THE HOST RESPONSE IN EXPERIMENTAL CORNEAL XENOTRANSPLANTATION

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SUMMARY

In addressing the worldwide shortage of human donor cornea for transplantation, animal cornea may be a substitute if mechanisms of xenogeneic (cross-species) rejection can be identified and controlled. Xenotransplantation of solid organs is followed by hyperacute rejection within minutes due to humoral graft rejection. In an experimental model corneal xenografts in rats survived for 2–3 days, depending on the phylogenetic disparity of the donor animal. Endothelial injury was the specific cause of graft failure, probably mediated by humoral rejection mechanisms. A later cell-mediated rejection response was seen. The potent humoral response is the most important feature differentiating xenograft from allograft rejection.

Xenogeneic transplantation is the transplantation of tissues from a member of one species to that of another. In the past, clinical xenotransplantation of baboon and monkey kidneys and hearts in a small number of reported cases was followed in each recipient by short graft survival due to uncontrolled humoral and cellular rejection.[1–4] The improved survival of clinical organ allografts in the 1980s, due largely to successful immunosuppression, has led to a shortage of human donor organs (in particular heart and liver) and recent renewed interest in xenotransplantation.[5,6] In view of the recent and imminent advances in xenografting of solid organs, animal cornea would be a possible alternative to human donor material for corneal replacement, should control of the host xenogeneic response be possible. As in the case of solid organs, the use of xenogeneic tissue might alleviate the shortage of donor corneas.

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Experimental Organ Xenograft Rejection

Early general experimental studies illustrated that, in general, xenografts were always rejected faster than allografts when similar types of tissues were transplanted under similar circumstances, and the phylogenetic disparity between donor and recipient species was related to the rapidity of rejection.[7,8] At around the same time as this became evident, it was recognised that pre-existing 'natural' antibody and subsequently complement components, platelets and vasoactive substances were involved in the immediate hyperacute rejection observed in many experimental xenograft models.[9] In more recent investigations of experimental xenograft rejection, it has become apparent that mechanisms vary among different animal models and that graft survival may depend more on the organ or tissue transplanted than on the species disparity (reviewed by Auchincloss[5]).

Models of xenotransplantation of immediately-vascularised organs, such as kidney or heart, demonstrate invariable hyperacute antibody-mediated rejection within minutes of graft in discordant donor–host combinations. The characteristic pathological feature of hyperacute rejection is intragraft interstitial haemorrhage with margination of leucocytes and thrombosis clearly shown to occur in donor vessels.[10,11] The major cell surface antigen recognised by pre-formed natural antibody is the terminal disaccharide α-1–3 galactose epitope, i.e. galactose in an (α1–3) linkage with galactose.[12–14] This is abundantly expressed on the various cells of non-primate mammals, possible xenograft donor animals. The antibody which reacts with this antigen is present in all humans. Binding of pig endothelial cell surface oligosaccharides by human pre-formed antibody has been demonstrated.[14,15] The roles of pre-formed natural antibody and complement in initiating hyperacute rejection have been clarified to

an extent. The consensus of opinion is that hyperacute rejection is mediated by pre-formed antibody via activation of the classical or alternative pathways of complement.[16–20] The molecules which protect autologous cells from complement-mediated injury are membrane-bound complement regulatory proteins CD46 (membrane cofactor protein), CD55 (decay accelerating factor) and CD59 (protectin). Unlike complement, these molecules are highly species-specific: graft injury by recipient complement is likely to occur unless recipient complement regulatory proteins are expressed on xenograft cells. It is clear from all reports that hyperacute rejection must be understood and controlled or circumvented if xenotransplantation is to reach clinical practice.

In contrast to vascular organs, survival of some tissue xenografts can be achieved even in species combinations that demonstrate hyperacute rejection of immediately-vascularised organs. Calne examined the effect of xenografts in dogs of goat kidney and skin. Renal graft rejection at 30–90 minutes contrasted with skin survival for 6 days.[21] The cornea, being avascular, might be considered likely to undergo rejection in a pattern more similar to skin than to immediately-vascularised organs.

Corneal Xenotransplantation

The first recorded human transplant operation was in fact a corneal xenograft performed by Kissam in New York in 1838. The patient was blind from staphyloma and the donor cornea was from a 6-month-old pig. It appears from the report that the graft perforated within 1 month.[22] It was to be more than a century before the human xenotransplantation of any other tissue was reported, but all reported lamellar and penetrating corneal grafts performed until the late nineteenth century were xenografts. Von Hippel reported in 1888 a clear lamellar corneal xenograft in a young girl in which the donor was full-thickness rabbit cornea excised using a clockwork trephine which he had himself designed.[23] However, all other available reports indicate that xenografts failed. With the recognition of the superior survival of human donor cornea, reports of clinical corneal xenografts have been only anecdotal in the last century.

