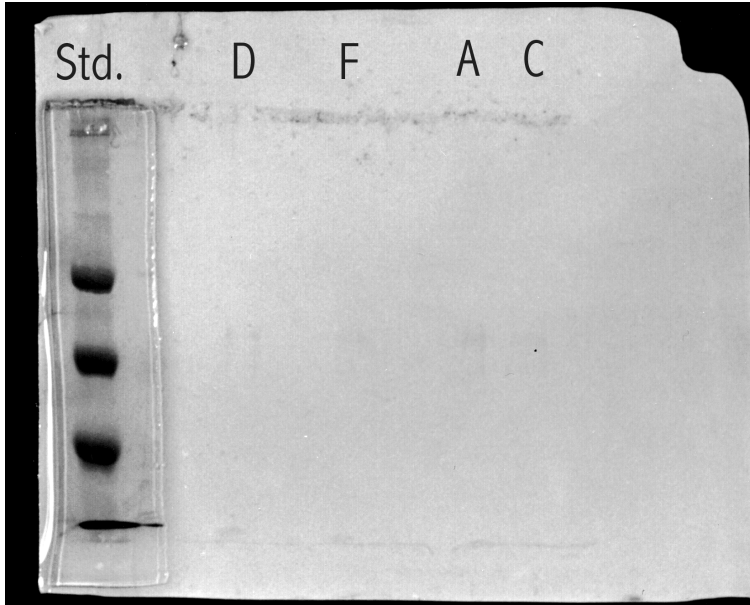
Figure 6: Western Blot of serum of patient 6 (coeliac patient)

Std.:   Sample with standard molecular weight markers (α-lactalbumin:

             14,4 kDa, trypsin inhibitor: 20,1kDa, carbonic anhydrase:

             30,0 kDa)

D: Fibroblast-sonicate of skin biopsy sample of patient D (control)

F: Fibroblast-sonicate of skin biopsy sample of patient F (control)

A: Fibroblast-sonicate of skin biopsy sample of patient A (coeliac

             patient)

C: Fibroblast-sonicate of skin biopsy sample of patinet C (coeliac

             patient)

The serum of treated coeliac patients (3 and 4) failed to produce any visible blots.

Similarly no band and no reaction developed in connection with control serum of healthy individuals (7 - 10). Even after a few repeat runs no change on the membrane could be observed with one exception: when serum of control 7 was used there was once a reaction with fibroblast-samples of patients C (coeliac) and D (control) (see figure 7): in the lane of patient D a single rather weak band developed in the middle molecular weight area. A similar band, only far more intense, was seen with the sample of patient C. Additionally another blurred band could be identified in the lower molecular

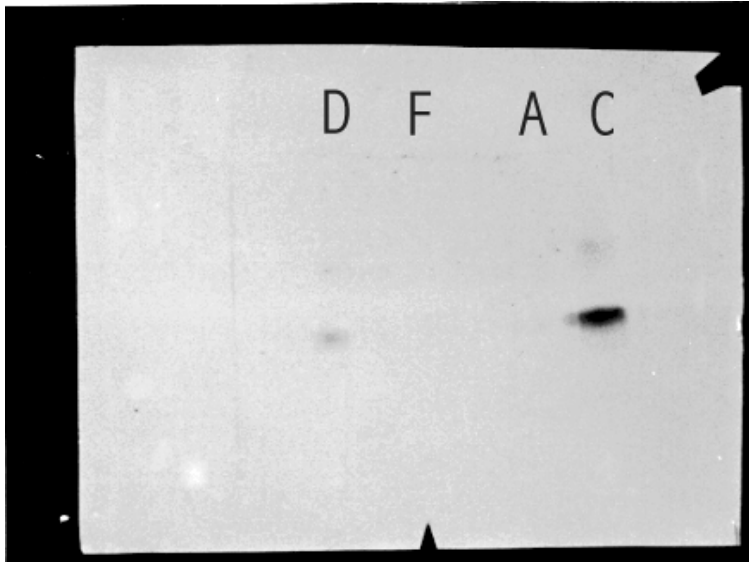weight area in the same patient. These band(s) could not be reproduced in several repeated experiments.

