OUTLINE OF A RISK ASSESSMENT: THE WELFARE OF FUTURE XENO-DONOR PIGS

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Abstract *Animal Welfare 2003, **12**: 219-237*

The welfare of transgenic animals is often not considered prior to their generation. However, we demonstrate here how a welfare risk assessment can be carried out before transgenic animals are created. We describe a risk assessment identifying potential welfare problems in transgenic pigs generated for future xeno-donation of organs. This assessment is based on currently available information concerning transgenic animal models in which one or more transgenes relevant to future xeno-donation have been inserted. The welfare risk assessment reveals that future xeno-donor pigs may have an increased tendency toward septicaemias, reduced fertility and/or impaired vision. The transgenic animal models used in generating hypotheses about the welfare of xeno-donor pigs can also assist in the testing of these hypotheses. To ensure high levels of welfare of transgenic animals, analogous risk assessments can be used to identify potential welfare problems during the early stages of the generation of new transgenic animals. Such assessments may form part of the basis on which licenses to generate new transgenic animals are granted to research groups.

Keywords: *animal welfare, organ donor, risk assessment, transgenesis, xeno-donor, xenotransplantation*

Introduction

In response to the shortage of human organs for allotransplantation, substantial efforts have been made over the last decade to develop transgenic donors for animal-to-human xenotransplantation. The aim has been to overcome obstacles to xenograft rejection by developing animal donors carrying various transgenic modifications that render their organs compatible with the human immune system. However, the reduction of xenograft rejection should not be the only area of interest in the development of new transgenic animals. Optimisation of animal welfare can also be an important target of research, as we shall explain here.

Xenotransplantation technology holds the promise of life-saving transplantations for tens of thousands of people in need of organs (UNOS 2001; Eurotransplant 2001; ITCS 2001), but the public is rather critical of the technology. Xenotransplantation raises several ethical issues (Olsson 2000; Vanderpool 1998) and these have a major effect on the public's opinion

of the technology. This is clearly shown by a survey of European attitudes toward modern biotechnologies conducted in 1996 in which, out of five applications of modern biotechnology, xenotransplantation was the least supported (Durant *et al* 1998). Despite the fact that 51% saw it as useful, only 15% definitely agreed and 23% tended to agree that the technology should be encouraged (Figure 1). The survey indicates that low levels of support for xenotransplantation result from its being perceived as morally unacceptable and/or risky: more than half of those asked (53%) found the technology to some extent morally unacceptable, and a similar proportion (59%) assessed the technology as risky. Support for transgenic animal production is also limited. Public opinion on xenotechnology and the production of transgenic animals differs from that on genetic technologies applied for medical purposes, such as gene testing and medicine production. The latter are the most enthusiastically supported of the six applications of biotechnology assessed in the survey.

Some of the scepticism about xenotransplantation can be explained by the fact that xenotransplantation technology requires transgenic animals to be used. As noted above, the production of transgenic animals is among the least supported applications of modern biotechnology, and the technology also shares a low level of moral acceptability (Durant *et al* 1998). It is obvious that the development and refinement of xenotransplantation technology has involved, and will continue to involve, the use of animal experimentation, and this is also likely to affect public attitudes to it, since such experimentation is generally viewed critically (Pocard 1999). Public hostility to xenotransplantation was investigated further in a series of focus-group interviews carried out in Denmark during 1999 and 2000[1].

An overview of the most frequent arguments about xenotransplantation expressed in the interviews appears in Table 1. It is noteworthy that moral considerations dominate the critical arguments, those relating to what could be called 'human interference with nature' being the most prevalent. This argument could be interpreted as a first reaction to a complicated new technology with consequences that cannot readily be foreseen. Critical arguments relating to the technical or biological risks of xenotransplantation (eg xenozoonoses) were few, although this does not necessarily indicate that such risks are considered less important. More probably, it indicates the current unawareness of such risks by the public. Most arguments about xenotransplantation revolve around the fact that the targets of the organs are humans; therefore, disputes often reflect general debates about human-to-human allotransplantation. Only two participants addressed animal welfare issues. One argued that xenotransplantation violates animal rights because animals, reduced to suppliers of organs, are ranked lower than humans. The other participant argued that the technology threatens the welfare of the animals used for producing spare-parts for humans.

One of the most striking findings of the Danish focus-group interviews was that only two of the six groups took up the issue of xenotransplantation on their own initiative. This indicates that xenotransplantation is not seen as being closely related to biotechnology/genetic engineering. It also indicates that, so far, xenotransplantation is not a

[1] Six focus-group interviews were conducted between September 1999 and April 2000. Six to nine individuals participated in each group, having been recruited in such a way that a variety of socio-economic and demographic backgrounds were represented. During the interviews, participants were asked to evaluate selected uses of gene technology in food and non-food areas. Conceptions of risk and ethical concerns, including the benefits of gene technology, were the foci. The interviews were carried out as part of the EU project 'Life Sciences in the European Public', by the Danish Team: Professor Arne Thing Mortensen (Roskilde University), Assistant Professor Erling Jelsøe (Roskilde University), Mercy Wambui Kamara and Assistant Professor Jesper Lassen (The Royal Veterinary and Agricultural University).

prominent issue in the public debate. This probably reflects the fact that xenotransplantation has not been given the same attention in the media as other uses of genetic engineering, such as food biotechnology and other medical issues. Thus, it is obvious that the consideration of animal welfare is not yet an issue in xenotransplantation. Given that discussions of the welfare of laboratory and transgenic animals are already receiving broad attention, however, it is likely that the intensified focus on the welfare of animals used for human purposes will also eventually cover the welfare of xenotransplantation donor pigs. Interest in the subject of animal welfare in xenotransplantation may also be brought forward by non-governmental organisations (CRT 2001).