Other interesting early studies in xenotransplantation involved the eye. Sixty years ago Harrison described xenotransplantation of the optic cup in salamander embryos. Recipients developed into xenogeneic chimaeras with entire eyes derived from other species.[24] The natural tolerance of embryos was taken for granted. Of course these embryological experiments and the earlier clinical corneal xenografts pre-dated the understanding of the immunological basis of graft tolerance and rejection. First investigations of the immunological response to corneal xenografts were reported in 1952. In experimental studies of *interlamellar* corneal xenografts, partial thickness donor cornea was transplanted into a pocket in the recipient rabbit cornea.[25–29] Rabbit interlamellar corneal xenografts survived for a mean 9.9, 10.2 and 8.8 days for guinea pig, human and chicken donors respectively in a later study.[30] In 1962, Kuwabara described *lamellar* rabbit xenotransplants of chicken, cat, dog and human tissue.[31] Orthotopic, *penetrating* grafts are most relevant to human corneal transplantation. In 1964, a series of 10 patients in Thailand were described who received penetrating grafts of gibbon cornea.[32] Five of the grafts were stated to remain transparent for more than 5 months. Vascularisation, inflammation and graft necrosis was found on pathological examination of failed grafts.

More recently, Ross and colleagues reported 10 rat penetrating xenografts of guinea pig donor cornea. These survived between 6 and 9 days – presented as evidence that hyperacute rejection of guinea pig-to-rat corneal xenografts does not occur.[33] In the same donor–recipient combination, heart xenografts survived 17±4 minutes in 17 rats, underlining the disparity in survival between cornea and heart xenografts. We have developed another rat model of penetrating corneal xenotransplantation, using several donor–recipient combinations, in which the immunopathology of rejection has been examined in an attempt to define the mechanism of rejection responsible for eventual graft loss.

EXPERIMENTAL MODEL

Donor–Recipient Combinations

Experimental animals, surgical and examination procedures are reported in detail elsewhere[34,35] and described here in brief. Outbred guinea pigs and chickens were used as corneal xenograft donors. Inbred adult Fischer 344 (F344) and congenitally athymic CBH *rnu/rnu* rats were used as corneal graft recipients.

A unilateral 3 mm diameter penetrating corneal graft was performed on each recipient rat. No immunosuppressive agent was given at any time. Recipient eyes were examined daily following graft for up to 14 days and the day of rejection was defined as that on which, in a graft that had previously been clear with or without intact graft epithelium, graft transparency was lost to the extent that iris vessels were not visible through the graft (Fig. 1).

Characteristics of Rat Corneal Xenograft Rejection

Xenograft rejection was observed in all cases. Survival of chicken donor and cornea was slightly shorter than that of guinea pig (median survival 2 and 3 days respectively). Corneal graft epithelial defects and anterior chamber haemorrhages were

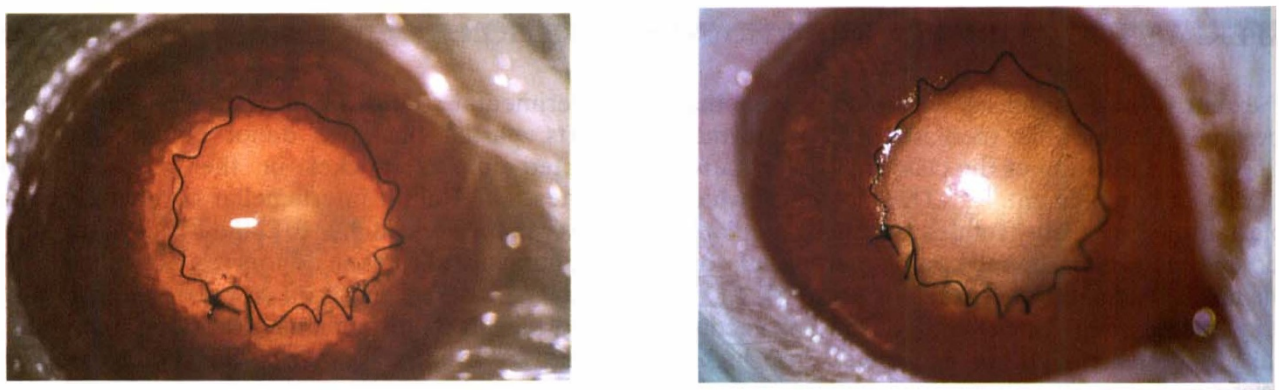

Fig. 1. *Left: Guinea pig donor corneal xenograft 3 days following graft. Donor and recipient corneal oedema is seen. Right: The same xenograft 7 days following graft, showing graft oedema and recipient corneal vascularisation.*