Figure 7: : Western Blot of serum of patient 7 (control)

D: Fibroblast-sonicate of skin biopsy sample of patient D (control)

F: Fibroblast-sonicate of skin biopsy sample of patient F (control)

A: Fibroblast-sonicate of skin biopsy sample of patient A (coeliac patient)

C: Fibroblast-sonicate of skin biopsy sample of patinet C (coeliac patient)

5.) **Discussion:**

5.1.) SDS Polyacrylamide Gel Electrophoresis

Many different proteins were found in the media. As there were many bands in every molecular weight area, proteins and possible antigens of every molecular seize were present in the media.

5.2.) Medium

The same band was seen using serum of patient 2 and control serum (7). Therefore an antigen was picked up not only by coeliac serum, but also by control serum, so that this antigen is very likely not to be specific for coeliac disease. Patient 1 had a similar band. Unfortunately this band is probably not significant as it was found when probing media from cultivated coeliac fibroblasts and in fresh media in which no cells had been cultivated. This means that the antigen(s) are already in the uncultered medium. It is most likely they are in the foetal calf serum, a major biological component of the cultured medium. Therefore other methods and cultivation essays should be used in future that do not use foetal calf serum as a component. Both coeliac and control sera recognize the antigens(s). Subtraction of the bands found with the fresh uncultivated medium did not reveal additional band, which means there is no any evidence of protein secreted by the fibroblasts when using this way of cultivation. Nervertheless the question of the secretion of a protein into the medium can only be answered sufficiently, if a method without foetal calf serum is used, as the many bands produced by foetal calf serum are in some parts a little bit faded. Only a renouncing of foetal calf serum could produce a picture without those bands and could uncover bands of possibly secreted proteins. The possibility that the Blot itself was insufficient and a possible protein was lost on that part of the essay can be ruled out, as staining of the SDS Polyacrylamide Gel after the blotting took place did not reveal any proteins in the gel anymore. Therefore all proteins

were successfully transfered to the membrane. So when using the described essay, it has to be concluded that no antigens are secreted by the fibroblasts into the medium. Therefore the question of tissue-transglutaminase existing intra- and/or extracellularly has to remain unanswered and both therories last further [30] [53] [54].

5.3.) Fibroblast - Sonicates

The results produced in this part of the study are most complicating, especially those concerning blots developed with the serum of patient 1. A „ladder-like" arrangement of bands that developed in fibroblast-sonicates of coeliac patients only and not in control fibroblasts was seen when serum of patient 1 had been used. This appearance was a constant finding in several repeat experiments. Patient 1 is a coeliac with a past history of alcoholism and at the time his serum was collected he had a high serum IgA level, high serum IgA gliadin antibodies and abnormal liver biochemistry. There is no explanation for the presence of the IgA antibodies in his serum. Interestingly they only reacted with sonicates from coeliac fibroblasts. At least 10 seperate bands could be identified with molecular weights between 15 and 25 kDa. As this picture could not be reproduced in combination with any other coeliac or control serum it is very likely that this variety of bands is rather related to this patients's high alcohol intake than his coeliac disease. On the other hand the presence of this reaction only with sonicates from coeliac fibroblasts indicates a strong relation of these antigens to coeliac disease, too. Therefore it would be interesting to examine further alcoholic coeliacs, non-alcoholic coeliacs and alcoholic non-coeliac to determine if antibodies that appeared in those bands are related to alcoholic liver damage or alcoholic damaged coeliac liver only. Similar results or coeliac antigens related to alcohol liver damage have not been reported yet in literature.

When serum of patient 2 was used for the first time a reaction developed in the high molecular weight area. This band was not shaped like a typical band, but appeared more to be organized in a vertical way instead of horizontally. Furthermore it could not be repeated, so it was very likely to be a non-specific reaction and was therefore not related to a possible antigen of coeliac disease, although it only occured in coeliac fibroblast-samples.

