Glaxo/MRS Young Investigator Prize

Xenotransplantation: immune barriers beyond hyperacute rejection

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1. The use of organs from animal donors (xenotransplantation) is a potential solution to the chronic shortage of allogeneic organs and currently the pig is thought to be the most suitable donor for man. However, porcine organs are rejected rapidly by a vascular process called hyperacute rejection which has so far prevented clinical xenotransplantation. Although it is likely that this barrier will be overcome in the near future by the application of novel strategies, probably involving the use of organs from transgenic pigs, data from animal models indicate that multiple other immune mechanisms will contribute to the rejection of xenografts.

2. We have described two aspects of these immune mechanisms. First, the phenomenon of ‘accommodation’, whereby xenografts acquire in vivo resistance to vascular rejection, has been explored in an in vitro model utilizing immortalized porcine endothelial cells. The results indicate that human anti-pig antibodies induce a concentration-dependent and time-dependent change in porcine endothelial cells compatible with the development of accommodation.

3. Secondly, the in vitro human anti-porcine T-cell response has been documented in detail, with particular emphasis on quantitative and qualitative comparisons with the in vitro T-cell alloresponse. The results of this work, which indicate that the response to porcine xenografts is likely to be significantly stronger than that against allografts, have important implications for the level of conventional immunosuppression that may be necessary to prevent xenograft rejection, and provide an important basis for the development of strategies to promote xenograft-specific immunosuppression and tolerance.

INTRODUCTION

Because of the severe shortage of donor allografts for transplantation, thousands of patients every year, throughout the world, suffer significant morbidity and mortality awaiting a suitable kidney, heart, liver or lung transplant. Xenotransplantation, the use of animal organs, would solve this problem and the pig, for a variety of reasons (Fig. 1), is currently perceived to represent the most suitable donor for man.

However, pig organs are ‘discordant’ in man [1] (and other primates), and suffer an immediate vascular rejection process called hyperacute rejection. Although this has been hitherto regarded as the most formidable barrier to clinical xenotransplantation, there are now several strategies, including the use of organs from transgenic pigs expressing human regulators of complement activity [2], which offer real hope that this aspect of xenograft rejection will be overcome [3].

After the recent Kennedy report to the Department of Health [4], a specialist committee has been established by the British government to oversee the introduction and development of clinical xenotransplantation in the U.K. There are a number of questions, most prominently the risk of infection from xenogeneic organs, that need to be addressed before clinical trials using porcine organs can begin (see Fig. 2). Other issues concern the length of time that xenografts transplanted into humans will survive and the adequacy of their performance, although these may be difficult to address in detail before the first patients have been transplanted. Eventually, xenografts will have to survive as long as allografts to solve the problem of organ shortage; short-term survival, with xenografts used only as ‘bridging organs’ (until an allograft becomes available), may worsen the present situation.

Key words: T-cell xenoresponse, xenograft accommodation, xenotransplantation.

Abbreviations: DCs, dendritic cells; HNG, human normal globulin; IPECs, immortalized porcine endothelial cells; MHC, major histocompatibility complex; SLA, swine leucocyte antigen; VCAM-1, vascular cell adhesion molecule-1.

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Advantages of xenografts compared to allografts

- Inexhaustible supply if the chosen donor breeds rapidly and has large litters.
- Organs available for people currently excluded from waiting lists.
- Extension of the therapeutic possibilities of transplantation (to include diabetes mellitus, for example).
- Opportunity for genetic manipulation of donor organ to influence recipient rejection responses.
- Planned, unhurried operations.
- Species differences in susceptibilities to disease may make xenografts useful for transplants in specific patient groups (e.g. HIV or Hep. B+ patients).

Pigs as donors compared to other species

- Pigs have a gestation period of 3 months and produce two or more litters each year, each with an approximate size of 6-9 piglets.
- Pigs have fewer human-like qualities than other species such as primates and are therefore ethically more acceptable to most people.
- Pigs are already farmed on a worldwide basis for food and undergo intensive breeding programmes.
- Pigs are anatomically and physiologically similar to humans.