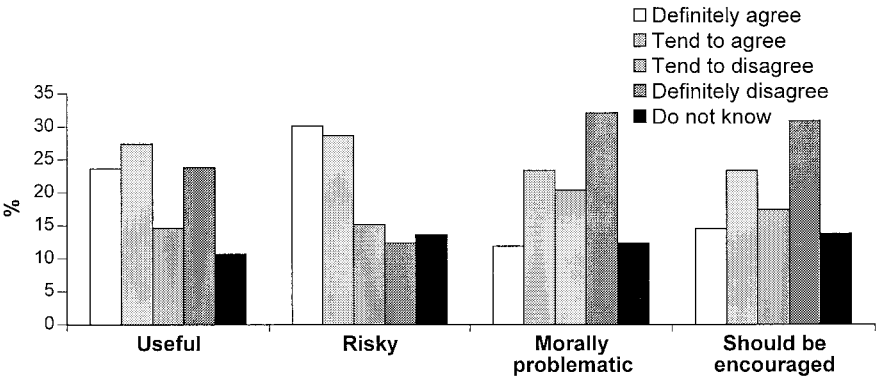

Figure 1 **Attitudes to xenotransplantation in Europe. In a major international Eurobarometer survey conducted in 1996, the European population was found to be critical of xenotransplantation when asked about its usefulness, its risks, its moral acceptability and whether the introduction of human genes into animals (eg pigs) to produce organs (eg hearts) for human transplants should be encouraged. Although 51% found the technology useful to some extent, only 38% tended to agree or definitely agreed that it is a technology worth encouraging (Durant *et al* 1998).**

Table 1 **Arguments about xenotransplantation put forward in a series of focus-group interviews on attitudes to modern biotechnology during the winter of 1999–2000.**

Critical	Interference with nature	Xenotransplantation is unacceptable because it is in conflict with natural functions/processes in the body.
	Slippery slope	Xenotransplantation is a step down a slippery slope.
	Animal welfare	Xenotransplantation is unacceptable because it violates the rights of animals and/or causes suffering to the animals.
	Unknown consequences	Xenotransplantation is unacceptable because we don't know the consequences.
	Alternatives	Xenotransplantation is wrong because there are alternatives.
Positive	Progress	Xenotransplantation is an expression of progress, and progress is fundamentally good.
	Relief	Xenotransplantation is a means to handle the shortage of organs and thus to relieve suffering.

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It therefore seems appropriate at this point — before the actual xenograft donor has been developed — to investigate the impact of transgenesis on the xeno-donor animal. For this purpose, risk assessment techniques may be introduced as a means of evaluating potential reductions of animal welfare. There are at least two ways to perform a welfare risk assessment on an as-yet non-existent xenograft donor animal. One is to assess the consequences of transgenesis using existing theoretical knowledge of the transgenes, and, on this basis, to develop new hypotheses with respect to animal welfare that can be experimentally tested on the transgenic animal in question. Another way is to base the risk assessment on currently existing models of future transgenic-donor animals, such as transgenic α-gal-deficient knockout mice, which are fully or partly transgenically equivalent to future xeno-donor pigs, or mice and pigs transfected on the key loci involved in rejection of xenografts (see later). In this way, important information on the welfare of future xeno-donor animals can be acquired and potential problems can be identified, which will help to ensure that recommendations securing a high level of animal welfare are put forward.

The second approach to the evaluation of animal welfare can be applied, not just to future xeno-donor pigs, but more widely. To date, the development of transgenic animals has in general preceded any discussion of the animals' welfare. It would be reasonable to reverse this order and, prior to the development of any transgenic animal, to make a welfare risk assessment by collecting all available data on potential consequences of the particular transgenes vis-à-vis health, physiology and behaviour. The following risk assessment of the likely welfare status of future xeno-donor pigs is therefore also intended as a role model for other welfare assessments that precede the development of transgenic animals. Such assessments can be carried out by adapting a model of risk assessment that has been applied in connection with food safety or environmental hazards. In this context, the assessment will involve three elements: first, the identification of hazards in relation to the welfare of future xeno-donor pigs; second, a preliminary evaluation of the impact of the identified problems; and third, a strategy for investigating the identified risks. The risk assessment will provide a more solid basis for identifying and solving potential problems relating to the welfare of xeno-donor animals. It may also generate new hypotheses concerning what may be expected of the xeno-donors not only regarding the welfare of the animals but also regarding other aspects of their biology. Decisions regarding the welfare of future xeno-donor animals may be based on scientific risk evaluations, but they should also involve ethical reflection in which different approaches to the idea of animal welfare are recognised. Indeed, the identification of potential risks calls for an ethical evaluation, because this judgement itself reflects an ethical point of view. It therefore seems important to discuss what the idea of animal welfare involves and how the welfare of animals involved in xenotransplantation technology may be affected.

