Circe, Cassandra, and the Trojan Pigs: Xenotransplantation¹

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Suppose YOU NEED a transplant, a new heart or kidney. Rather than wait for an altruistic human donor to die or a living relative to volunteer a kidney, you may one day book in for elective surgery to receive an organ freshly taken from a specially bred pig. The transplant surgeon, assisted by the anti-rejection potion prepared by immunologists, represents our modern day Circe, who conjures the metamorphosis of the patient whose mind remains "as human as ever." I have found myself playing the role of Cassandra, prophesying doom largely unheeded by the surgeons.

Ideally, the source animal will be reared in specific pathogen-free conditions and should therefore be much less of an infection hazard than a "free range" human donor who might be infected by HIV or hepatitis viruses, and certainly will be carrying several kinds of herpesvirus. But certain porcine viruses, the paleontological ones, cannot be eliminated; they reside within the pigs' DNA, but like the Greeks hidden inside the Trojan horse, they may emerge once the pig tissue or organ is taken into the human body.

Xenotransplantation—the transfer of animal cells, tissues, or organs into humans—poses a number of problems: ethical, physiological, immunological, and microbiological. Nevertheless, xenotransplantation is being explored in several ways with the aim of improving human health. Bovine and porcine heart valves have been used in cardiac surgery for more than thirty years, but these valves do not represent living tissues; they are pickled first, contain very few cells, are not rejected, and do not release viruses. More recently, clinical trials of live cellular therapies have been undertaken. Fetal pig neurons inoculated into the human brain might ameliorate degenerative conditions such as

¹Read 15 November 2003.

PROCEEDINGS OF THE AMERICAN PHILOSOPHICAL SOCIETY VOL. 148, NO. 3, SEPTEMBER 2004



FIGURE 1. "Circe offered Odysseus's men a potion mixed with cheese, honey and wine. And when they had emptied their bowls, they grew pigs' heads and bristles; but their minds were as human as ever" (Homer, *The Odyssey*, translated by E. V. Rieu, Penguin Classics, 1946).

Parkinson's and Huntington's diseases (Fink et al. 2000). Cells from the islets of Langerhans of the pig pancreas may be useful in treating type 1 diabetes, since porcine insulin works in humans (Groth et al. 1994). Pig liver cells have been used extracorporeally as the equivalent of a dialysis machine in order to tide over patients with fulminant liver failure until either their own liver recovers or a human transplant becomes available (Chen et al. 1997). Whole organs from animals, however, present greater technical hurdles for controlling rejection, although genetically modified pigs may well provide the answer (Platt 2003)—and additional infection hazards (Weiss 1998).

In fact, the transfer of animal cells into humans has been practised experimentally for centuries. In the sixteenth to eighteenth centuries, sheep blood was occasionally transfused into patients (fig. 2), if only to replace the human blood extracted by the application of leeches. Samuel Pepys gives a graphic account of the treatment of a mental affliction in his diary for 1667 (Scott 2004). Fellows of the Royal Society in London "differ in the opinion they have of the effects of it; some think that it may have been a good effect upon him as a frantic man; others, that it will not have any effect at all." Pepys later reports that the treatment did the patient no harm although he was still "cracked a little in the head" and "he had but 20 shillings for his suffering it." The ethics of paying the man to undergo experimental therapy was of no concern to Pepys, only how little money he received.



FIGURE 2. A man receiving a blood transfusion from a sheep. The anatomical plates of Pietro da Cortona (1596–1669), published by Fausto Amideo, Rome, 1741.

In the United States, animal to human blood transfusion was attempted during the eighteenth and nineteenth centuries (Schmidt 1968). Such transfusions probably did more harm than good because humans have a blood group incompatibility with domestic animals that causes hyperacute rejection. In 1901, Karl Landsteiner published his Nobel Prize-winning discovery of the ABO histo-blood group incompatibilities. The ABO system comprises carbohydrate antigens on the surface of red blood cells and other tissues. Humans make natural antibodies to the blood group they do not possess. Thus a group A person makes anti-B, a group B person anti-A, and a group O person both anti-A and anti-B. We also make antibodies to a blood group called α Gal, which is expressed on the tissues of nearly all mammalian species other than Old World primates. Just as we reject all cells and tissues that express A or B if we are not compatible, so we reject animal tissues in a process whereby antibodies in conjunction with blood factors called complement destroy cell membranes (Galili 1993).