Since almost nothing is known about how porcine organs will function in a human environment, it is possible that physiological incompatibilities may limit the survival of these organs. However, it is most likely that immunological rejection will provide the biggest threat to the long-term survival of these organs. Because hyperacute rejection has been such a formidable barrier to breach, the precise nature of the rejection mechanisms beyond this stage is only just emerging from various studies. This article will describe some of these other immunological hurdles and briefly discuss some of the opportunities for novel immunosuppressive strategies.

**REJECTION OF XENOGRAGTS: BEYOND HYPERACUTE REJECTION**

Figure 3 lists the factors important in the pathophysiology of hyperacute rejection and the strategies that may be successful to overcome this clinically. There is a realistic chance that combinations of these will be effective and enable xenografts to survive beyond one or two days. However, there are several other immunological barriers beyond hyperacute rejection [5] (Fig. 4).

**DELAYED XENOGRAGT REJECTION**

The pathophysiology of delayed xenograft rejection is still incompletely understood [6]. Hist-
logical studies in rats receiving guinea pig hearts show IgG and IgM xenoreactive natural antibodies deposited along graft endothelium, fibrin deposition, platelet aggregation and a prominent infiltration of inflammatory cells, most commonly polymorphonuclear cells, monocytes and natural killer cells. Several lines of evidence indicate that three factors may be involved in the development of delayed xenograft rejection: binding by xenoreactive natural antibodies, activation of graft endothelium and intrinsic natural killer cell activity against xenogeneic tissues.

**ACCOMMODATION OF XENOGRAFTS**

Transplanted xenografts, protected from hyperacute rejection and delayed xenograft rejection by depletion of xenoreactive natural antibodies or inhibition of complement can sometimes continue to

<table>
<thead>
<tr>
<th>The pathophysiology of HAR</th>
<th>Strategies to prevent HAR (clinically relevant)</th>
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<tbody>
<tr>
<td><strong>A: Xenoreactive natural antibody</strong></td>
<td>Manipulation of the recipient</td>
</tr>
<tr>
<td>Predominantly IgM</td>
<td>Immunoabsorption by perfusion of carbohydrate columns</td>
</tr>
<tr>
<td>Dominant epitope is the galα(1-3) gal carbohydrate, found on a variety of glycoproteins and glycolipids</td>
<td>Systemic treatment with purified carbohydrate</td>
</tr>
</tbody>
</table>

| **B: Complement** | | |
| Activated via the classical pathway by XNA bound to graft endothelium | Systemic anti-complement agents such as soluble CR1 | Pigs transgenic for human regulators of complement activity |
| Responsible for the vigour of HAR | | |

| **C: Endothelial cell activation** | | |
| Loss of integrity of vascular wall as endothelial cells retract | Anti-coagulants and anti-platelet agents | Induction of 'accommodation' |
| Exposure of tissue factor on subendothelial cells | | Pigs transgenic for human thrombomodulin |
| Initiation of coagulation | | |
| Activation and recruitment of platelets and inflammatory cells | | |

*Fig. 3. Hyperacute xenograft rejection (HAR). *Not yet possible with pigs as embryonic stem cells have proved difficult to isolate.*
Fig. 4. Immune barriers beyond hyperacute rejection (HAR). Further details are given in the text.

Fig. 5. Effect of HNG, a commercial preparation of human IgG on IPECs. (a) Single experiment, analysed to show the influence of the time that IPECs are exposed to HNG. Graphs show lysis in human serum after incubation with two concentrations of HNG, 100 µg/ml and 1000 µg/ml, for 72, 96, 120 and 144 h. Shorter incubation periods have not been systematically examined. The resistance to lysis that develops is time dependent. (b) The same experiment as in (a), analysed to show the influence of the concentration of HNG. Baseline cells were grown in the absence of added HNG. Each graph shows cytotoxicity of IPECs incubated in HNG for either 72, 96, 120 or 144 h. The resistance to lysis that developed was largely independent of the concentration of HNG. Dashed lines on the bottom two graphs are to indicate the percentage serum needed to cause 50% lysis.
function despite the return of anti-donor antibody and complement back to pre-transplant levels [7]. This phenomenon, termed graft ‘accommodation’ [8] has also been described in allografts transplanted across both ABO and human leukocyte antigen (HLA) barriers [9, 10].