With serum of patient 5 no antigen could be detected in any of the fibroblast samples, as there was no reaction at all visible.

The sera of patients 6 and 1 are most interesting. Those sera are the only ones which recognize a protein band of the molecular weight of 20 - 25 kDa. As this particular antigen is elaborated by both coeliac and non-coeliac fibroblasts it would appear unlikely to be a specifically modified coeliac autoantigen. However, it should be noted that cultured coeliac fibroblasts produce quantitatively more of this antigen, that means the bands were more intense. Further quantitative study is needed to confirm this observation.

It has been hypothesized that skin fibroblasts might elaborate tissue-transgluatminase and that coeliac tissue-transglutaminase might be structurally modified and resulting in an enhancemenrt of antigenicity [58]. Unfortunately the published molecular weights of human tissue-transglutaminase (80-82 kDa) [30] [56] and the active (50 kDa) or the pre-enzymatic form (78 kDa) of epidermal tissue transglutaminase [57] are not in the range of 20 - 25 kDa. Thus a method of isolating and characterising coeliac and non-coeliac tissue-transglutaminase has not been found. Nevertheless the possibilty remains that the antigens discovered are other fragments of tissue-transglutaminase. It could also be that the protein that was picked up at 20 - 25 kDa has a fragment of tissue-transglutaminase as a component. It is known that tissue-transglutaminase tends to form irreversible aggregates when isolated under native conditions [57]. Therefore it could be that the found antigen got its reacting part in the blot from a fragment of tissue-transglutaminase, but the whole molecule is so modified that it could be classed with the heterogenous group of tissue-

transglutaminases. Marttinen et al. purified fibroblast-derived celiac antigen molecules and found that four single polypeptides in the range between 17 and 39.5 kDa reacted with Iga seperated from sera of coeliac children [50]. However, at that time (1993) tissue transglutaminase was not yet discovered to be the or a coeliac antigen. Secondly in their study fibroblasts were purified from fetal lung tissue and not from human skin biopsies as in this study and sera were taken from children rather than adults. Giving regard to these points it is well possible that the 20 – 25 kDa antigen found in this study is similar or even identical with one of the antigens found by the group of Marttinen. If so, fetal lung tissue is not needed any more as a source of fibroblasts.

Sera from patients 2 thru 5 did not recognise an antigen of 20 - 25 kDa molecular weight. This is possibly related to the amount of gluten in the diet at the time the serum was collected.

Patient 2 had been on a gluten free diet for several months before the serum sample used in the study was collected. Patient 3 claimed to be on a good gluten free diet when phlebotomised (patients frequently improve their dietary compliance when notified they have to attend outpatient clinic). Patient 4 was on a strict gluten free diet. Patient 5 is a newly diagnosed Non Insulin Dependant Diabetes Mellitus (NIDDM) obese patient, whose father is a coeliac. She had positive gliadin antibodies, but these were only slightly raised. It was a surprise to find her small bowel mucosa so severely damaged. She has silent coeliac disease. The absence of the 20 - 25 kDa antigen recognition could relate to the dietary status in patients 2 thru 4 and the poor antibody development in the NIDDM patient (5). Obviously more study of antibodies to this band and its relationship to dietary gluten and gliadin antibody status is required. Gliadin antibodies may persist in the circulation longer than antibodies to the 20 - 25 kDa antigen. However, it could be possible to differentiate between clinically manifested and latent or potent coeliac disease with this test as demonstrated in patient 5. When using this method it becomes obvious that it is difficult to identify "the typical coeliac" with "the typical antibody-status". Apparently many antigens reacted

with different sera, but in consideration of the dietary status the 20-25 kDa antigen appears to be the important one.

Only a single reaction was observed with non-coeliac serum in the Western Blot. This was probably an artefact, as it was not found on two repeat runs and was therefore considered non-significant. Thus cultured fibroblasts (coeliac or non-coeliac) do not elaborate an antigen which evokes the production of antibodies in non-coeliac serum.

