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Hydrolase participation in allograft rejection in rat penetrating keratoplasty

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Abstract • Background: A rat model of orthotopic corneal graft rejection was used to investigate the alterations in hydrolase activity within the corneal graft or within cellular infiltrates during acute rejection. • Methods: The distribution of the lysosomal enzymes [acid phosphatase (AP), *N*-acetyl-β-D-glucosaminidase (NAG), β-glucuronidase (β-Gluc), β-galactosidase (β-Gal), dipeptidylpeptidase II (DPPII)] and of the membrane-bound proteases [aminopeptidase M (APM), aminopeptidase A (APA), γ−glutamyltransferase (GGT), alkaline phosphatase (ALP), dipeptidylpeptidase IV (DPPIV)] were investigated by histochemical methods in the grafts at 3, 5, 8, 10 and 12 days following allogeneic transplantation. Serial sections of the grafts were also examined for RT1b, CD4, CD4+, CD8, CD11b/c and CD45, in order to determine hydrolase activity within infiltrating cells. • Results: Allogeneic grafts were invaded by macrophages, CD4- and CD8-positive lymphocytes. In contrast, syngeneic grafts, performed as a control, contained occasional lymphocytes and focal aggregations of macrophages around suture sites. The allogeneic cellular infiltrate stained intensely for AP and ALP; moderately for β-Gluc, NAG and β-Gal; and mildly for GGT, DPPII and APM in grafts at all postoperative times. Serial sectioning indicated that the majority of the lysosomal hydrolases were located in macrophages; AP, APM and GGT were, however, observed in lymphocytes. Vessel ingrowth could be observed with enzyme staining for AP, β-Gluc, NAG, ALP, APA and APM. Hydrolase activity in the corneal endothelium served as an indicator of endothelial function during the rejection process. • Conclusion: Changes in normal hydrolase activities in corneal grafts in the rat model indicate decreasing corneal function during the rejection process. Hydrolases released from infiltrating cells contribute to the morphological disruption and, possibly, to graft rejection.

Introduction

As immunologic rejection remains the leading cause of graft failure in human corneal transplants [16], various animal models have been used to study the mechanisms of the rejection process. The rabbit model, used by earlier investigators [2, 3, 15, 17, 20, 27], allowed the examination of rejection in an orthotopic graft. A disadvantage of the rabbit model was the impossibility of ascertaining the histocompatibility differences between donor and recipient [36]. Although the well-defined immunogenics of inbred mice offered a solution to this problem, orthotopic grafts were very difficult due to the relatively small mouse eye. Heterotopic corneal grafts to

the thoracic cage were, therefore, used to study the immunobiology of corneal allografts in the mouse [32]. In 1985, Williams and Coster developed a rat model of penetrating orthotopic keratoplasty which combined the advantages of the rabbit and mouse models [36]. Although some histopathological studies of the corneal graft in this model have been undertaken [1, 6, 13, 23, 36], investigations of the participation of hydrolases following keratoplasty in the rat are few. Located either within lysosomes or on the cell membrane, hydrolases break down tissue components and play a significant role in a number of processes, including phagocytosis [31], collagen turnover [24] and, in pathology, both the inflammatory and immune responses [35].

The alterations in corneal activities of five lysosomal enzymes [acid phosphatase (AP), β-glucuronidase (β-Gluc), *N*-acetyl-β-glucosaminidase (NAG), acid-β-galactosidase (β-Gal), dipeptidylpeptidase II (DPPII)] and five membrane-bound proteases [aminopeptidases M (APM) and A (APA), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), dipeptidylpeptidase IV (DPPIV)] were examined in a rat model of orthotopic corneal graft rejection. Serial sections of the grafts were also examined immunohistochemically for RT1b, CD4, CD4+, CD8, CD11 b/c and CD45 to determine the hydrolase activity of infiltrating cells.

Methods

Female Lewis rats ($RT1^l$) and Lewis-Brown Norway ($RT1^{lxn}$), approximately 15–20 weeks old and weighing between 200–300 grams, were purchased from the Central Institute for Laboratory Animal Breeding, Hannover. The Lewis rats were used as recipients in all cases; the Lewis-Brown Norway rats served as donors. The animals were treated according to the provisions established by the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Surgical procedure

Anaesthesia of both donor and recipient rat was achieved with an intraperitoneal injection of Rompun (Bayer; 2 mg/kg) and Ketanest (Parke-Davis; 25 mg/kg). Twenty minutes prior to surgery, the Lewis rats also received 0.5 mg/kg of atropine (Eifelfango) subcutaneously. Neosynephrine-POS (5%; Ursapharm) eyedrops were also placed on the operative eye.