In human-to-human transplantation, most cells and tissues (other than red cells) require further tissue matching of antigens called the major histocompatibility complex (MHC) class 1 and 2. MHC antigens also differ between animals and humans, so it is remarkable that a chimpanzee kidney xenografted to a patient survived for nine months (Reemtsma 1969). MHC molecules are essential for presenting foreign antigens to the immune system, but, apart from identical twins, human individuals are so varied that it can be difficult to find a tissue match. Immunosuppressive drugs like cyclosporin A and tacrolimus help to overcome partial mismatches. These drugs have been crucial for the success of human "allotransplantation" between one person and another and would also be used in xenotransplantation. Another early attempt at clinical, whole organ xenotransplantation was conducted with livers from ABO, α Gal compatible baboons by a member of the American Philosophical Society, Dr. Thomas Starzl, and his team in Pittsburgh (Starzl et al. 1993).

Although large monkeys such as baboons and great apes such as chimpanzees would appear to be the most suitable source species for physiological and immunological compatibility, the use of chimpanzees is precluded because of their rarity (they are on the CITES list), and both kinds of primate may harbour dangerous pathogens that cannot easily be eliminated through quarantine procedures. Domesticated animals present lesser ethical and microbiological concerns; they can be sterilely delivered by cesarean section and raised and maintained in a specific pathogen-free environment. Pigs in particular are favored as donors for human transplantation; these animals provide organs of approximately human size; they are easily bred, producing large litters; and recently transgenic and cloning technology has allowed them to be genetically engineered to resist hyperacute rejection (Langford et al. 1994; Lai et al. 2002; Platt 2003). Moreover, unlike sheep and cattle, pigs are not known to harbor agents of transmissible spongiform encephalopathy (mad cow disease, scrapie) or lentiviruses related to HIV (Maedi-Visna virus of sheep, caprine arthritis encephalitis virus of goats, and bovine immunodeficiency virus of cattle). Nonetheless, all animal-to-human transplants carry some risk of disease transmission, or zoonosis, if unknown or ineradicable pathogens are carried by the donors.

Two years ago the gene encoding $\alpha(1-3)$ galactosyltransferase was "knocked out" and the cloning of a pig without the α Gal xeno antigen was announced with much fanfare (Lai et al. 2002; Phelps et al. 2003). A cartoon appeared similar to figure 3, with Pooh's comment, to which I have added Piglet's riposte. So what viruses might Piglet "have along" with him? They range from small viruses such as foot-and-mouth disease virus and parvovirus to very large ones (on the virological scale) such as African swine fever virus and porcine cytomegalovirus. Recently



Pooh: "I only have you along in case I need a transplant." Piglet: "But you don't know what I have along with me."

FIGURE 3. The changing relations between species

discovered porcine viruses include Nipah virus, which originated in fruit bats (flying foxes), infected pigs in 1998, and moved on to kill more than a hundred pig farmers in Malaysia and abattoir workers in Singapore, rather as SARS jumped from civet cats to humans two years ago in China. Although such outbreaks occurred far away from the herds bred for xenotransplantation, their capacity to move with the rapidity of human air travel was manifest in the transfer of SARS from Guangzhong via Hong Kong to Toronto in a matter of days. Even the cleanest U.S. herds of swine were found to be contaminated with two other "novel" porcine viruses. One is a relatively new pathogen of pigs, porcine circovirus type 2, whereas the other, porcine hepatitis E virus, which may infect humans (Meng 2003), is probably an ancient infection of pigs that is new to human knowledge. Being new, neither infection was initially included in the repertoire of screening tests required for testing potential donor animals.