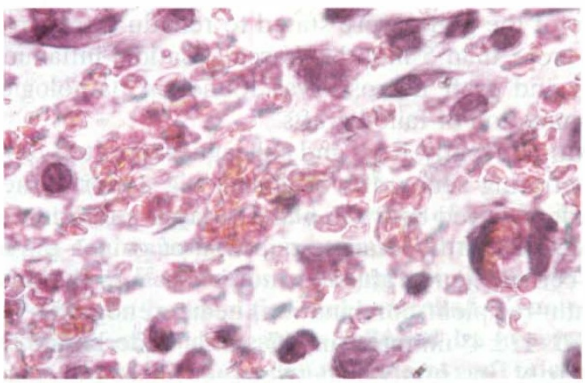

Fig. 2. *Erythrocyte extravasation in recipient cornea 4 days following graft. (H&E, original magnification ×1000).*

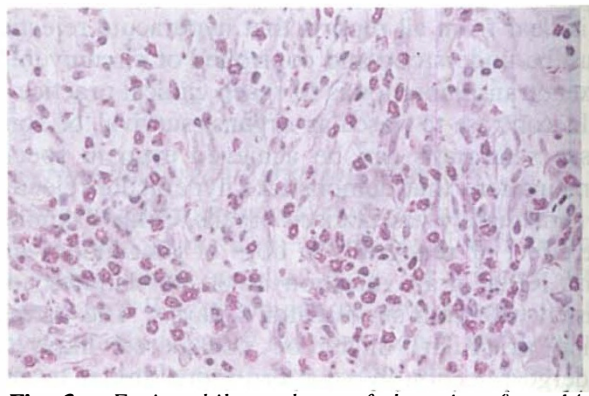

Fig. 3. *Eosinophils at the graft–host interface, 14 days following chicken donor xenograft. (H&E, original magnification ×400)*

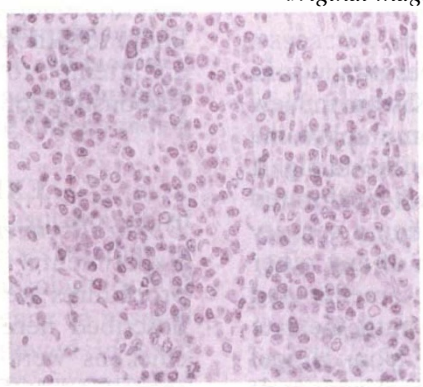

Fig. 4. *Massive infiltration of immature, blast lymphocyte forms 14 days following graft. These cells stained with CD2 monoclonal antibody on immunoperoxidase staining, confirming their T cell lineage. (H&E, original magnification ×400)*

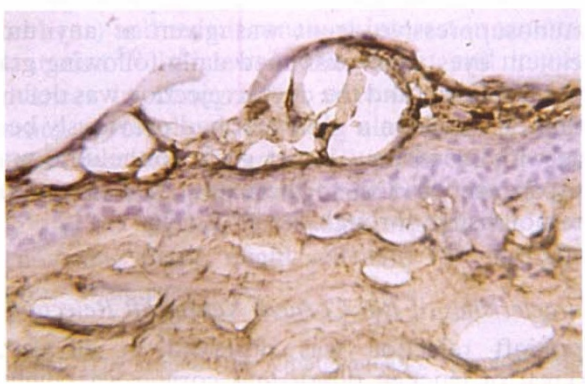

Fig. 5. *Chicken donor corneal xenograft 12 hours following transplantation. Intense superficial epithelial staining with mouse anti-rat IgG_{2a} monoclonal antibody is shown. (Original magnification ×400)*

Fig. 6. *Corneal endothelial silver stain 4 days following xenograft. Only peripheral graft cellular staining is seen. (Original magnification ×100)*

evident in many grafted eyes. Graft survival was similar in athymic and euthymic rats.