Although the precise mechanisms underlying accommodation are still not clear, it is thought to involve changes in xenograft endothelial cell physiology, rendering the cells resistant to the effects of complement [11]. It could be due, at least in part, to recovery from the ischaemia that the graft is subject to during the period between donor and recipient. However, there is evidence that healthy endothelial cells can accommodate in vitro [12].

**EFFECT OF ANTI-XENOGRoF ANTIBODIES ON ENDOTHELIUM. A PARADOX?**

The evidence from some models of xenotransplantation supports a role for xenoreactive natural antibodies in the initiation of both hyperacute rejection and delayed xenograft rejection, and consistent with this is the notion that antibody depletion is crucial for accommodation to occur [13]. However, evidence from other models suggests that anti-xenograft antibody may promote accommodation [14]. How can this apparent paradox be explained?

An attractive hypothesis is that accommodation might arise because the endothelium is exposed to a low level of antibody. This was first suggested in general terms (i.e. exposure to low level of stimulus) by Bach et al. [15], and it might explain why accommodation is associated with antibody depletion [13]. An alternative is that IgM and IgG xenoreactive natural antibodies each have a different effect on endothelial cell physiology. The basis of this might be signalling through different membrane glycoproteins that each isotype is thought to bind. If this was the case, the outcome of rejection or accommodation would be determined by the relative concentrations of each during the period after transplantation. We investigated the first of these possibilities in an in vitro model of porcine endothelial cell accommodation [16].

In order to ensure that experiments were performed on a phenotypically stable population of endothelial cells, we generated cloned immortalized porcine endothelial cells (IPECs) from primary aortic endothelial cells using a retroviral construct containing the SV40 large T-variant SV-U19 [17]. Clones were chosen for study if they behaved like primary cells during culture and after stimulation with human cytokines. A commercially available preparation of human immunoglobulin [human normal globulin (HNG), BPL, Herts, U.K.] prepared from pooled serum was confirmed to be a rich source of human anti-pig IgG antibodies, as we had expected, since human anti-pig IgG accounts for approximately 1% of total IgG in most individuals [18].

Two concentrations of HNG, 100 µg/ml (nonsaturating binding by IgG xenoreactive natural antibodies) and 1000 µg/ml (near-saturating) were chosen for investigation. Separate cultures of IPECs were established and maintained for 6 days, and HNG was added at different times to individual flasks throughout the culture period. On day 6 all monolayers were harvested simultaneously and the cells examined for changes in phenotype.

One series of experiments examined the sensitivity of these IPECs to the effects of human complement. It was clear that cells incubated in HNG developed a resistance to lysis, demonstrated using standard 51Cr release assays (Fig. 5). After 120 h the concentration of human serum required to give 50% lysis was increased by a factor of 10. However, there was no apparent relationship between the concentration of HNG with which the IPECs were incubated and the degree of resistance that developed. These experiments were repeated several times to establish that the effect of HNG was real and reproducible. In a full analysis of all these assays (results not shown), the relationship between the length of exposure to HNG and the development of resistance to lysis was still obvious, but best seen under conditions where lysis was limited by the dilution of human serum. The optimal exposure time for development of maximal resistance appeared to be 120 h. In contrast, the concentration of HNG appeared to have
little influence on the resistance that developed so that incubation with 100 µg/ml had a similar effect to 1000 µg/ml.

A possible explanation for this protective effect was that preincubation with HNG was saturating xenoreactive antibody binding sites, so that in the cytotoxicity assay complement-fixing antibodies present in the human serum were prevented from binding to IPECs. However, this was not the case. Maximal binding by complement-fixing IgM to HNG-incubated IPECs was equivalent or higher than the binding of IgM to control IPECs (results not shown). The implication is that the resistance to lysis by human serum developed because of a real change in the behaviour of IPECs induced by HNG. One possibility that we are currently investigating is that complement regulatory proteins such as CD59, which protect cells against lysis by complement, may be upregulated by exposure to human anti-pig antibodies.