Two donor corneal buttons (3.5 mm) were harvested from the donor rat using a trephine and curved Castroviejo scissors. The donor animal was then killed by ether inhalation. The left eye of the recipient Lewis rat was prepared by removing a central 3.0-mm button as above. A drop of methylcellulose was placed over the 3.0-mm corneal opening, before the donor cornea was fixed with 10–12 interrupted sutures (11–0 nylon, Ethicon). The anterior chamber was not re-established following surgery. Prior to closure of the left eyelids with 3–4 interrupted sutures (7–0 nylon, Ethicon), Polyspectran (Alcon Thilo) eye ointment was placed over the operated eye. This antibiotic coverage was also administered on removal of the eyelid suture and was the only form of treatment that the animals received postoperatively. A total of 25 penetrating keratoplasties were performed in the allogeneic group; a control group of seven Lewis rats received syngeneic grafts. The corneal sutures were not removed.

Postoperative care

The eyelid suture was removed 48 h following surgery, allowing for the first assessment of the cornea on the slit-lamp microscope by a masked observer. Slit-lamp evaluations were performed every 2–3 day under Ketanest anaesthesia, with assessment of the corneas following scoring system of graft opacity, oedema and vascularization [13]. Graft opacity was scored from 0 to 4:0 = clear cornea; 1 = slight haze; 2 = increased haze with view of anterior chamber; 3 = increased haze without view of anterior chamber; 4 = opaque cornea. Graft oedema was scored from 0 to 4:0 = no corneal thickening; 1 = slight corneal thickening; 2 = diffuse oedema; 3 = diffuse oedema with microcystic oedema of epithelium; 4 = diffuse oedema with bullous keratopathy. Graft neovessels were scored from 0 to 4:0 = no vascularization; 1 = peripheral cornea; 2 = neovessel growth to wound edge; 3 = vessels on graft; 4 = total vascularisation. Thirteen animals were killed by ether inhalation when corneal rejection was considered to have occurred (rejection score greater than 6). In addition, three animals were killed on each of postoperative days 3, 5, 8 an 10. The eyes of all animals were enucleated and immediately frozen in liquid nitrogen-cooled Isopentane (J.T. Baker) using the embedding medium Tissue Tek (Miles, USA) and stored at −60°C. The syngeneic group were evaluated every 2–3 days as above on the slit-lamp microscope for the first 14 days and then weekly until the end of the trial (40 days). The animals were then killed and the eyes prepared for histological examination as described above. Animals in which technical problems occurred were excluded from the study.

Histochemistry

Cryostat sections (10 μm) were transferred to semipermeable membranes (Sigma) and examined using the simultaneous azocoupling technique for the following lysosomal enzymes: AP [naphthol AS-BI-phosphate (Sigma) and coupling agent, rosanilin (Sigma)]; β-Gluc [naphthol-AS-BI-glucuronide (Sigma) and rosanilin]; NAG [naphthol-AS-BI-*N*-acetyl-β-D-glucosaminide (Sigma) and rosanilin]; and DPPII [Lys-Ala-4-methoxy-2-naphthylamide (MNA; Sigma) and Fast Blue B (FBB; Sigma)] [19]. β-Gal was demonstrated by the indigogenic method using 4-Cl-5-Br-3-indolyl-β-galactoside (Sigma) as a substrate and a ferri-ferro mixture as coupling agent [19]. The specimens were incubated at 37°C for 12 h, fixed in 4% formaldehyde, rinsed in distilled water and mounted in a glycerol-gelatin mixture [19].

The simultaneous azocoupling method was also used to demonstrate the presence of APM [substrate L-Ala-MNA (Sigma, Australia) and FBB]; APA [α-glutamyl-4-MNA (Sigma) and FBB]; GGT [γ-glutamyl-MNA (Sigma) and FBB]; DPPIV [Gly-Pro-4-MNA (Sigma) and FBB]; and ALP [5-Br-4-Cl-3-indoxylphosphate (Bissendorf Biochemicals, Germany) and tetranitro tetrazollium blue chloride (TNBT; Serva)] [19]. Serial cryostat sections (10 μm) were collected on slides and incubated for 1 h at 37°C [19]. The slide preparations were fixed in 4% formaldehyde, rinsed in distilled water and mounted using Kaiser's gelatine solution (Merck, Germany). The histochemical controls undertaken included the omission of respective substrates and coupling agents; changes in the pH of buffer solutions required for the dissolution of each substrate and coupling agent; and variation of the incubation period.