Any discussion of animal welfare will need to explore different ideas of welfare if it is to be relevant to the public debate. A strictly scientific definition of welfare will often fail to provide answers to the questions raised (Stafleu *et al* 1996; Tannenbaum 1991). The questions asked by society about the way we treat animals will eventually involve an ethical debate about our duties towards animals and an ethical evaluation of what is good for the animal — in other words, of what animal welfare is. Conceptions of animal welfare currently in circulation in society need to be reflected in the notion of animal welfare used by scientists and animal welfare researchers (Fraser *et al* 1997). It is often much easier to agree on what constitutes bad rather than good animal welfare. Most people will agree that welfare problems are present in sick and starved animals and those in pain. The presence of healthiness, and the lack of sickness, pain and suffering, could therefore characterise positive

animal welfare. Health can be measured by qualitative and reproducible measurements of physiological parameters, clinical appearance, reproductive abilities and behaviour. However, this does not encompass all aspects of animal welfare, for these parameters have to be compared to a reference set of values that is not automatically given. For instance, reproductive capacity could be much higher in captive animals than in wild animals, but even though the parameter has been agreed upon as a reliable means of welfare measurement, it would not necessarily be agreed that captive animals experience higher levels of welfare. Hence, before choosing measurements and reference values, it is necessary to have an idea of animal welfare itself — in other words, to describe welfare — in order to know what to look for.

Animal welfare can be described in at least two very different ways (Appleby & Sandøe 2001). One approach is to think of the animal's opportunity to realise its various potentials as proportional to its level of welfare. For instance, realising the potential to reproduce is, from this point of view, essential to a good animal life. The various biological processes taking place in wild animals are expressions of the potentials of the particular animals. Captive animals need to be offered the same opportunity to realise their potentials if high levels of welfare are to be assured. This also implies that sickness in wild animals does not necessarily equate to low levels of welfare. Realising potential, or 'flourishing', is what matters from what may be called a 'perfectionist's' point of view. With regard to transgenic animals, different perfectionist views can be taken. Some may feel that if the transgenic animal does not have the same potentials as the original animal, its welfare will be reduced. Others may think that the transgenic animals are novel varieties of animal, and that the newly formed potentials of these animals are what matter (Rollin 1995).

The 'hedonistic' idea of animal welfare draws attention to what are thought to be good or bad experiences of the animal (Duncan 1996). Negative experiences quite obviously include pain and suffering. Some activities, such as playing and eating, will evoke feelings that are positive. According to the hedonist, the aggregate 'weighting' of positive and negative feelings in the life of an animal forms the basis for a welfare evaluation. This hedonistic approach to welfare, however, has certain disadvantages. Weighing positive and negative feelings against each other is difficult. For instance, the experience of eating may be more positive when an animal is hungry, but how hungry does the animal need to be in order to keep the balance on the positive side?

Thus, anticipating that ethical issues regarding animal welfare will have a major effect on public opinion about xenotransplantation, it is important to recognise the existence of different views of animal welfare and to incorporate this recognition into any further research of xenotransplantation. Investigations of the impact of xenotransplantation on the animals involved should also be an area of intensified focus, and research in this area should be sensitive to the ethical context of these potential problems.

Xeno-strategies

Impetus for developing a transgenic donor of organs

Transplantation between discordant species may give rise to a hyperacute rejection (HAR). HAR occurs within minutes or hours. By contrast, transplantation between concordant species may cause a delayed xenograft rejection (DXR). DXR occurs over several days (Chen *et al* 2000). Man is concordant with Old World primates but discordant with all other animals. The pig has, however, been preferred as a potential donor of organs for several reasons. Amongst these is the availability of the pig, in comparison with the endangered

status of many primates. Their high rate of reproduction in captivity and our extensive knowledge of their husbandry are also reasons to favour pigs as potential donors. Furthermore, the risk of transmission of xenozoonotic diseases from primates to humans is considered greater than that of transmission from pigs to humans (Julvez *et al* 2000). Finally, the use of primates for xenotransplantation purposes may pose ethical problems. Such use of pigs may be more acceptable (Nuffield Council on Bioethics 1996).

However, pigs and humans are discordant species, and thus pig organs will evoke HAR if transplanted into the human body. An antigen known to activate HAR via the classical pathway is the α1,3-galactosyl epitope (α-gal) (Galili *et al* 1988). The effect of this epitope may be reduced or eliminated by various transgenic approaches (Table 2). Currently, the most common methods for transgenic modification of animals are microinjection of DNA into the cell, thereby adding genes to the genome (Gordon *et al* 1980), and targeted mutation in embryonic stem cells, allowing genes to be removed from the genome (Gossler *et al* 1986). Transgenic modifications through targeted mutation have only recently become available in pigs by means of cloning techniques. Using this method, four heterozygous α-gal knockout pigs have been produced (Lai *et al* 2002). It is still not clear whether a homozygous α-gal knockout pig will be viable. From the limited data on the four heterozygous pigs, it has not been possible to deduce any information on the impact of the gene knockout on the pigs' welfare. The microinjection technique is available in both mice and pigs and has been used to introduce genes coding for complement-regulatory factors (CRFs), elements that identify the tissue as homologous with the complement system (Table 3). The elimination of the α-gal epitope by targeted mutation will probably reveal the impact of other HAR-related xeno-antigens (Cooper 1998). Consequently, the most likely future xenograft-donor is a multiple-knockout pig with some CRF genes inserted into its genome.