In a second series of experiments we examined the phenotype of cells exposed to HNG. This was done by flow cytometric analyses using monoclonal antibodies specific for E-selectin, vascular cell adhesion molecule (VCAM)-1 and major histocompatibility complex molecule (MHC) class I, chosen primarily because of the availability of pig-specific antibodies. These experiments indicated that a significant downregulation of VCAM and MHC class I expression occurred on IPECs in response to HNG (Fig. 6). It was also apparent that 100 µg/ml HNG induced greater changes in VCAM and MHC class I than 1000 µg/ml, and that incubation periods longer than 72 h were required to induce any change in the expression of either molecule. Experiments were also performed to investigate whether incubation with HNG influenced the ability of IPECs to respond to submaximal doses of human recombinant tumour necrosis factor α. Although the reduction in VCAM expression induced by HNG could be overcome by incubation with tumour necrosis factor α, expression remained significantly lower than on control IPECs [16].

Finally, to investigate the possibility that factors other than IgG anti-pig antibody in the HNG may have been influencing the responses of IPECs, the immunoglobulin fraction was isolated by adsorption on to a recombinant protein G column. Incubation experiments were repeated with this purified immunoglobulin and it was clear that the VCAM modulating ability of HNG resided within the eluted IgG fraction (results not shown).

![Diagram of Hyperacute Rejection, Delayed Xenograft Rejection, and Accommodation](image)

**Fig. 7. Role of xenoreactive natural antibodies in accommodation; a hypothesis.** IgM xenoreactive natural antibodies (XNA) promote hyperacute rejection in the presence of complement. Endothelial cell activation, after XNA binding and complement activation, leads to recruitment of platelets and inflammatory leucocytes. Individual cells are caused to retract, with consequent loss of monolayer integrity resulting in oedema and exposure of subendothelial tissue factor. This initiates coagulation and leads to widespread intravascular thrombosis. In the absence of complement, XNA deposition is one of the factors thought to promote delayed xenograft rejection during which the endothelium becomes activated and inflammatory monocytes and cells infiltrate into the graft, using Fc-receptor-dependent and porcine-accessory-molecule natural killer (NK) dependent mechanisms. Based on our experimental observations and the observations from in vivo models, we propose that low concentrations of IgG XNA mediate changes in endothelium leading to accommodation several days later. In the absence of complement, hyperacute rejection does not occur. Inflammatory cell traffic into the graft is predicted to be influenced by two things, the absence of significant IgG binding and the downregulation of accessory cell molecules such as VCAM on accommodated cells. Accommodated grafts are therefore also protected from delayed xenograft rejection.
Our results imply that IgG anti-pig antibodies can mediate the development of an accommodated phenotype that is characterized by the development of resistance to complement-mediated lysis and by the downregulation of expression of molecules such as VCAM and MHC class I. Fully accommodated cells developed only after exposure to low concentrations of IgG for at least 4 days, supporting the low-level stimulus hypothesis (Fig. 7). The implications are that the development of accommodation in vivo may be crucially determined by the titre of IgG anti-pig antibodies in the immediate post-transplantation period. This in vitro model is expected to be of value in characterizing further the process of endothelial cell accommodation.

T-CELL RESPONSE TO PORCINE XENOGRAFTS

The initiation of T-cell-mediated graft rejection begins with the recognition of graft antigens by specific T-cells (for review see [19]). Two pathways by which transplantation antigens are recognized have been described [20, 21]. These mechanisms are illustrated in Fig. 8. In the first, the ‘direct’ pathway, MHC-expressing allo- or xenogeneic bone-marrow-derived specialized antigen-presenting cells provide all the signals required for T-cell activation, including that through the T-cell receptor. Two factors determine the vigour of ‘direct’ T-cell responses; first, on a cell-to-cell level, the efficiency of interaction between the crucial molecular ligand pairs necessary for T-cell activation; second, at the level of the whole organism, the proportion of T-cells with receptors specific for intact allo- or xeno-MHC. In the case of allogeneic stimulators, efficient molecular interactions and a high frequency of specific T-cells [22] provide the basis for significant primary in vitro alloresponses and strong in vivo cellular responses [23].