Histopathological Correlates. In all xenograft specimens at day 4, interstitial haemorrhage was seen in recipient corneal stroma (Fig. 2). Significant numbers of xenograft stroma-infiltrating macrophages and granulocytes were observed from day 7, with CD4+ cells aggregated on the endothelium. At day 14, the stroma of donor cornea and the host–xenograft interface were largely replaced by inflammatory cells. Eosinophils were a prominent constituent of the infiltrate in all xenografts in immunocompetent recipients, in maximum numbers at the host–graft interface (Fig. 3). Massive xenograft infiltration was a uniform finding: cells comprised blast lymphocyte forms (Fig. 4) (staining with the T cell surface marker CD2), CD4+ cells and macrophages. Specimens from athymic xenograft recipients at 7 and 14 days post-graft showed epithelial loss, endothelial loss and interstitial haemorrhage similar to corneas from euthymic rats. In striking contrast, however, there was only a very sparse graft infiltrate and negligible eosinophils.

Rat Immunoglobulin Deposition on Xenografts. Strong and diffuse IgG_{2a} monoclonal antibody staining was demonstrated by immunoperoxidase staining on xenograft surfaces at 12 and 24 hours post-graft, suggesting early recipient antibody-mediated damage to graft surfaces (Fig. 5).

Xenograft Endothelial Damage. Corneal whole mount endothelial silver staining at day 3 post-graft showed central graft endothelial loss (Fig. 6).

Rat Antibody to Guinea Pig and Chicken Cells. Sera from peripheral blood samples taken pre- and post-graft were tested for reactivity with unfixed guinea pig and chicken white blood cells (WBC) by indirect immunofluorescence on flow cytometry. All euthymic rats examined exhibited natural antibodies reactive with up to half of the peripheral WBC of both the guinea pig and chicken.

DISCUSSION

Clinical and histological examinations indicate a similar host response to guinea pig and chicken donor cornea. Possibly reflecting greater species disparity and higher host levels of pre-formed antibody, the response to chicken was observed to be more intense. Chicken donor corneal survival was shorter, epithelial loss more extensive and frequently seen, intraocular haemorrhage more frequent, and graft infiltration evident earlier on histological examination in all cases. Survival of guinea pig cornea was similar in euthymic and athymic rat recipients.

Corneal Xenograft Rejection Effector Mechanisms are Primarily Humoral

(1) The finding of pre-formed antibody to guinea pig and chicken leucocytes in all recipient specimens examined, is the first evidence from this model suggesting a role for humoral mechanisms in graft rejection. Pre-formed rat antibody to guinea pig surface antigens has been detected by some investigators (Reding *et al.* by radioimmunoassay of anti-RBC antibodies,[36] Leventhal *et al.* by lymphocytotoxicity and platelet membrane-targeted immunoassay[37]), but negligible levels of antibody have been reported by others (Terasaki *et al.* by lymphocytotoxicity,[38] Miyagawa *et al.* by haemagglutination[16]). We suggest that pre-formed antibody cross-reacts between leucocytes and corneal cells and is responsible for the early corneal xenograft injury found. Xenogeneic pre-formed antibodies have recently been shown to display cross-reactive idiotypes[39] and can bind to multiple ligands:[40] this is in contrast with most antibodies that arise after immunisation, which are monoreactive and bind only to the immunogen. Our finding of pre-formed antibody in athymic rats is consistent with the observation that pre-formed antibodies derive in part or predominantly from B cells expressing the CD5 glycoprotein.[40,41]

(2) Defects in the graft epithelial layer almost certainly represent clearance of dead cells, at an interval after cell death. It is possible that death of epithelial cells occurred in all xenografts, with only those in which it was extensive developing clinically manifest defects, partial or complete. This is the second piece of evidence which suggests humoral, hyperacute rejection, mediated by pre-formed antibody in the tear film which is in direct contact with the epithelial layer.[42] Replacement by recipient epithelium was, however, observed in all cases. The endothelium also manifested cell damage, in this instance probably due to damage by antibody in the aqueous humour. Unlike the tear film, this fluid is in normal circumtances protein-free due to the effect of the blood–aqueous barrier through which large molecules do not penetrate even if plasma concentrations are high.[43] Following surgery, circulating pre-formed antibody might enter the aqueous and reach the graft endothelium via Schlemm's canal or leaking uveal vessels.[44]

(3) Deposition of rat IgG and IgM on epithelial and endothelial xenograft surfaces and the anterior chamber exudate within 24 hours of graft further support the major role of antibody in rejection.

(4) Indirect evidence of humoral rejection is the equivalent xenograft survival in athymic and euthymic recipients. Median survival time for guinea pig donor cornea was 3 days in both immunocompetent F344 and athymic CBH *rnu/rnu* rats.