Table 2 **Transgenic animals developed for xenotransplantation research. MI, microinjection technique; ES, embryonic stem cell technique; CRF, complement regulatory factor; KO, knockout.**

Transgene			Mouse		Pig	
Full name	*Abbreviation*	*Function*	*Transfection method*	*Reference*	*Transfection method*	*Reference*
Functional genes						
Human decay accelerating factor	H-DAF CD55	CRF Inhibition of C3 →C5	MI	van Denderen *et al* 1997	MI	Rosengard *et al* 1995
α1,2-fucosyl-transferase (H-transferase)	HTF	Competitive H-substance synthesis	MI	Chen *et al* 1996	MI	Koike *et al* 1996
CD59	CD59	CRF Inhibition of C6 →C7 → C8 →C9	MI	Cowan *et al* 1996	MI	Fodor *et al* 1994
Human membrane cofactor protein	MCP CD46	CRF Inhibition of C3 →C5	MI	Ijzermans *et al* 1996		
α1,3-galactosidase	α1,3-GT	Degalactosi-dation of α-gal epitope	MI	Ikematsu *et al* 1993		
Knockout genes						
α-galactosyl-transferase (gal-transferase)	α-gal KO	Lacking α-gal epitope synthesis	ES	Tearle *et al* 1996; Thall *et al* 1995	Knockout technique not available	

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Table 3 **Combined transgenic animals used in xenotransplantation research. KO, knockout (homozygotic).**

Transgene combinations	Method	Mouse	Pig
α-gal KO × H-DAF	Cross-breeding	van Denderen *et al* 1997	-
α-gal KO × CD59	Cross-breeding	Costa *et al* 1999	-
α-gal KO × HTF	Cross-breeding	Costa *et al* 1999	-
α-gal KO × HTF × CD59	Cross-breeding	Costa *et al* 1999	-
CD 59 × H-DAF	Microinjection	Cowan *et al* 1998a	Byrne *et al* 1997
CD 59 × HTF	Cross-breeding	Costa *et al* 1999	-
CD 59 × H-DAF × HTF	Microinjection	—	Cowan *et al* 2000

Removal of the α-gal epitope by knockout of the α-gal transferase gene

As some mammalian species, such as humans and Old World primates, naturally lack the α-gal epitope, life is obviously possible without it. However, it is not clear whether animals without the α-gal epitope possess complementary and compensatory epitopes. Two research groups have reported that they have successfully produced α-gal knockout mice (Tearle *et al* 1996; Thall *et al* 1995) by knocking out the gene for the α-gal-forming enzyme, α-gal transferase, using targeted mutation. Apart from the cataracts described below (Tearle *et al* 1996), the homozygous α-gal knockout mice seem to have the same size, appearance and clinical chemistry as their wild-type litter mates, and their organs do not seem to differ macroscopically or microscopically (Tearle *et al* 1996; Thall *et al* 1995). In a colony of α-gal knockout mice, observations over two years showed that the mice had normal health status and life spans (Pearse *et al* 1999). Studies of these mice have so far provided very little information on any welfare problems (LaTemple & Galili 1998).

Cataracts

In one evaluation of α-gal knockout mice it has been reported that the mice develop cortical cataracts associated with significant membrane leakage at the age of 4–6 weeks (Tearle *et al* 1996). No such changes are reported by Thall *et al* (1995). The impact of cataracts on animal welfare has not yet been investigated. It is also unknown whether α-gal knockout pigs will suffer from the same type of cataracts as α-gal knockout mice. Cataracts have previously been found in Australian sows with a prevalence range 8–40%. However, an examination of two of these sows revealed normal levels of gal-transferase (Cargill *et al* 1983). Weakening of the lens capsule in the α-gal knockout mice is probably the cause of the cataracts. The lens capsule of the pig is thin (El-Bab *et al* 1982) and it may therefore be suspected that pigs are prone to cataract development. Visual impairment is likely to result from cataracts. The vision of mice is poor, whereas their sense of smell is very well developed (van der Meer *et al* 1996), olfaction being used to detect food and predators as well as for social organisation. In mice, therefore, cataracts may not have a major impact: mice already depend more on olfactory than on visual cues, and they may be able to compensate for reduced visual ability. Like mice, pigs are social animals with a highly developed sense of smell; they

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also appear to depend less on vision (eg blinding pigs with contact lenses had no effect on the hierarchy formation in a group of pigs [Ewbank 1985]). However, pigs appear to discriminate between a familiar handler and a non-familiar person primarily on the basis of visual cues (Koba & Tanida 1999). Furthermore, pot-bellied pigs with visual impairment caused by accumulation of hypertrophied periocular fat have been observed becoming more aggressive and displaying fear–biting behaviour as their vision deteriorates (Andrea & George 1999). When their vision is restored through a surgical procedure, the pigs become less aggressive and socialisation with humans improves (Andrea & George 1999). If vision gradually becomes impaired in xeno-donor pigs, and if this leads to increased aggressiveness, this may very well represent a welfare problem. It therefore seems appropriate to investigate the nature of cortical cataracts and the presence of the α-gal epitope in the pig lens, so that the likelihood of cataracts developing in α-gal knockout pigs can be predicted. Also, it is important to investigate the effect of cataracts on the behaviour and welfare of α-gal mice in order to anticipate whether the knockout will lead to welfare problems in α-gal pigs.