The second recognition pathway, used by CD4+ T-cells, is called ‘indirect’ to reflect the necessity for processing of allo- or xenoantigens before presentation of the peptides generated on cell-surface class

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Fig. 8. Diagrammatic representations of direct (A) and indirect (B) xenorecognition. (A) The types of molecular interactions necessary for efficient direct xenorecognition are numbered 1–3. 1, Cognate interaction between T-cell receptor on responder T-cell and MHC molecules on xenogeneic antigen-presenting cells (APCs). 2, Non-cognate interaction between co-receptors CD4 and membrane proximal domains of MHC class II, and CD8 and α3 domains of MHC class I. 3, Non-cognate interactions between accessory and co-stimulatory molecules. Important interactions are between B7 family members (on APCs) and CD28 (on T-cells), leukocyte-function-associated antigen-3 (on APCs) and CD2 (on T-cells), vascular cell adhesion molecule-1 (on APCs) and very-late-expressed antigen-4 (on T-cells) and intercellular adhesion molecule-1 (on APCs) and leukocyte-function-associated antigen-1 (on T-cells). (B) Diagrammatic representation of indirect xenorecognition. Xenantigens (4) released by xenogeneic cells are taken up and processed (5) into peptide fragments by specialized antigen-presenting cells (6) before binding to MHC class II molecules (7) and display on the cell surface (8) for presentation to xenospecific self-MHC class II-restricted T-cells.
Fig. 9. Experiments to illustrate the direct human anti-pig xenoresponse. A. Proliferative response by $1 \times 10^5$ human T-cells against porcine dendritic cells at high stimulator numbers. Graphs show [H]thymidine incorporation expressed as mean c.p.m. ± SEM. □, no antibody; ○, anti-SLA-DQ 0.3 μg/ml, anti-SLA-DR and anti-HLA-DR 1 μg/ml; ◇, anti-SLA-DQ 1 μg/ml, anti-SLA-DR and anti-HLA-DR 3 μg/ml; △, anti-SLA-DQ 3 μg/ml, anti-SLA-DR and anti-HLA-DR 10 μg/ml. These results indicate that the response is directed predominantly against SLA-DR molecules expressed on porcine DCs. Representative of six experiments. B. Proliferative response of human T-cells from two responders (□ and ○) to allogeneic or porcine DCs. Responses to DCs prepared from a standard protocol from peripheral blood mononuclear cells were examined using a fixed number ($5 \times 10^5$) of human T-cells. The number of stimulators per well was corrected for number of DCs (determined by flow cytometric phenotype). At low responder/stimulator ratios (stimulators in excess), the proliferative response is similar to both human and pig DCs. At high ratios, approximately ten times fewer human allogeneic DCs are needed to generate a proliferative response. Representative of two experiments.

Table 1. Results of experiments to determine the precursor frequency (pf) (expressed as a reciprocal, 1/lf) of human interleukin-2-producing T-cells recognizing porcine DCs by direct xenorecognition