(5) Finally, the rapid tempo of rejection, with demonstrable signs of graft failure within 2–3 days of graft in most xenograft recipients, suggests humoral rather than cell-mediated effector mechanisms. Rejection at this interval post-graft is much earlier than would be expected in primarily cell-mediated rejection, and earlier than reported by other investigators.[33]

While the dominant role of humoral mechanisms in corneal xenograft rejection is proposed, the relative contributions of natural antibody and complement to corneal xenograft tissue injury have not been delineated in this model. Such a study would necessitate selective antibody or complement depletion. Many of the complement pathway components have been shown to be present in tears,[45] aqueous humour[45] and cornea[46] of normal humans. Strong expression of each of the complement regulatory proteins CD46, CD55 and CD59 has been demonstrated in human corneal epithelium but not endothelium:[47] this suggests the possibility of chicken and guinea pig corneal epithelial injury by rat complement.

Cellular Immunity and Eosinophils are Involved in the Later Response to Corneal Xenografts

Our finding of similar graft survival in athymic and immunocompetent mice is evidence that T cells or other thymic factors do not play a major role in corneal xenograft rejection. This conflicts with previous studies proposing cell-mediated responses to have a major role in xenograft rejection, such as one report of indefinite skin xenograft acceptance in athymic mice.[48] Nevertheless, early graft injury was followed in immunocompetent rats by evidence of a cell-mediated response. The graft-infiltrating cell population included CD4+ and CD8+ cells, macrophages and neutrophils. Eosinophils were the prominent cell type at the host–graft interface in specimens at day 14 post-graft. An eosinophil infiltrate has previously been reported in interlamellar sheep-to-rabbit xenografts.[25] Eosinophils have not been reported in human or experimental corneal allograft rejection, but have recently been reported in liver allograft rejection.[49] Eosinophils in the cornea could be directly involved in tissue damage or might be a non-specific indicator of substantial immune activation. These cells are antibody-dependent cytotoxic granulocytes and their presence in rejected xenogeneic cornea may be due to differentiation or migration as a result of local lymphokine release. Absence of eosinophils in athymic recipient corneas suggests that migration of tissue eosinophils is dependent on T cells or other thymus factors absent in athymic rats: this finding concurs with the absence of an eosinophil response to helminth infection in athymic mice.[50] Interleukin-5 is the stimulant most specific for the eosinophil lineage, inducing late proliferation and differentiation in blast cells.[51] Notwithstanding, selective eosinophil production in the setting of a massive xenogeneic response may be less likely than multilineage stimulation by GM–CSF or IL-3, which induce differentiation of neutrophils and macrophages in addition to eosinophils.[52]

Recipient Vascular Injury is a Component of Xenogeneic Rejection

Interstitial haemorrhage from graft vessels is the pathognomonic histological feature of hyperacute rejection. In rejected immediately vascularised organ grafts haemorrhage from donor vessels is clearly seen.[10,11] The finding in this model of interstitial haemorrhage from corneal vessels and contemporaneous haemorrhage from the iris is of particular interest, indicating damage not to xenograft but to recipient vessels. It is direct evidence of this phenomenon in graft rejection. Haemorrhage from recipient vessels has not been formally demonstrated in any other tissue. The pathogenesis of corneal recipient vascular damage is uncertain. The transient nature of haemorrhage from host vessels (observed at days 2–4 only) and the absence of associated host tissue damage support the concept that local recipient vascular leakage is a necessary component of the xenogeneic response which allows humoral factor and inflammatory cell access to the avascular graft. Activation of the alternative complement pathway may by some mechanism be induced, followed by deposition of complement components on recipient corneal vascular endothelium.

Xenograft Rejection Differs Substantially from Allograft Rejection, with Implications for Immunosuppression Regimes

The strength and the prominence of humoral rather than cell-mediated mechanisms of rejection are the dominant features which distinguish organ xenograft from allograft rejection. These studies have confirmed this to be the case also for xenografts of cornea, with rejection in all cases and shorter graft survival. Loss of corneal transparency due to endothelial damage was evident in this study as early as day 2–3 following graft. The experimental model demonstrates evidence for multiple component mechanisms of xenograft rejection. Suppression of both humoral and cell-mediated components of the recipient immune system would be required to allow long-term corneal xenograft survival, and such pan-immunosuppression would not be justifiable in clinical management with present drugs. An alternative approach would be to prevent xenoantigen recognition and rejection by modification of the donor cornea, using gene-based or other techniques.

If humoral rejection can be controlled it will then be necessary to identify the potential donor animal species with the most appropriate corneal size and refractive index. Corneal xenotransplantation is likely to reach clinical practice eventually, but the immunological factors that need to be understood and overcome to allow its application are formidable.

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Key words: Cornea, Rejection, Xenotransplantation.

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