The α-gal epitope in the process of fertilisation

The α-gal epitope is situated on the zona pellucida of normal mouse oocytes, and spermial transferases capable of producing the epitope play an important role in the attachment of sperm to the zona pellucida (Shur & Hall 1982a,b). The binding of spermial gal-transferases to *N*-acetylglucosamine residues in the zona pellucida, which releases inhibitors of binding of other capacitated sperm, is an important capacitation step that triggers several other reactions relating to final penetration (Shapiro & Eddy 1980). Difficulties in producing homozygotic α-gal knockout animals are therefore to be expected, as α-gal-deficient sperm may not be able to bind to the zona pellucida. One research group does report that matings between heterozygous α-gal knockout mice do not result in the expected 1:2:1 ratio (ie the transmission of the knockout allele to the offspring is mildly but significantly reduced [Tearle *et al* 1996]). A potential reduction in fertilisation rates is therefore to be taken into account when evaluating α-gal knockout animals. However, extensive variation is known to exist between species in capacitation and acrosome reactions. As the impact on fertility occurs at oocyte–sperm adhesion level (Tearle *et al* 1996), no pain or distress can be said to be involved in reduced fertility. Thus, this fertility reduction does not give rise to welfare problems in the hedonistic sense. However, reduced ability to reproduce may pose welfare problems when considered from a perfectionist's point of view: it may hamper transgenic animals in realising their full reproductive potential.

Increased sensitivity to sepsis

The depletion of α-gal epitopes is associated with the presence of anti-gal antibodies. These occur naturally in humans and in α-gal knockout mice. In humans, immunoglobulin G (IgG) antibodies against the α-gal epitope account for up to 1% of total circulating IgG antibodies (Galili *et al* 1984). These antibodies probably derive from a reaction to members of the intestinal flora, especially *Enterobacteriaceae* spp., but other types of infectious agent also possess the α-gal epitope as a structural element in their cell walls (Galili *et al* 1988; Table 4). It is interesting that α-gal antibodies in human blood do not seem to initiate immune-mediated lysis of α-gal epitope containing bacteria. Nor do they provide a protected site for complement factor C3 deposition. On the contrary, these human antibodies seem to protect the bacteria against lysis via the alternative complement pathway. This is also consistent with the finding that *Enterobacteriaceae* cultivated from blood derived from septic human patients bind anti-gal antibodies more frequently than *Enterobacteriaceae* isolates

from the faeces of healthy humans (Hamadeh *et al* 1992). Furthermore, bacteria isolated from the gallstones of patients with sepsis have been shown always to express the α-gal epitope (Wetter *et al* 1994). There are major differences in the level of expression of the α-gal epitope, even between bacteria of the same genus. In certain bacteria, such as *Escherichia coli* O86, the epitope is situated in the capsule or glycoprotein portion of the bacterial wall (Galili *et al* 1988). In other bacteria, such as *Neisseria meningitidis*, it is situated on the pili (Hamadeh *et al* 1995). The epitope is here continuously exposed as an antigen, but in yet other bacteria, such as *Klebsiella* strain 18022, it is situated under the capsule in a state that is probably not directly accessible to an antibody (Galili *et al* 1988). The α-gal epitope is also expressed by some viruses, such as the human papilloma virus responsible for invasive cervical carcinoma in women (Tremont-Lukats *et al* 1997). The expression of the epitope probably depends on the gal-transferase-production capability of the cells of origin. For example, retroviruses produced from human cells are resistant to inactivation by human complement. On the other hand, α-gal antibodies *do* react with retroviruses produced in porcine cells expressing porcine gal-transferase (Takeuchi *et al* 1996) and with viruses produced in human cells manipulated into expressing gal-transferase (Reed *et al* 1997). The same phenomenon can be shown with the Eastern equine encephalitis virus (Repik *et al* 1994).

Table 4 Microorganisms known to express the α-gal epitope.

Species	References	Infecting Humans	Infecting Pigs
Bacteria			
Enterobacteriaceae			
Citrobacter spp.	Wetter *et al* 1994	+	+
Enterobacter spp.	Wetter *et al* 1994	+	+
Escherichia coli	Galili *et al* 1988	+	+
Klebsiella spp.	Galili *et al* 1988	+	+
Salmonella spp.	Galili *et al* 1988	+	+
Serratia spp.	Galili *et al* 1988	+	+
Enterococcus spp.	Wetter *et al* 1994	+	+
Neisseria meningitidis	Hamadeh *et al* 1995	+	–
Pseudomonas spp.	Wetter *et al* 1994	+	+
Parasites			
Leishmania (American)	Towbin *et al* 1987	+	–
Trypanosoma cruzi	Gonzalez *et al* 1995	+	–
Trypanosoma rhodesiense	Towbin *et al* 1987	+	–
Viruses			
Eastern equine encephalitis virus	Repik *et al* 1994	+	–
Human influenza virus	Galili *et al* 1996	+	–
Human papilloma virus	Tremont-Lukats *et al* 1997	+	–
Retroviruses type C	Takeuchi *et al* 1996	+	+

If α-gal antibodies are capable of protecting *Enterobacteriaceae* spp. from complement-mediated lysis in α-gal knockout pigs, this could pose a problem in colonies of such pigs. Septicaemias caused by *Enterobacteriaceae* spp. are among the most common causes of death in pig herds, and may occur in both piglets and young pigs (Bertschinger & Fairbrother 1999). As many as 2% of the population may die from this (Cutler *et al* 1999). *E. coli* is the most frequent cause, but another α-gal epitope expressing bacterium, *K. pneumoniae*, is also a common cause (Glastonbury 1977; Nielsen *et al* 1975). In the pig, all components of both

the native and the acquired immune system develop *in utero* and are functional, although less than fully efficient, at birth (Hammerberg *et al* 1989). Hence, an inability to lyse septicaemic bacteria by complement may increase the incidence of septicaemias dramatically. Septicaemia is associated with fever and various secondary infections such as meningitis, arthritis and serositis. An increase in susceptibility to septicaemia may therefore be associated with reduced welfare. Various methods of preventing septicaemia are available. However, it is very unlikely that even advanced barriers known from laboratory animal production will offer effective protection from *Enterobacteriaceae*, as these bacteria are present in the intestines of all — even barrier-protected — mammals (Hansen 1992; Hansen 1998). If pigs are to be kept free from *Enterobacteriaceae* is it more likely that isolation units will be used. Husbandry of this sort will have an impact on animal welfare, because pigs are social animals that benefit from contact with other pigs. Keeping pigs individually will reduce animal welfare from both a perfectionist's and a hedonist's point of view.