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>1/lf</th>
<th>95% Confidence limits</th>
<th>$\chi^2$ value</th>
<th>P value</th>
</tr>
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<td>Medium</td>
<td></td>
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<td>239645</td>
<td>77105–744824</td>
<td>4.2</td>
<td>&lt;0.5</td>
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<tr>
<td>2</td>
<td>115299</td>
<td>58091–228845</td>
<td>8.89</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>3</td>
<td>468705</td>
<td>117351–1872038</td>
<td>0.9</td>
<td>&lt;0.98</td>
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<tr>
<td>Alveolar DCs</td>
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<tr>
<td>4</td>
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<td>5</td>
<td>6191</td>
<td>4744–8081</td>
<td>15</td>
<td>&lt;0.02</td>
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II MHC molecules. In these responses, crucial molecular signals leading to T-cell activation and proliferation, provided by recipient antigen-presenting cells, are always efficient and do not limit the vigour of the response. However, in vitro primary indirect alloresponses are difficult to detect for two reasons. First, they are usually obscured by stronger direct responses [24]. Second, the precursor frequency of T-cells specific for processed alloantigen is either undetectable or very low, and although the frequency increases in a secondary mixed lymphocyte reaction [25] it remains approximately 100 times lower than that of T-cells activated by direct allorecognition.

For many years the common perception was that T-cell xenoresponses were much weaker than those to alloantigens, a view that arose from in vitro experiments using human and murine mixed lymphocyte cultures, where a series of cross-species molecular incompatibilities are known to limit direct xenorecognition [26]. Very few in vitro studies have been specifically designed to examine the indirect xenoresponse, and until recently no studies had examined the specific human T-cell response to porcine tissue.

Using adaptations of protocols designed for use in other species, we isolated and characterized dendritic cell populations from the lungs and peripheral blood of pigs. Dendritic cells (DCs) were chosen as stimulator cells in these experiments for two reasons. First, they correspond to the lineage that is most likely to play a major role in provoking a direct response in vivo after clinical xenotransplantation [19]. Second, because it was possible to isolate human and porcine DCs simultaneously from peripheral blood using the same protocols, a meaningful comparison could be made between allo- and xenogeneic responses.

The results of these experiments are illustrated in Fig. 9 and Table 1. Under conditions where stimulator cells were in excess, these 'direct' anti-pig xenoresponses appeared equivalent in strength to

<table>
<thead>
<tr>
<th>Stimulators per well (x10^-4)</th>
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<tbody>
<tr>
<td>ADAPc26 +anti-SLA-DR</td>
</tr>
<tr>
<td>0.5-3.1</td>
</tr>
<tr>
<td>1µg/ml</td>
</tr>
<tr>
<td>3µg/ml</td>
</tr>
<tr>
<td>10µg/ml</td>
</tr>
<tr>
<td>No antibody</td>
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Table 2. Results of an experiment to determine the precursor frequencies (pf) of CD4+ T-cells with specificity for SLA-DRc or HLA-DR1, compared with control DAP3. Representative of two experiments with ADAPc26, 0.5-3.1, DAP3 clone transfected with HLA-DR1, ADAPc26, DAP3 clone transfected with SLA-DRc.

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>1/pf</th>
<th>95% confidence limits</th>
<th>( \chi^2 ) value</th>
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Fig. 11. Experiments to illustrate the indirect human anti-pig xenoresponse. A. Proliferative response of human peripheral blood mononuclear cells (PBMCs) to porcine stimulators, chosen because of their inability to stimulate a direct proliferative response. Graphs show $[^{3}H]$thymidine incorporation expressed as mean c.p.m. ± SEM, and are representative of >10 experiments with six responders. (i) shows the loss of proliferative response after depletion of antigen-presenting cells (APCs), indicating that human APCs are required in the response. (ii) shows that an anti-HLA-DR monoclonal antibody inhibits the proliferative response, indicating that this is a true indirect response involving presentation of porcine antigens on HLA-DR molecules. (iii), (iv) and (v) confirm the absence of any direct xenorecognition as antibodies specific for SLA class I and II molecules failed to inhibit the proliferative response by PMBCs. Only the saturating dose of each is shown. Representative of four experiments with four responders. (vi) Single experiment comparing the proliferative response of $1 \times 10^6$ PMBCs against a porcine SLA class-I-negative endothelial cell line and human fibroblast line. An indirect response is observed only with porcine cells, not allogeneic cells. Representative of four experiments with different responders (all HLA-mismatched for the allogeneic cell line). B. Graphs show $[^{3}H]$thymidine incorporation expressed as means c.p.m. ± SEM. (i) Comparison of the indirect proliferative response by $1 \times 10^6$ PMBCs, CD8-depleted PMBCs or APC-depleted PMBCs against porcine stimulators. CD8+ T-cells were depleted to <2% of population (by flow cytometric analysis). Representative of three experiments. (ii) Comparison of the indirect proliferative xenoreponses by $1 \times 10^6$ whole PMBCs and B-cell-depleted PMBCs against porcine stimulators. CD19 labelled B-cells were depleted using magnetic beads to <1% of the PMBC population, as assessed by staining with anti-CD19. Representative of three experiments. (iii) Proliferative response of umbilical cord mononuclear cells (UCMCs) to porcine stimulators. UCMCs ($1 \times 10^7$) or APC-depleted UCMCs were used. Representative of three experiments.
direct alloresponses, with a high precursor frequency of pig-specific T-cells. These responses were mediated by human CD4⁺ T-cells recognizing swine leucocyte antigen (SLA)-DR molecules [27]. SLA is the name given to porcine MHC molecules. However, under conditions where the numbers of stimulator cells were limiting, 'direct' proliferative anti-pig xenoresponses appeared weaker than alloresponses [28, 29]. The implication was that there might be qualitative ‘defects’ in the molecular interactions involved in the human anti-pig direct xenoresponse, so experiments were performed to investigate the provision of cognate and non-cognate interactions to human T-cells by porcine ligands.