Immunocytochemistry

Cryostat serial sections (10 μm) were transferred to glass slides and examined immunohistochemically using a standard proce-

Table 1 Monoclonal antibody (MoAB) specifications

MoAB	Clone designation	Specificity
RT1b	OX-6	Rat MHC class II antigen, I-A equivalent on peripheral B cells, thymic cortical epithelial and medullary reticular cells and a subset of thymocytes [10, 22]
CD4	OX-38	CD4 antigen on T-helper cells, thymocytes and macrophages; similar epitope of CD4 as that recognised by W3/25 moAb [14]
CD4+	OX-40	Reacts with activated CD4+ T blasts [25]
CD8	OX-8	Reacts with CD8 antigen on thymocytes and T-suppressor/cytotoxic cells; not with T-helper cells [5]
CD11 b/c	OX-42	Recognizes the rat homologue of the iC3b receptor found on most granulocytes, monocytes and macrophages [30]
CD45	OX-1	Reacts with all molecular forms of CD45 (leucocyte common antigen) [33]

dure [8]. Briefly, the specimens were initially fixed in acetone for 10 min before being incubated in the primary mouse anti-rat monoclonal antibodies, which included RT1b, CD4, CD4+, CD8, CD11 b/c and CD45 (Pharmingen, Germany) (Table 1). The secondary antibody used was rat anti-mouse (DAKO, Denmark). Visualisation of the binding sites was performed using the APAAP complex (DAKO, Denmark). Specimens were then counterstained with haematoxylin and mounted using Kaiser's gelatine solution (Merck, Germany). The immunohistochemical controls undertaken included omission of primary antibodies and substitution with either TBS or non-immune sheep serum.

All specimens were evaluated by two independent observers without knowledge of their postoperative age. Specimens were graded for the intensity of enzyme staining within the cornea: 0 = negative, 1 = mild, 2 = moderate, 3 = strong, 4 = intense. The number of infiltrating cells per square millimetre was also determined in the peripheral, central anterior and central posterior cornea by the observers using a 10 × objective (Zeiss microscope).

Results

Syngeneic group

On removal of lid sutures in both the syngeneic and allogeneic groups, the grafts were clear and characterised by very mild postoperative oedema and no vascularisation. In six of the seven syngeneic grafts, the oedema resolved within the first 7–10 days; the last animal was excluded due to cataract formation. Weekly slit-lamp examination until the 6th postoperative week revealed clear grafts with minimal oedema and peripheral vascularisation only. Histological examination of the grafts after 40 days revealed mildly swollen grafts with focal cellular infiltrates, consisting of macrophages around the sutures (Figs. 1, 2). The endothelium was intact (Fig. 1). In contrast to the allogeneic grafts (Figs. 3 and 4), a large influx of CD45 positive cells or an alteration in the normal corneal Class II antigen distribution was not observed in the syngeneic corneal graft. Hydrolase distribution and activity throughout the cornea was similar to that observed in the normal Lewis rat [9]; however, strong AP, β-Gluc, β-Gal and ALP activities were observed in the few inflammatory cells present.

Allogeneic group

Within the allogeneic group, the average corneal rejection occurred on the 12th postoperative day. The changes in enzymatic activity and the corresponding immunohistochemical changes observed on the 3rd, 5th, 8th, 10th and 12th postoperative days are described below (Table 2).

Postoperative day 3

Clinically, the graft by the 3rd day was observed to be transparent with minimal oedema and completely avascular (rejection rating of 0 or 1). On cryostat sectioning, the anterior one-fifth of the stroma was penetrated by a mild cellular infiltrate, numbering 80 cells/mm^2 in the peripheral cornea and 60 cells/mm^2 centrally. Although these cells extended across the graft, concentration of cells did occur around suture sites. Cells within the infiltrate stained intensely for AP (Fig. 5) and ALP; moderately for β-Gluc, NAG and β-Gal (Fig. 6); and mildly for GGT (Fig. 7), DPPII and APM. Immunocytochemistry suggested that the cellular infiltrate in the graft 3 days after operation consisted of T-helper (CD4) cells, T-suppressor/cytotoxic (CD8) cells and macrophages in a ratio of 1.5:1:1.3. Serial sectioning revealed that the many infiltrating cells expressed class II antigens. In both the periphery and the central-anterior cornea, CD45-positive cells were apparent (Fig. 3); CD4+ blasts were rare in the grafts at all postoperative times.