Increased sensitivity to autoimmune disease

Elevated titres of α-gal antibodies have been found in human patients suffering from diseases with autoimmune elements (Table 5). This might be a result of exposure to cryptic α-gal epitopes that are present in the cells of certain tissues, such as the thyroid gland (Etienne-Decerf *et al* 1987). The thyroid cells of human beings and other human cells express only low levels of the α-gal epitope, which could indicate that the supposed autoimmune reaction is an artefact caused by α-gal epitopes present on thyroid tissues used for bioassays (Thall *et al* 1991a). Alternatively, the binding of α-gal antibodies to the epitope on the surface of thyroid cells may lead to the rearrangement and subsequent increased expression of the epitope (Thall *et al* 1991b). The interaction of bacterial wall components and α-gal antibodies may contribute to inflammatory processes, and these, in addition to a reaction to invading bacteria, may result in damage to the human tissue. Interaction of this kind is seen when fragments of *E. coli* O86 adhere to normal fibroblasts, thereby mediating the binding of α-gal antibodies to their surface (Galili *et al* 1988). Galili *et al* (1988) also hypothesise that gal-transferase present in α-gal-positive bacteria may initiate the expression of the normally suppressed α-gal epitope on human cells, thereby exposing the antigen to the α-gal antibodies. α-gal antibodies have been implicated in the clearance of senescent human erythrocytes. They might be synthesised on senescent human erythrocytes by gal-transferases of bacterial origin translocated into the circulation during commensal colonisation of the gut by gram-negative bacteria. For instance, *Klebsiella pneumoniae* synthesises at least four gal-transferases capable of adding an α-gal epitope to human cell surface acceptor structures. Three of these may form α-gal structures on human erythrocytes that bind α-gal antibodies, thereby creating 'autoimmune' senescence-associated epitopes (Hamadeh *et al* 1996). Elevated titres of α-gal antibodies have also been found in rheumatoid arthritis patients with kidney damage after treatment with gold and/or D-penicillamine (Malaise *et al* 1986). It is not clear whether α-gal antibodies are involved in the development of autoimmune diseases; raised titres may simply be an artefact. Where this is not the case, they may be a non-aetiological symptom. With few exceptions, such as isoimmune purpura thrombocytopenia in piglets (Nielsen *et al* 1973), autoimmune diseases have not generally been described in short-lived meat-production pigs. Even if the typical meat-production pig lived longer, autoimmune disease would probably occur too seldom and in too sophisticated a manner to attract further attention. Further studies are therefore needed, both to clarify the reality of the problem and to assess whether autoimmune disease is likely to occur in the pig at all. The

Table 5 **Human autoimmune diseases known to correlate with raised titres of α-gal antibodies.**

Name of disease	Disorder	References
Chagas' disease	Infection with *Trypanosoma cruzi* leading to eg autoimmune endocarditis	Avila *et al* 1988
Rheumatoid arthritis	Chronic inflammatory reaction against synovial joints	Malaise *et al* 1986
Henoch-Schönlein purpura	Small vessel vasculitis in children	Davin *et al* 1987
IgA nephropathy	Mesangial glomerulonephritis with predominant IgA deposits	Davin *et al* 1987
Grave's disease	Autoimmune thyroid dysfunction	Etienne-Decerf *et al* 1987

potential impact of such disease on animal welfare is heavily dependent on the nature of the disease, and generalisations about it are therefore unsound.

Given the fact that species-specific differences in the α-gal epitope do exist, it is important not to extrapolate results obtained in one species to another without careful consideration. The differences can be exemplified by the different patterns of α-gal expression in pigs and mice (Tanemura & Galili 2000) and the different levels of α-gal antibodies produced in naive α-gal knockout mice and primates (Latemple & Galili 1998). Moreover, several cases of different phenotypes resulting from identical genetic manipulations in animals of different genetic backgrounds (Doetschman 1999) emphasise the importance of sound analysis of species-specific differences.

Transformation of the α-gal epitope by the H-antigen and H-transferase

Instead of being knocked out, the α-gal epitope could be transformed by inserting a gene coding for an enzyme that processes the epitope molecule. The H-transferase enzyme produces the H-antigen by fucosylation of *N*-acetyl-lactosamine. The H-antigen may be further synthesised into A- or B-substance, ie the human ABO blood types (Table 6). While humans and higher primates are fully devoid of the α-gal epitope, lower primates and non-primate mammals are not fully devoid of A-, B- and H-antigens. Because these animals possess in their endodermal cells another fucosyl transferase, Se-transferase, A- B- and H-antigens are found in their ectodermal and endodermal (but not vascular endothelial) cells and in their erythrocytes (Oriol *et al* 1993). Introducing H-transferase into, for example, a pig may therefore not involve the introduction of a totally new structure. The pig will probably, like humans, be immunotolerant to the H-antigen, and natural antibodies to the H-antigen are unlikely to occur in transgenic or wild animals, although the bacteria giving rise to antigenic stimulation contain this structure as well as many other structures.