Examination of accessory and co-stimulatory signals revealed that porcine VCAM interacted efficiently with human VLA-4 to promote T-cell adhesion and proliferation under certain conditions [16]. Similarly, porcine B7 was found to provide efficient co-stimulatory signals for human T-cell proliferation and interleukin-2 production (see the legend to Fig. 8 for a definition of these accessory molecules). Overall, porcine cells were able to support human T-cell proliferation at least as well as human accessory cells, implying adequate provision of non-cognate interactions by pig ligands [28]. Results from other groups corroborate this conclusion [30, 31].

The molecular basis of the weakened direct response was traced, at least in part, to an inefficient interaction between SLA-DR and human T-cells. This is illustrated in Fig. 10 and Table 2. Human T-cell proliferative responses to transfectant murine DAP.3 clones expressing SLA-DR were always reduced compared with those expressing HLA-DR1. Limiting dilution analyses revealed that approximately 3-5 times fewer human T-cells had specificity for xeno- compared with allogeneic MHC. Comparison of the responses to these two MHC class II molecules was particularly informative because of their remarkable similarity; the predicted primary amino acid sequence of the α1 domain of c-haplotype SLA-DR is more like HLA-DR1 than many other HLA-DRβ alleles [32], and SLA-DRc probably represents the most ‘human-like’ of the porcine MHC molecules. By implication, the human T-cell responses to SLA-DRc will be the most ‘allo- geneic-like’ of the human responses to porcine SLA-DR.

The other mechanism by which T-cell responses to xenografts may be initiated is by ‘indirect’ recognition of xenoantigens. We examined the indirect response to pig antigens using SLA-class II-negative or co-stimulator-deficient porcine stimulator cells that were unable to provoke a direct response [27, 33, 34]. These results, illustrated in Fig. 11 and Table 3, indicate that the frequency of HLA-class II-restricted CD4⁺ T-cells with specificity for pro-

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### Table 3. Results of six experiments to determine the precursor frequency (pf) of human interleukin-2-producing T-cells with indirect specificity for porcine xenoantigens

<table>
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<th>P value</th>
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A: Problems with systemic immunosuppression

i) Relatively ineffective at preventing chronic rejection; chronic rejection of xenografts may be more aggressive than that of allografts

ii) Associated with the significant complications of infection and neoplasia

iii) Systemic therapy may exacerbate the risks of zoonoses from xenografts

B: Graft-specific immunosuppression may be easier to achieve with xenografts compared to allografts

i) Donor-recipient pairs identified before any operation

Scope for manipulating the donor as well as the recipient

Time to plan treatment strategies

Time to test the success of each strategy before transplantation

ii) Use of pig-specific reagents such as monoclonal antibodies which will have little effect on the recipient’s general immune responses

iii) The potential of developing transgenic pigs that are resistant to post-HAR rejection

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Fig. 12. Incentives for graft-specific immunosuppressive strategies in xenotransplantation
cessed pig xenantigens was much higher than the equivalent frequency of cells specific for processed alloantigens. Consequently, primary indirect proliferative responses were easily observed against porcine, but not against allogeneic stimulators.