As the Western Blot is a very sensitive method to detect antigens ELISA plates were prepared using the fibroblast sonicates. In these plates larger numbers of coeliac and non-coeliac sera were tested. Coeliac sera were more frequently positive than by Western Blot. Even some of the sera from treated coeliac patients that had been negative in Western Blot were positive in the ELISA. The reason for the disparity is unclear. The basic reaction in the ELISA is a more simple one than the preparation, gel separation and transfer procedures. It is possible the 1,5 ml of fibroblast-sonicate solution used contains insufficient antigen to produce positive Western Blots, but it would be enough to cause a positive reaction in the ELISA. On the other hand it is not possible to identify an antigen by size when ELISA is used instead of Western Blot. Using an ELISA only allows an answer to the question of whether there is an antigen-antibody-reaction or not without any information about the reacting antigen. In order to get a bigger amount or a bigger concentration of antigen it would be neccessary to purify the molecules produced by the fibroblasts. This was described by Marttinen and colleagues: After they had found that fibroblasts synthesize and secrete proteins that bind to IgA-class anti-reticulin and anti-endomysium but not to anti-gliadin antibodies in coeliac disease patients [59] they developed a method to purify these antigen molecules from fibrolblast culture medium [60]. To achieve this High Performance Liquid Chromatography (HPLC) was used which requires bigger amounts of medium produced by fibroblasts or fibroblast sonicates (about 2l). Consequently it must be concluded that these amounts should be used together with the superior method of HPLC in further studies in order to identify the reacting antigen in the ELISA and the

disparity of the results in the ELISA and the Western Blot as described above. Unfortunately both the big amount of produced medium and HPLC was not available for this study. However, as described above it must be noticed that although at the time Maki and Marttinen published their results tissue-transglutaminase has not yet been discovered to be the or a reacting antigen in coeliac disease the reported sizes of the found molecules do not stand in dispairity to the antigens found in this study. As the proteins described by Marttinen were obtained from fetal lung tissue and the protein in this study was produced by fibroblasts derived from human skin this tissue deserves further study with the methods described above.

6.)   Conclusions:

1.)   Cultivated skin fibroblasts do not secrete an antigen into their culture medium.

2.)   The thesis that cultured human skin explants elaborate tissue-transglutaminase has to be rejected.

3.)   It is possible that cultured human skin explants elaborate an antigen. This could be a fragment of tissue-transglutaminase .

4.)   These preliminary results suggest cultured fibroblasts contain a coeliac antigen which deserves further study.

7.) References:

1.   Feighery, C. Fortnightly review: coeliac disease. *Bmj* **319**, 236-9. (1999).

2.   Maki, M. & Collin, P. Coeliac disease. *Lancet* **349**, 1755-9. (1997).

3.   Godkin, A. & Jewell, D. The pathogenesis of celiac disease. *Gastroenterology* **115**, 206-10. (1998).

4.   Dicke, W.K. MD Thesis, University of Utrecht (1950).

5.   Jewell, D.P. Coeliac Disease. in *Oxford Textbook of Medicine*, Vol. Vol. 2 (ed. Weatherall D.J., L.J.G.G., Warrel B.A.) 1916-20 (Oxford Medical Publications / Oxford University Press, 1996).

6.   Walker-Smith, J.A., Guandalini, S., Schmitz, J., Shmerling, D.H. & Visakoppi, J.K. Revised criteria for diagnosis of coeliac disease. *Archives of the Disabled Child* **65**, 909-11 (1990).

7.   Catassi C., e.a. Screening of Coeliac Disease. in *Coeliac Disease: Proceedings of the 7th international symposium on coeliac disease, September 5-7, 1996* (ed. Mäki, M.) 23-24 (, Tampere, Finland, 1996).