Consequential to the cellular infiltrate was a disruption in the stromal morphology in the anterior one-fifth of the cornea with an apparent reduction in the central keratocyte activity for AP, β-Gal, β-Gluc, DPPII, ALP, GGT and APM. In the peripheral (recipient) corneal epithelium, an increase in GGT activity was observed. No changes were observed in the corneal enzyme activities of DPPIV and APA. While APA is weakly present in some anterior and peripheral keratocytes in the normal Lewis rat cornea, DPPIV activity is limited to the corneal endothelium, where it stains strongly [9]. The posterior cornea was virtually free of infiltrate and, consequently, the anatomical arrangement was relatively normal.

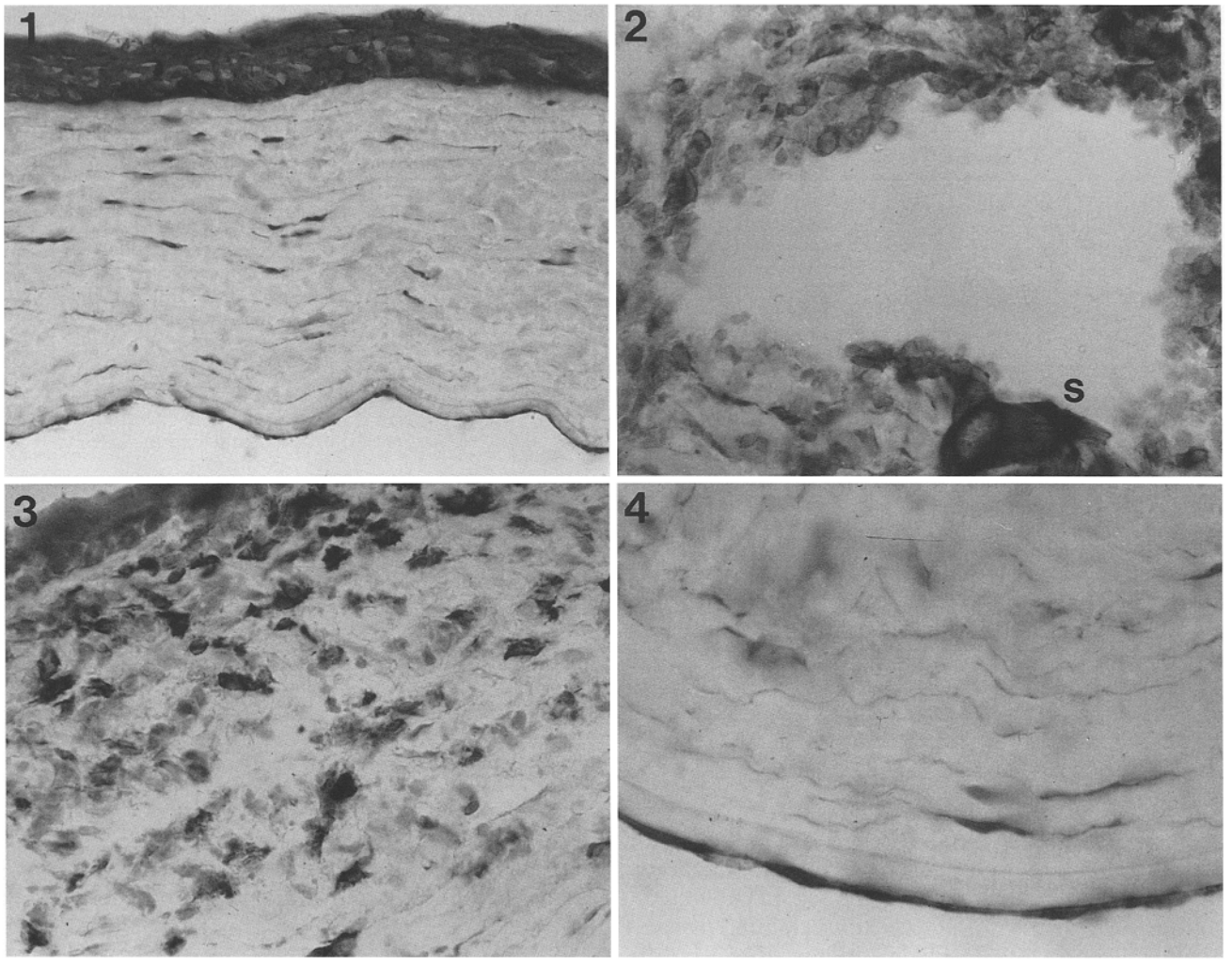


Fig. 1 Syngeneic corneal allograft (40 days postoperative; ×10 objective)

Fig. 2 CD11-positive cells accumulated around a suture site (*S*) in a syngeneic graft (40 days postoperative; ×63 objective)

Fig. 3 Antero-centrally situated CD45-positive infiltrating cells in an allogeneic allograft (5 days postoperative; ×40 objective)

Fig. 4 The endothelium of an allogeneic allograft staining for RT1b (class II) (5 days postoperative; ×40 objective)

Postoperative day 5

Clinical observations of the graft on postoperative day 5 usually included slight haziness, slight or focal oedema and vascularisation of the peripheral cornea (rejection rating of 3). On histology, the width of the cellular infiltrate had increased, occupying the anterior third of the graft with resulting increased morphological disruption of the anterior and central keratocytes. Similarly, the number of cells per square millimetre increased to 100–120 and consisted of CD4 and CD8 cells and macrophages in a ratio similar to that mentioned above. An increased number of CD45-positive cells was also observed centrally. Consistent hydrolase activity, as described above, was observed in the infiltrate. While decreased hydrolase activity was observed in anterior keratocytes, posterior keratocytes retained their hydrolase activities. The endothelium remained morphologically – and, for most hydrolases, enzymatically – intact on the 5th postoperative day (Fig. 8). In the majority of specimens, the corneal endothelium was also RT1b-positive by postoperative day 5 (Fig. 4).