Several possibilities were considered to account for strong indirect xenoresponses. First, the presence of xenospecific B-cells, responsible for production of xenoreactive natural antibodies, might have acted as efficient antigen-presenting cells for porcine antigens. The unique antigen-concentrating abilities of antigen-specific B-cells have been well documented [35]. However, B-cell-depleted human responders were equally as efficient as B-cell-replete populations at mounting indirect anti-pig responses, effectively ruling out this explanation. These results do not address the question of whether the presence of xenoreactive natural antibodies contributes to antigen uptake and processing by conventional Fc-receptor-expressing antigen-presenting cells.

The second possibility was that T-cell xenoresponses may have been primed by exposure to antigens shared between environmental pathogens and xenostimulator populations or by exposure to antigens present within food. Oral priming after ingestion of antigens has been documented in some animal models [36]. However, naive T-cells, obtained from umbilical cord specimens, mounted indirect responses as effectively as those from adults, implying that priming was not necessary.

We have concluded therefore that indirect xenoresponses are stronger than alloresponses mainly because of the greater number of xenantigens compared with alloantigens. This in turn is most probably due to the high degree of protein polymorphisms across different species, in contrast to the limited polymorphism between individual humans.

**SUMMARY OF HUMAN T-CELL ANTI-PIG XENORESPONSES**

Direct recognition of pig stimulators by human T-cells appears efficient and results in significant primary proliferative responses, although under certain conditions the response is reproducibly weaker than equivalent alloresponses. Our observation that porcine MHC class II molecules are recognized less efficiently than their allogeneic counterparts is interesting and clearly illustrates the general point that intercellular molecular interactions tend to be less efficient across species barriers, but it is probably without any direct practical relevance. *In vivo*, where stimulator cell numbers are unlikely to be limiting, the vigour of anti-xenograft rejection will probably resemble the vigour of anti-allograft rejection.

Human HLA-DR-restricted T-cells also mount significant indirect proliferative responses to processed porcine xenantigens, and these are likely to play a major role in initiating and perpetuating acute cellular rejection *in vivo*.

The available evidence therefore suggests that a formidable T-cell response to porcine antigens will develop in recipients of pig organs.

**XENOGRAFT-SPECIFIC IMMUNOSUPPRESSION**

Whether post-hyperacute rejection immune barriers will eventually yield to conventional immunosuppressive therapies is still uncertain; the newer, more potent pharmacological agents may be required routinely in xenograft recipients [26].

However, if xenotransplantation becomes a reality, there will be exciting opportunities to develop new methods of immunosuppression, with an emphasis on tailored, graft-specific approaches allowing a shift away from complete reliance on systemic immunosuppression (Fig. 12).

For instance, the results of our *in vitro* studies, along with those from other groups [37], have provided insights into the potential mechanisms underlying accommodation, and it may be possible in the future to manipulate the xenograft to induce accommodation before, or at the time of transplantation. In addition, our detailed examination of the *in vitro* human anti-pig cellular xenoresponses, like those from other groups [38, 39], has established a rationale for the design of tailored immunosuppressive strategies. In this context, targeting and tolerizing xenospecific T-cells may be easier to achieve than has been possible for allospecific cells; for example, pig-specific monoclonal antibody therapy, targeting molecules involved in T-cell activation, may be an effective means of inducing tolerance in graft-reactive T-cells (see Fig. 12 for other examples).

The next major challenge in xenotransplantation research, now that hyperacute rejection has been overcome, is to exploit these opportunities in full to achieve long-term graft survival and reduce the need for systemic immunosuppression.

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