Little is known about the function of the H-antigen. The presence of structures of the human blood groups, including the H-antigen, may correlate with the development of diseases of an infectious or oncological nature (Garratty 1995). The H-transferase gene has been introduced using microinjection techniques into both mice (Sharma *et al* 1996) and pigs (Koike *et al* 1996). In the Golgi apparatus of these transgenic animals the α-gal-transferase enzyme and the H-transferase enzyme should compete for the substrate *N*-acetyl-lactosamine. Cells from H-transferase transgenic mice as well as from H-transferase

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Table 6 **Formation of the α-gal epitope on endothelial cell walls and the formation of blood group antigens on human cell walls.**

	All mammals	All mammals other than humans and Old World primates	Humans and Old World primates	Humans with blood type A	Humans with blood type B	Humans with blood type O
Compound	*Gal β1,4 GlcNAc-R N-acetyl-lactosamine*	*Gal β1,4 GlcNAc-R N-acetyl-lactosamine*	*Gal β1,4 GlcNAc-R N-acetyl-lactosamine*	*Gal β1,4 GlcNAc-R N-acetyl-lactosamine*	*Gal β1,4 GlcNAc-R N-acetyl-lactosamine*	*Gal β1,4 GlcNAc-R N-acetyl-lactosamine*
↓						
Enzyme		*α1,3 galactosyl-transferase* ***α-gal transferase***	*α1,2 fucosyl-transferase* ***H-transferase***	*α1,2 fucosyl-transferase* ***H-transferase***	*α1,2 fucosyl-transferase* ***H-transferase***	*α1,2 fucosyl-transferase* ***H-transferase***
↓						
Epitope		*Gal α1,3 Gal β1,4 GlcNAc-R* ***The α-gal epitope***	*Gal β1,4 GlcNAc-R α1,2 Fuc* ***H substance***	*Gal β1,4 GlcNAc-R-α1,2 Fuc* ***H substance***	*Gal β1,4 GlcNAc-R-α1,2 Fuc* ***H substance***	*Gal β1,4 GlcNAc-R-α1,2 Fuc* ***H substance***
↓						
Enzyme				*α1,3 N-acetyl-galactosaminyl-transferase* ***A-transferase***	*α1,3 galactosyl-transferase* ***B-transferase***	
↓						
Epitope				*GalNac α1,3 Gal β1,4 GlcNAc-R α1,2 Fuc* ***A substance***	*Gal α1,3 Gal β1,4 GlcNAc-R α1,2 Fuc* ***B substance***	

transgenic pigs show increased resistance to human sera (Koike *et al* 1996). In mice produced by Chen *et al* (1996), it was found that expression of the α-gal epitope was nearly eliminated in cells in which the expression was also low in non-transgenic mice, such as the endothelial cells lining the renal tubular sinusoids. However, no reduction was observed in the arterial endothelial cells of the heart and the kidney, which normally express high levels of the epitope. H-transferase mice have so far been produced using the H-$2K^b$ promoter, but higher expression levels might be achieved using the ICAM-2 promoter, which has been shown to give high endothelial expression levels (Cowan *et al* 1998b). Although no pathological or other types of deviation in H-transferase transgenic animals have been reported, it has been impossible to produce homozygotic H-transferase animals (Pearse *et al* 1999). Too high an expression of H-transferase in gal-transferase-producing animals seems to be toxic. The mechanisms underlying this are not fully understood, but lectin binding studies show that the structure of the cell walls of H-transferase transgenic animals is changed in ways that go beyond the sole deficit of the α-gal epitope and presence of the H-antigen. Some crypt antigens, Tn and Forssman, may even become exposed on the cellular wall, and this in turn may increase the risk of DXR of organs from an H-transferase transgenic donor (Pearse *et al* 1999). The H-transferase approach to elimination of the α-gal epitope has not identified any potential welfare problems, but it seems to be problematic in other respects and it is doubtful whether it offers a feasible way to produce a xenograft donor. The inability to breed homozygously will increase the cost of producing donor pigs.

Processing the α-gal epitope by α1,3-galactosidase

Another way of transforming the α-gal epitope is by inserting the gene coding for α1,3-galactosidase into the genome. This enzyme can transform the α-gal epitope so that it is unrecognisable by anti-gal antibodies. Mice transgenic for α1,3-galactosidase tend to secrete

more proteins in their urine than do wild-type mice. Furthermore, low body weights, partial damage to hair growth and early death occur more frequently in these mice (Ikematsu *et al* 1993). These negative consequences are probably associated with the inserted gene but could also result from an insertion mutation (the inserted gene obscuring the host gene function), estimated to occur in 5% of microinjections (Rijkers *et al* 1994). It is unlikely that the α1,3-galactosidase approach will be pursued further because of its drastic impact on the viability of the animals. The impact of α1,3-galactosidase insertion on pigs has not been elucidated.

Insertion of complement regulatory factors (CRFs) modulating the xenograft rejection

Both mice and pigs transgenic for CRFs have been produced (Table 2), and the transgenic animals have been combined with one another and with animals bearing mutations of α-gal-related genes (Table 3). None of the studies report any pathological, behavioural, clinical or welfare-related problems (van Denderen *et al* 1997; Cowan *et al* 1996; Ijzermans *et al* 1996). Animals transgenic for such genes will express both the added and their own species-specific CRFs. Because the immune system will develop self-tolerance towards epitopes present at early neonatal stages, the inserted human CRFs will not cause the animals to reject their own organs, and the function of the pig's own CRFs will still be to differentiate between pig and non-pig. Addition of CRF transgenes seems unlikely to cause problems because of the suspected inertness of the CRFs on the pig cell surface. However, one must remember that problems caused by insertion of transgenes have been known to occur: for example, the insertion and excess expression of one transgene in pigs caused them to develop lameness, lethargy and gastric ulcers (Pursel *et al* 1990). These problems could, however, have been expected, given the known function of the transgene inserted (Pursel *et al* 1990). Therefore, the insertion of CRF transgenes seems less likely to affect the welfare of the animal than the removal of epitopes, but closer examination of CRF transgenic animals will be necessary to see whether animals are affected and, if so, to what extent.