Postoperative day 8

Clinically, the grafts on the 8th postoperative day were observed to have increased haze; however, the iris was still visible. The grafts had become diffusely oedematous with vascularisation approaching the wound rim (rejection rating of 5). Histologically, the observations were similar to those detailed above; the cellular infiltrate,

Table 2 Histological changes observed during acute rejection in the rat penetrating keratoplasty model (*PO* Postoperative, *CD*4 T-helper cells, *CD*8 T-suppressor/cytotoxic cells, *Mø* macrophages)

PO day	Rejection rating	Width of infiltrate	Cells per mm^2	Cell ratio (CD4:CD8:Mø)	Hydrolases in infiltrate	Corneal hydrolases
3	0–1	Anterior 1/5 of cornea	60–80	1.5:1.3:1.3	Strong: AP, ALP Moderate: β-Gluc, NAG, β-Gal Mild: GGT, DPPII, APM	Decreased anterior keratocyte activity for AP, βGal, β-Gluc, DPPII, ALP, GGT and APM. Increased epithelial GGT (periphery). Normal endothelial hydrolase staining
5	3	Anterior 1/3 of cornea	100–120	1.5:1.3:1.3	As above	Decreased keratocyte activity in infiltrated area for hydrolases above. Normal hydrolases in posterior keratocytes and endothelium (now RT1b positive)
8	5	Anterior 2/3 of cornea	100–120	1.5:1.3:1.2	As above	Neovessels positive for AP, β-Gluc, NAG, ALP, APA and APM neor wound rim
10	6	Anterior 4/5 of cornea	100–120	1.5:1.3:1.3	As above	Noevessel growth ti rim Weaker hydrolase activity in endothelium
12	>6	Entire corneal width	100–120	1.5:1.5:1.2	As above	Advanced neovascularization Weak or absent endothelial activity (ALP, DPPIV)

however, occupied the anterior two-thirds of the graft. An increase in the cell number per square millimetre or a significant change in the cell ratios was not apparent. The ingrowth of vessels towards the region of the graft could be observed, with some sections staining particularly for the enzymes AP (Fig. 5), β-Gluc, NAG (Fig. 9), ALP, APA and APM. Lymphocytes were apparent within or surrounding the lumina of the neovessels (Fig. 10).