8.   Riestra, S., Fernandez, E., Rodrigo, L., Garcia, S. & Ocio, G. Prevalence of Coeliac disease in the general population of northern Spain. Strategies of serologic screening. *Scand J Gastroenterol* **35**, 398-402. (2000).

9.   Troncone, R. *et al.* Latent and potential coeliac disease. *Acta Paediatr Suppl* **412**, 10-4. (1996).

10.   Hin, H., Bird, G., Fisher, P., Mahy, N. & Jewell, D. Coeliac disease in
       primary care: case finding study. *Bmj* **318**, 164-7. (1999).

11.   O'Farrelly, C. Is villous atrophy always and only the result of gluten
       sensitive disease of the intestine? *Eur J Gastroenterol Hepatol* **12**,
       605-8. (2000).

12.   Trier, J.S. Celiac sprue and refractory sprue. in *Gastrointestinal and
       liver disease (pathophysiology / diagnosis / management)*, Vol. Vol. 2
       (eds. Feldman, M., Scharschmidt, B.F. & Sleidinger, M.H.) 1557-73
       (W.B. Saunders Company, 1998).

13.   Bhattacharyya, A., Patel, M.K. & Tymms, D.J. Coeliac disease in
       adults: variations on a theme. *J R Soc Med* **92**, 286-9. (1999).

14.   Martinelli, P. *et al.* Coeliac disease and unfavourable outcome of
       pregnancy. *Gut* **46**, 332-5. (2000).

15.   Gasbarrini, A. *et al.* Recurrent spontaneous abortion and intrauterine
       fetal growth retardation as symptoms of coeliac disease. *Lancet* **356**,
       399-400. (2000).

16.   Bardella, M.T. *et al.* Alopecia areata and coeliac disease: no effect of
       a gluten-free diet on hair growth. *Dermatology* **200**, 108-10 (2000).

17.   Jennings, J.S., Wyatt, J.I. & Howdle, P.D. A new cause of 'non-
       responsiveness' in coeliac disease? *Postgrad Med J* **76**, 227-9.
       (2000).

18.   Selby, W.S., Painter, D., Collins, A., Faulkner-Hogg, K.B. & Loblay,
       R.H. Persistent mucosal abnormalities in coeliac disease are not
       related to the ingestion of trace amounts of gluten. *Scand J
       Gastroenterol* **34**, 909-14. (1999).

19. Savilahti, E. *et al.* IgA antigliadin antibodies: a marker of mucosal damage in childhood coeliac disease. *Lancet* **1**, 320-2. (1983).

20. Maki, M. The humoral immune system in coeliac disease. *Baillieres Clin Gastroenterol* **9**, 231-49. (1995).

21. Russo, P.A., Chartrand, L.J. & Seidman, E. Comparative analysis of serologic screening tests for the initial diagnosis of celiac disease. *Pediatrics* **104**, 75-8. (1999).

22. Lenander-Lumikari, M., Ihalin, R. & Lahteenoja, H. Changes in whole saliva in patients with coeliac disease. *Arch Oral Biol* **45**, 347-54. (2000).

23. Lahteenoja, H., Toivanen, A., Raiha, I., Syrjanen, S. & Viander, M. Salivary antigliadin and antiendomysium antibodies in coeliac disease. *Scand J Immunol* **50**, 528-35. (1999).

24. Vitoria, J.C. *et al.* Antibodies to gliadin, endomysium, and tissue transglutaminase for the diagnosis of celiac disease. *J Pediatr Gastroenterol Nutr* **29**, 571-4. (1999).

25. Karska, K., Tuckova, L., Steiner, L., Tlaskalova-Hogenova, H. & Michalak, M. Calreticulin--the potential autoantigen in celiac disease. *Biochem Biophys Res Commun* **209**, 597-605. (1995).

26. Dieterich, W. *et al.* Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* **3**, 797-801. (1997).

27. Bowron, A. *et al.* Cost-effective strategy for the serological investigation of coeliac disease. *Ann Clin Biochem* **37**, 467-70. (2000).