Discussion and animal welfare implications

The aims of this paper are threefold: first, to evaluate our knowledge of the transgenes and transgenic models currently being discussed in connection with the development of xeno-donor pigs, with the aim of identifying potential welfare problems; second, to show how different views on animal welfare may influence conclusions reached about the welfare of transgenic animals; and third, to put this kind of welfare assessment forward as an example to show that hypotheses about animal welfare can be formulated and sometimes even tested prior to the generation of transgenic animals. Other things being equal, the collection of information on the welfare of transgenic animals before their generation will help to improve their welfare.

Research in the area of xenotransplantation is progressing, and the possibility that a xeno-donor pig will be available in the future seems likely, even though alternative technologies — such as the transplantation of autografts developed by therapeutic cloning (Becht *et al* 1991) and mechanical devices such as heart pumps (Frazier 2000) — will be further refined. Debate about the risks involved in xenotransplantation has so far revolved around the risk of xenozoonosis. Moreover, public debate about xenotransplantation has been limited to the issue of what harm the technology may do to humans. However, this may change when people realise that there are implications for the animals used in the research and/or use of the technology, and when it is recognised that animal welfare is an ethical issue

that may affect public opinion on xenotransplantation in the same way that ethical issues have affected the public's attitude to other biotechnologies.

An understanding of the ethical perspectives involved in this debate is fundamental to fruitful communication between scientists and the rest of society. A significant number of people will conclude that the clearly positive intentions of those researchers developing techniques for improving and sustaining many peoples' lives do not outweigh the negative impact of the suffering of transgenic animals held in laboratories. Indeed the practice of such weighing may not be acceptable at all. In science, the traditional anthropocentric way of thinking places very few limits on what may be done to help humans; on the other hand, a perfectionist approach to animal welfare will offer reasons for limiting human action.

To evaluate the potential implications for animal welfare, a risk assessment has been used that exploits knowledge gained from observation of existing transgenic animals developed in xenotransplantation research. As has been discussed, any future transgenic animal donors of organs to humans are likely to be pigs that have been depleted of the α-gal-forming enzyme, possibly knocked out for other epitopes as well, and further modified by inserted human CRFs. These genetic modifications may bring about an increased tendency to septicaemia, reduced vision because of cataracts, and possibly autoimmune diseases and reduced fertility.

If transgenes inserted into pigs cause an increase in the prevalence of septicaemias, this will quite obviously reduce their welfare by any plausible standard. However, if the presence of septicaemia could be substantially reduced, for example by management, then being a xeno-donor would not necessarily be associated with reduced welfare from a hedonistic point of view — provided that the management routine is compatible with the presence of positive feelings and the absence of negative feelings. From a perfectionist's point of view, an increased tendency to develop disease could constitute a welfare problem because of the inherent imperfection that disease causes to the animal. Similar arguments apply in the case of lowered fertility. No pain or other negative experiences are associated with the condition because reproduction is reduced at an embryonic level. Therefore, this condition does not represent a welfare problem from a hedonistic point of view. However, it may be perceived from a perfectionist's point of view as an inbuilt malformation of the animal, hampering its natural ability to reproduce and thus constituting a welfare problem. Because both views are represented in our society, both conclusions on welfare should be recognised and debated.

The cataracts in α-gal knockout mice supposedly cause visual impairment. It is not known whether this will also be the case in α-gal knockout pigs. Studies and comparisons of the molecular constitution of the lenses of the pig and the mouse could assist in determining this. If impaired vision in future donor pigs causes aggression, this would by any standard lead to reduced levels of welfare. Even without any accompanying behavioural changes, changes in the lenses of xeno-donor pigs could also be perceived as a problem from a perfectionist point of view, as they may hamper the animals' natural ability to see.

This theoretically based evaluation of the welfare of future xeno-donor pigs has raised questions that can be answered by practical investigation of existing models of the xeno-donor, such as α-gal knockout mice. Whether or not future xeno-donor pigs will, in fact, be prone to septicaemias should be tested in α-gal knockout mice, as should the welfare consequences of reduced vision. Throughout, the differences between these two species will obviously have to be borne in mind. As has already been stated, the conclusions of a welfare assessment will depend to a very great extent on the notion of animal welfare that is the basis for the evaluation. However, it is essential to recognise the ethical nature of the concept of

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animal welfare if the results of an evaluation are to satisfy the questions raised and a meaningful debate on the subject is to be commenced.

Hypotheses concerning the welfare of future xeno-donors have been derived in this paper from available knowledge of the transgenes currently in focus — the existing transgenic animals generated in xenotransplantation research, in combination with fundamental information on the impact of these modifications on animal welfare. Analogous evaluations may be performed in the same way prior to the generation of transgenic animals in the future so that potential welfare problems can be anticipated and responded to as early as possible in programs of transgenic research and development. Furthermore, an animal welfare risk assessment could provide solid argumentation when licenses to perform lawful research involving the generation of transgenic animals are under review.

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