Postoperative days 10–12

By the 10th postoperative day, the majority of grafts had increased haze, with a difficult view of anterior chamber structures, and growth of vessels to the graft rim (rejection rating of 6). Rapid deterioration of the graft occurred over the following 24–48 h, with further clouding and vascularisation of the graft (rejection ratings greater than 6). Histology revealed swollen grafts, which were totally occupied by a heavy cellular infiltrate consisting of T-helper (CD4) cells, T-suppressor/cytotoxic (CD8) cells and macrophages in a ratio of 1.5:1.5:1.2. Vessel growth around sutures, onto and into the graft could be followed with hydrolase staining. Weak or absent endothelial activity for most hydrolases was observed, indicating both morphological and functional disruption.

Discussion

The alterations in the activities of ten hydrolases, as well as the immunocytochemical findings, in the corneal

Fig. 5 Intense acid phosphatase (AP) activity in cells accumulated around a suture site (*S*) in an allogeneic allograft (8 days postoperative; red staining). On serial sectioning and immunohistochemical staining, the majority of these cells proved to be CD11-positive. Some neovessels (*nv*) are present inferior to the suture site, the endothelium staining for AP (×20 objective)

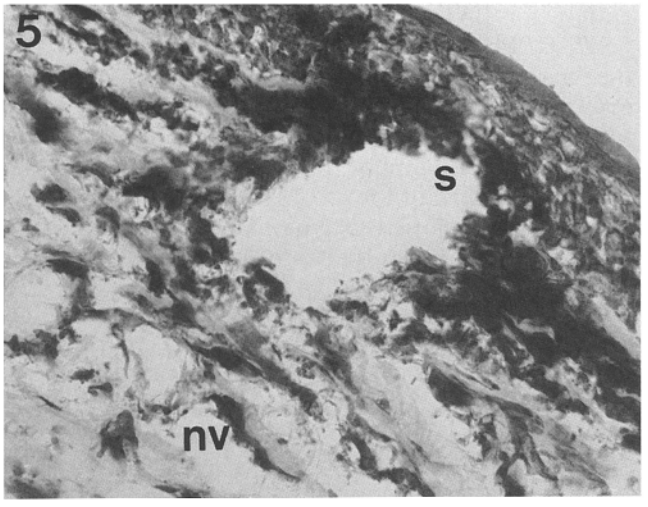
Fig. 6 β-Gal positive cells in the cellular infiltrate of an allogeneic allograft (5 days postoperative; turquoise-blue staining; ×40 objective)

Fig. 7 Mild activity of the membrane-located hydrolase γ-glutamyltransferase. On serial sectioning and immunohistochemical staining, these cells were also positive for CD4 and CD8 (10 days postoperative; gold-brown granular staining; ×100 objective)

Fig. 8 Alkaline phosphatase (ALP) staining of the endothelium of an allogeneic allograft on postoperative day 8 (×40 objective). The normal moderate ALP keratocyte activity is reduced (*arrowheads*), and ALP-positive infiltrating cells are apparent in the posterior allograft (*arrow*)

Fig. 9 NAG staining of a neovessel (*nv*) in an allogeneic allograft on postoperative day 10 (×63 objective)