It is the fossil viruses, however, the topic of this symposium, that have created the most concern about the infection hazards of xenotransplantation. As John Coffin has described, retroviruses can be maintained in their host populations by two quite distinct modes of transmission. The first, as in all other viruses and transmissible agents, is by infection. The second is by the virus's genes' becoming embedded or integrated in the DNA of the host's chromosomes (fig. 4). When the germ line—the cells destined to become eggs or sperm—integrate viral DNA, the latent virus gains a free ride to the next generation, and to



FIGURE 4. Replication cycle of a retrovirus. The virus particle (left) contains two RNA genomes that after entering the host cell are copied by the viral enzyme reverse transcriptase into DNA. Another viral enzyme, integrase, inserts the viral genome into host chromosomal DNA as a "DNA provirus." Progeny virus is produced by transcription of RNA from the DNA provirus. If the infected cell is in the germ line, the provirus can be passed on to the next host generation as a Mendelian set of viral genes.



FIGURE 5. Transmission of baboon and feline endogenous retroviruses. The baboon virus is transmitted vertically as an integrated provirus in baboon DNA, but it is expressed as potentially infectious virus particles in tissues such as the placenta. Infectious baboon virus was acquired by an ancestral cat and then became vertically transmitted again as a Mendelian provirus in the new host. This cross-species transmission event occurred after the evolutionary division of the genus *Felis* into tabby and spotted species, but before the speciation of the European wildcat and the ancestor of the domestic cat (Mediterranean sand cat). Adapted from Weiss (2001).

countless further generations through being inherited by the host as a Mendelian trait. We call these viral genomes endogenous retroviruses (Coffin 1982). This phenomenon has occurred in all vertebrate species studied; for example, thanks to the complete sequencing of the human genome we now realize that approximately 8% of our DNA represents the paleontological record of germ line infection.

Such a collection of fossils would be of no medical concern if they were truly dead relics, as almost all of the human endogenous retroviral genomes appear to be. But some of the endogenous retroviruses have maintained the capacity to awake, Rip van Winkle-like, and to emerge again as infectious agents. For example, baboons inherit a retroviral genome through the germ line that produces infectious particles in the placenta. This virus transferred by cross-infection to cats and in turn entered their germ line (Benveniste and Todaro 1974) (fig. 5). And, curiously, the cat retrovirus was discovered in cats only after it appeared as an actively propagating virus in a human tumor grown in a fetal cat brain as a xenograft (Coffin 1982). The two patients who received baboon livers (Starzl et al. 1993) also showed evidence subsequently of acquiring DNA sequences of the baboon endogenous retrovirus (Allan et al. 1998). Porcine endogenous retroviruses (PERV) could also re-emerge from xenografts in this way. The existence of PERV was first reported in the 1970s, but subsequently attracted little interest because they could not firmly be identified as causing any disease of importance to pig farmers or to veterinarians. An association with lymphoma was noted, but such tumors are rare in pigs, and it was not clear whether the virus plays an etiologic role in tumor formation, or whether the malignant state of the cell activates the virus. With the burgeoning interest in porcine xenotransplantation, however, Stove and Coffin (1995) sounded a warning note to the transplantation community.

When no one else appeared to take up the challenge to investigate whether activated PERV have the capacity to infect humans, I persuaded two young scientists in my laboratory, Clive Patience and Yasuhiro Takeuchi, to do just that. We soon found that pig kidney cells released PERV particles (fig. 6) and that two of three strains, PERV-A and PERV-B, could replicate in human cells in culture though not to high titers (Patience et al. 1997). The third strain, PERV-C, could not infect human cells because they lack an appropriate cell surface receptor for the virus. However, more recently, Patience has characterized a genetic hybrid virus that is mainly composed of PERV-C but has part of its envelope derived from PERV-A (Ericsson et al. 2003). This recombinant virus grows to a hundredfold high titer in human cells (table 1).

Approximately fifty copies of PERV DNA are present in the pig



FIGURE 6. Electron micrograph showing two budding particles and one mature particle of porcine endogenous retrovirus (PERV). The bar represents 120nm.

genome (Patience et al. 1997), although only a few of these copies represent full-length, potentially infectious genomes (Czauderna et al. 2000). Together with Jonathan Stoye's laboratory we observed that some of the PERV genomes are shared among all breeds of swine examined, including the Meishan breed that had been separately domesticated from wild boar in China to the European pigs (Le Tissier et al. 1997). Thus it will not be an easy task to breed swine that lack PERV sequences entirely, although it appears possible to breed pigs that do not release infectious, human-tropic PERV (Oldmixon et al. 2002; Scobie et al. 2004).