28. Rostami, K. *et al.* Should relatives of coeliacs with mild clinical complaints undergo a small-bowel biopsy despite negative serology? *Eur J Gastroenterol Hepatol* **12**, 51-5. (2000).

29. Johnston, S.D. *et al.* Genetic, morphometric and immunohistochemical markers of latent coeliac disease. *Eur J Gastroenterol Hepatol* **11**, 1283-8. (1999).

30. Aeschlimann, D. & Paulsson, M. Transglutaminases: protein cross-linking enzymes in tissues and body fluids. *Thromb Haemost* **71**, 402-15. (1994).

31. Folk, J.E. Mechanism and basis for specificity of transglutaminase-catalyzed epsilon-(gamma-glutamyl) lysine bond formation. *Adv Enzymol Relat Areas Mol Biol* **54**, 1-56 (1983).

32. Nunes, I., Gleizes, P.E., Metz, C.N. & Rifkin, D.B. Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. *J Cell Biol* **136**, 1151-63. (1997).

33. Bruce, S.E., Bjarnason, I. & Peters, T.J. Human jejunal transglutaminase: demonstration of activity, enzyme kinetics and substrate specificity with special relation to gliadin and coeliac disease. *Clin Sci (Colch)* **68**, 573-9. (1985).

34. Fasano, A. *et al.* Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet* **355**, 1518-9. (2000).

35. Molberg, O. *et al.* Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat Med* **4**, 713-7. (1998).

36. Schuppan, D., Dieterich, W. & Riecken, E.O. Exposing gliadin as a tasty food for lymphocytes. *Nat Med* **4**, 666-7. (1998).

37. Brusco, G. *et al.* Transglutaminase and coeliac disease: endomysial reactivity and small bowel expression. *Clin Exp Immunol* **118**, 371-5. (1999).

38. Lock, R.J., Gilmour, J.E. & Unsworth, D.J. Anti-tissue transglutaminase, anti-endomysium and anti-R1-reticulin autoantibodies-the antibody trinity of coeliac disease. *Clin Exp Immunol* **116**, 258-62. (1999).

39. Iltanen, S. *et al.* Expression of HSP-65 in jejunal epithelial cells in patients clinically suspected of coeliac disease. *Autoimmunity* **31**, 125-32. (1999).

40. van de Wal, Y. *et al.* Glutenin is involved in the gluten-driven mucosal T cell response. *Eur J Immunol* **29**, 3133-9. (1999).

41. Morris, M.A. *et al.* Coeliac disease and Down syndrome: associations not due to genetic linkage on chromosome 21. *Scand J Gastroenterol* **35**, 177-80. (2000).

42. Aldersley, M.A. *et al.* No polymorphism in the tissue transglutaminase gene detected in coeliac disease patients. *Scand J Gastroenterol* **35**, 61-3. (2000).

43. Sulkanen, S. *et al.* Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* **115**, 1322-8. (1998).

44. Dieterich, W. *et al.* Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* **115**, 1317-21. (1998).

45. Miller, A., Paspaliaris, W., Elliott, P.R. & d'Apice, A. Anti-transglutaminase antibodies and coeliac disease. *Aust N Z J Med* **29**, 239-42. (1999).

46. Chorzelski, T.P. *et al.* IgA anti-endomysium antibody. A new immunological marker of dermatitis herpetiformis and coeliac disease. *Br J Dermatol* **111**, 395-402. (1984).

47. Ladinser, B., Rossipal, E. & Pittschieler, K. Endomysium antibodies in coeliac disease: an improved method. *Gut* **35**, 776-8. (1994).

48. Whelan, A., Willoughby, R. & Weir, D. Human umbilical vein endothelial cells: a new easily available source of endomysial antigens. *Eur J Gastroenterol Hepatol* **8**, 961-6. (1996).

49. Rostami, K., Kerckhaert, J.P., Tiemessen, R., Meijer, J.W. & Mulder, C.J. The relationship between anti-endomysium antibodies and villous atrophy in coeliac disease using both monkey and human substrate. *Eur J Gastroenterol Hepatol* **11**, 439-42. (1999).