Fig. 10 New vessel growth in an allogeneic allograft on postoperative day 12 (×63 objective). CD4-positive cells can be observed within and surrounding the vessel lumen (*arrows*)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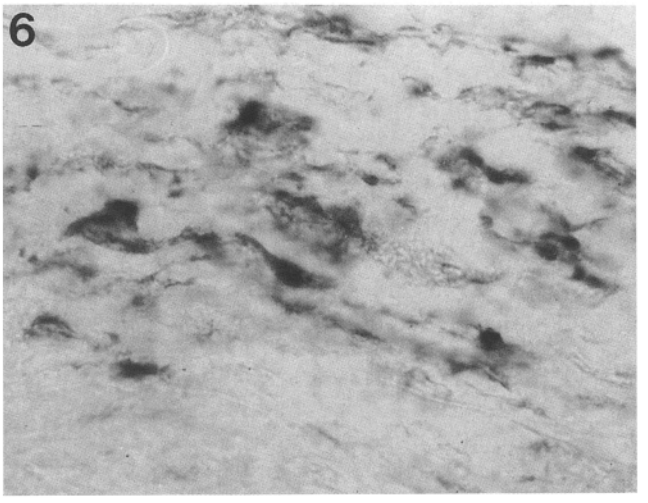
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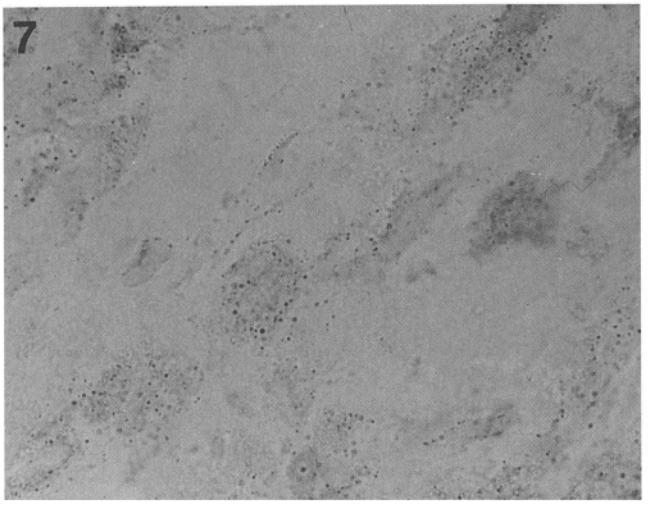
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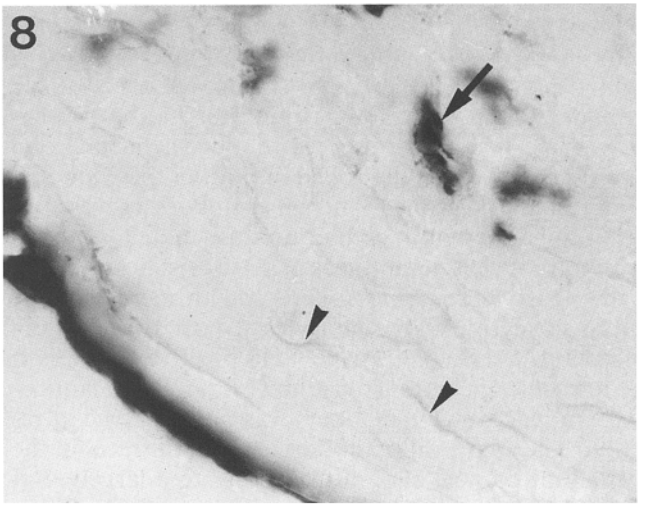
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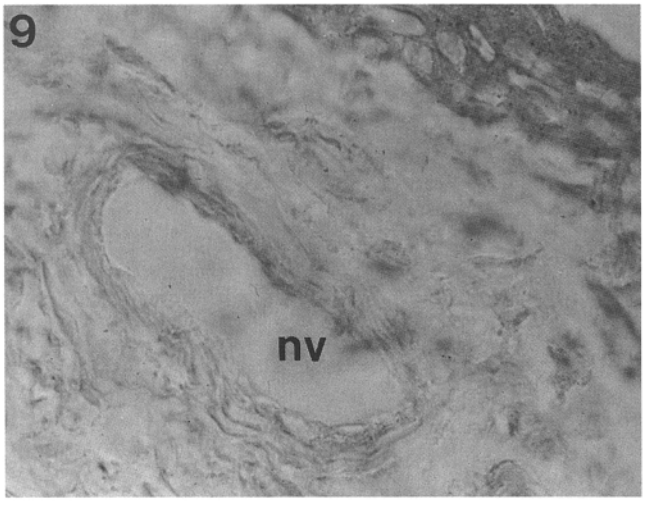
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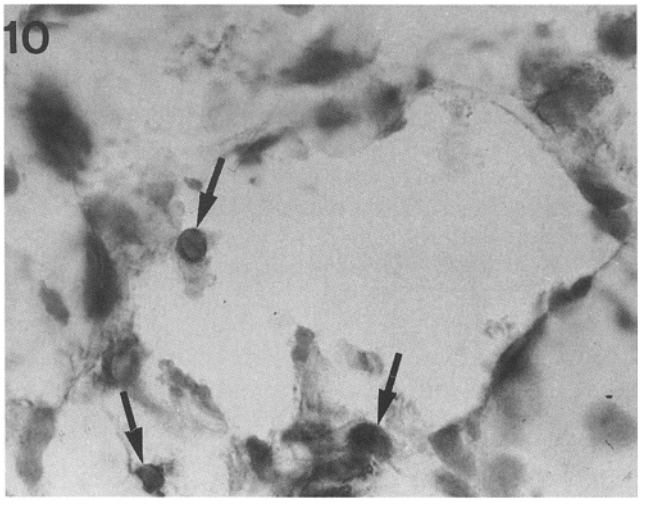
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graft following penetrating keratoplasty in the rat are described in the present study. The corneal rejection process has been described histologically by several authors in the rabbit [17, 27], mouse [31] and the rat [1, 6, 11, 13, 23, 36] models. Otsuka et al. [23] and Holland et al. [13] were the first to immunocytochemically examine corneal grafts in the penetrating keratoplasty rat model, describing the infiltrating cell subsets during the perioperative period. Whereas the syngeneic grafts were characterised by occasional T-lymphocytes and focal macrophage aggregations around the suture sites, both studies described the allogeneic grafts at rejection to be heavily infiltrated by macrophages and T lymphocytes. Otsuka et al. described a predominance of T-helper cells before rejection (postoperative days 2, 4 and 7), with a remarkable increase in T-suppressor/cytotoxic cells at rejection (day 14). These results agree with the observations of Holland and co-workers, who examined the grafts in the 4th postoperative week and reported a slight preponderance of T-suppressor/cytotoxic lymphocytes in the cellular infiltrate. In the present study, the cell subsets observed in the syngeneic group were similar to those described in both of these studies. In the allogeneic group, T-helper lymphocytes were observed to be present in greater number than T-suppressor/cytotoxic cells at all stages prior to rejection. Similar to the observations of Otsuka et al., an increase in T-suppressor/cytotoxic lymphocytes occurred at rejection, such that the ratio of CD4- to CD8-positive cells was 1. In contrast to the latter study, however, a significant decrease in macrophage numbers was not observed at rejection. The composition of the cellular infiltrates in the rejected rat corneal allograft is similar to a largely that reported in rejected human corneal allograft [26, 27].