A number of investigators in the United States and Germany soon confirmed and extended our studies, demonstrating that infectious

Virus Strain	Porcine Cells	Human Cells	Titer
PERV-A	+	+	4×10^{3}
PERV-B	+	+	2×10^{3}
PERV-C	+	_	6×10^{3}
PERV-A/C	+	+	8×10^{5}

TABLE 1. Infection of cells in culture by porcine endogenous retrovirus (PERV)

Data derived from Patience et al. (1997) and Ericsson et al. (2003).

PERV is released from normal pig cells including lymphocytes (Martin et al. 1998; Wilson et al. 1998; Czauderna et al. 2000; van der Laan et al. 2000). We went on to show that the three strains of infectious PERV utilize distinct cellular receptors (Takeuchi et al. 1998) and cloned two human genes that encode receptors for PERV-A (Ericsson et al. 2003). By examining the DNA of a variety of species of the pig family (e.g., European wild boar, Asian bearded pig, African bush-pig, and wart-hog), we found that PERV genomes are present in all species of Old World pigs, but are absent from New World peccaries (Patience et al. 2001). It is likely that PERV has been inherited in the porcine evolutionary lineage for at least 7.5 million years (Tönjes and Niebert 2003). It appears remarkable that intact, infectious viruses can still emerge from pigs after such a long sojourn in the host DNA. In addition, there are at least eight sets of more distantly related retrovirus genomes in pigs (Patience et al. 2001), but these contain numerous mutations and deletions and are probably of little or no consequence for xenotransplantation; they are genuine paleontological specimens inherited as junk DNA.

The publication of our first paper on PERV in March 1997 and an accompanying commentary (Allan 1997) made a dramatic impact on the debate on xenotransplantation and on public policy. Although these warnings were no more heeded by some enthusiasts for xenotransplantation than were those of Cassandra, the perception that Trojan pigs might carry viruses into the citadel of the human body alerted regulatory agencies to the need to more closely scrutinize clinical xenotransplantation. The U.S. Food and Drug Administration (FDA) had not included retroviruses in its list of porcine pathogens to regulate xenotransplantation trials. Now it rapidly convened a workshop of its advisory committee on transplantation. In October 1997, FDA placed all ongoing clinical trials on hold until the detection of infectious PERV could be satisfactorily examined in the xenotransplantation products and could be accurately monitored in patients (Bloom 2003). Similar regulations were set in place in Europe (although several European and Asian countries seem to turn a blind eye to alternative health clinics still practising inoculation of fetal lamb cell extracts). Using sensitive PCR detection methods for PERV genomes to discern whether any of the two hundred or so patients already exposed to live pig tissues had become infected, several groups including Novartis, the CDC, and ourselves found no evidence of PERV infection in human blood samples taken from individuals exposed to live pig tissues (Heneine et al. 1998; Patience et al. 1998; Paradis et al. 1999; Pitkin and Mullon 1999; Dinsmore et al. 2000; Cunningham et al. 2001). However, the frequency of infection by endogenous murine leukemia virus in human



FIGURE 7. Transmission of endogenous retroviruses between xenograft and host. Left, human tumor xenografts grown in immunodeficient mice occasionally become colonized by a host-to-graft infection by xenotropic murine leukemia virus (MLV-X). Right, PERV in a normal porcine xenograft might conversely transfer to the human host by graft-to-host infection, but this has not yet been observed in more than two hundred patients exposed to porcine tissues.

tumor xenografts grown in immunodeficient BALB/c mice is approximately 1%. Thus there is no room for complacency over the possibility of human infection, because if a host endogenous virus can infect human xenografts transplanted into animals, surely animal xenografts could be a source of infection to the human host (fig. 7).

What can we do to lessen or eliminate the likelihood of PERV colonizing humans via clinical xenotransplantation? The lack of evidence of human infection thus far shows that PERV is not highly contagious. However, HIV-1 took decades to get going as an epidemic infection, and there is evidence that zoonotic transfer of a coronavirus related to SARS has occurred frequently among civet cat handlers, but has spread alarmingly from person to person on only one occasion thus far. Accordingly, we must remain vigilant, and thanks to studying PERV, sensitive diagnostic reagents and tools are available to detect PERV in patients. We have also tested a number of existing anti-retroviral drugs already licensed for human use in order to treat HIV infection, but only one, zidovudine, effectively inhibits PERV at pharmacological doses (Qari et al. 2001). It should be feasible to develop a PERV vaccine if it were deemed desirable.