50. Marttinen, A. & Maki, M. Purification of fibroblast-derived celiac disease autoantigen molecules. *Pediatr Res* **34**, 420-3. (1993).

51. Rooney, D.E., Czepulkowski B.H. *Human cytogenetics - a practical approach*, (IRL Press at Oxford, University Press, 1992).

52. Hashim, Z. Cultured coeliac fibroblasts: a suitable source of tissue transglutaminase antigen? (University College Galway, Dept. of Medicine, not published, Galway, Ireland).

53. Sollid, L.M. & Scott, H. New tool to predict celiac disease on its way to the clinics. *Gastroenterology* **115**, 1584-6. (1998).

54. Schuppan, D. personal conversation. (1998).

55. Bazzigaluppi, E. *et al.* Comparison of tissue transglutaminase-specific antibody assays with established antibody measurements for coeliac disease. *J Autoimmun* **12**, 51-6. (1999).

56. Autuori, F., Farrace, M.G., Oliverio, S., Piredda, L. & Piacentini, M. "Tissue" Transglutaminase and Apoptosis. *Adv Biochem Eng Biotechnol* **62**, 129-36 (1998).

57. Wilhelm, B., Meinhardt, A. & Seitz, J. Transglutaminases: purification and activity assays. *J Chromatogr B Biomed Appl* **684**, 163-77. (1996).

58. Donlon, J. & Stevens, F. personal conversation. (1999).

59. Maki, M., Hallstrom, O. & Marttinen, A. Reaction of human non-collagenous polypeptides with coeliac disease autoantibodies. *Lancet* **338**, 724-5 (1991).

60. Marttinen, A., Sulkanen, S. & Maeki, M. Human fibroblast-derived molecules as antigens in enzyme-linked immunosorbent assay for coeliac disease-specific IgA. *European Journal of Clinical Investigation* **27**, 135-40. (1997).

Acknoledgements

I wish to express my sincere gratitude to:

Prof. Burkhard May for entrusting me to this thesis. He tutored my work under extraordinary circumstances.

Dr. Fiona Stevens, my supervisor from the Department of Gastroenterology of the National University of Ireland in Galway. She introduced me to the field of science and guided me during my stay in ireland. I am also very grateful to the whole laboratory of the Department of Clinical Medicine, especially to Miriam and Una for her generous support.

Dr. John Donlon, acting head of the Department of Biochemistry of the National University of Ireland in Galway, for his support in the laboratory of his department and his kind interest and valuable advice.

The whole laboratory of the Department of Biochemistry in Galway, especially Orla O'Donovan and Stephanie Ryan, for their outstanding patience and absolutely necessary aids to use the equipment and to perform the experiments.

Stewart Baker for taking the photographs and Uli Deppe for scanning them.

Prof. Hans Büning-Pfaue for encouraging me to write this thesis. He gave me the necessary insight into the right scientific strategies.

The fifth and sixth medical class of 1998/1999 of the National University of Ireland, Galway. They looked after me with so much kindness, cosiness and warmth, not to mention all the happy moments of laughter.

Curriculum vitae

Name: Reinsberger   Forename: Claus

Birthday: 04th of October 1974   Place of birth: Paderborn

Nationality: German   Marital status: unmarried

Education:

August 1981 - July 1985   **Primary school Marktschule**

Paderborn

August 1985 - May 1994   **Gymnasium Theodorianum**

Paderborn

final examination: Abitur

August 1994 – September 1995   **Institute of Sportsmedicine**

University of Paderborn

Community service

October 1995 – August 1998   **Ruhr-University of Bochum**

Study of medicine

in August 1997   Physikum

in August 1998   1. Clinical State Exam

September 1998 – September 1999   **National University of Ireland, Galway**

Study of medicine

(ERASMUS)

Since October 1999   **Ruhr-University of Bochum**

Study of medicine

in August 2001   2. Clinical State Exam