Are we making too much fuss about the infection hazards of xenotransplantation? My own view is similar to that of the FDA and the UK Xenotransplantation Interim Regulatory Authority, namely, that clinical xenotransplantation should indeed be encouraged to proceed, but only under carefully monitored conditions. For some, however, that is like closing the stable door after the horse has bolted. Following our first report on PERV infecting human cells in culture (Patience et al. 1997), Fritz Bach and colleagues concluded that the public were not yet ready for clinical xenotransplantation and proposed a moratorium while the debate matured (Bach et al. 1998). For others, such a proposed ban was considered overkill; the head of research and development for Novartis, P. Herrling, was quoted as saying, "Animals have transmitted viruses to humans throughout history. The added risk of xenotransplantation might be minimal" (Butler 1998). This is correct in that most epidemic viral diseases of humans, such as smallpox, measles, yellow fever, and, more recently, AIDS, West Nile encephalitis, and SARS, indeed originated naturally from animal-to-human infection (Weiss 2001). But I would argue that the risk of zoonosis is bound to increase if the physical barrier is removed by deliberately implanting animal tissues in patients, and by treating such patients with immunosuppressive drugs.

I would, moreover, add a further risk that is specific to xenotransplantation technology. The genetic modification of pigs allowing their tissues to appear less foreign to the human body in order to ameliorate hyperacute rejection will also coincidentally enhance the chance of infectious transmission of viruses (Rother and Squinto 1996). This increased risk derives from two separate points (Weiss 1998). One is that the mechanism of hyperacute rejection may have evolved to serve as a natural protection against enveloped viruses of animals. Both "knockout" pigs and transgenic pigs can release enveloped viruses that are relatively resistant to this type of innate immunity in humans (Takefman et al. 2002; Quinn et al. 2004; Magre et al. 2004). The other is that complement regulatory proteins such as decay-accelerating factor (CD55/DAF) and CD46 that down-regulate hyperacute rejection also happen to serve as cell surface receptors for viruses. If pigs are bred to express the human genes for such receptors, porcine viruses may take to using the human form of the receptor and thus become adapted to spread amongst humans. For example, CD55/DAF can act as an entry portal for small RNA viruses known as Coxsackie B viruses, which cause myocarditis. Swine vesicular disease is caused by a virus closely related to Coxsackie B5; if this porcine virus began to use human CD55/DAF as a receptor in transgenic pigs, the frequency of crossspecies transfer might increase. Similarly, CD46 can act as a receptor for morbilliviruses such as measles, and also for certain strains of herpesvirus and adenovirus. The ingenious immunologists who developed transgenic pigs for xenotransplantation were simply unaware that complement regulatory proteins are also virus receptors (Weiss 1998).



FIGURE 8. Assessing the ethics and safety of xenotransplantation

Thus there is much to ponder on the ethics and safety of xenotransplantation (fig. 8). Will society regard xenotransplant recipients as dangerous lepers and demand that they live in quarantine? For how long after xenotransplantation will they need to be monitored for infection? Can one require that their intimate partners also be tested for porcine infection? To what extent should the precautionary principle override the opportunity to make progress in medicine through advancing technologies? While surgeons tend to weigh concern about the infection hazard in xenotransplantation as a risk-benefit equation calculated for the individual patient, it behooves us to take the broader view and attempt to balance risk-benefit to the community at large (Bach et al. 1998; Chapman 2003). Although the likelihood of epidemic spread of a porcine virus appears even more remote than an individual patient's acquiring infection, the consequences would be more drastic. Surely we need a Hippocratic oath for public health that would minimize harm to the community resulting from the treatment of individuals? Overall, one can sum up xenotransplantation with the same aphorism that Joshua Lederberg applied to the debate on recombinant DNA technology at Asilomar nearly thirty years ago: it holds certain promise and uncertain peril.

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