The syngeneic grafts in this study displayed class II antigen distribution similar to that observed in the normal rat cornea [34]. Class II expression in the allogeneic grafts was extensive: in the majority of stromal infiltrating cells, in occasional invading cells within the epithelium, and in the endothelium in specimens from the 5th postoperative day. As reported by Callanan and coworkers, other anterior chamber structures in the allogeneic group contained inflammatory cells: in the absence of synechiae, T-helper cells and macrophages were observed in both the iris stroma and rat angular tissues.

Previous studies of hydrolase participation in corneal graft rejection have been conducted in the rabbit following intralamellar [2, 3] and penetrating [37] keratoplasty. Basu and Hasany demonstrated a relationship between the degree of graft opacity, cellular infiltration and lysosomal enzyme activity. In their xenograft model, both granulocytes and agranulocytes infiltrating the graft appeared to be the source of the lysosomal markers investigated, β-Gluc and AP [2]. In the present study, the cells infiltrating the corneal grafts were observed to stain intensely for AP and ALP, moderately for β-Gluc, NAG and β-Gal and mildly for GGT, DPPII and APM. Serial sectioning indicated that the majority of these hydrolases were located in macrophages; AP, APM and GGT were, however, observed in the lymphocytes. Although reported to be positive in human lymphocytes – and to be an important indicator in certain forms of lymphoma [18] – DPPIV was not present in the lymphocytic infiltrate in the Lewis rat following penetrating keratoplasty. This protease is, however, known for its considerable variation in activity among vertebrate tissues [21].

Invading capillaries could be marked with the hydrolases AP, β-Gluc, NAG, ALP, APA and APM. The last-named enzyme has been observed in neovascular growth during the wound healing process in the alkali-burned cornea [24]. Consequential to the vessel ingrowth, to the cell infiltration and to the subsequent release of hydrolases, a disruption in the stromal morphology and a corresponding decrease in keratocyte hydrolase activity was observed. Most of the above-mentioned lysosomal enzymes, including APM, are implicated in collagen metabolism [24, 29]. The weak APA keratocyte activity observed in the grafts at all stages following transplantation agrees with the suggestion that the contribution to corneal peptide degradation by APA in the normal and pathological cornea is insignificant in some species [4, 24].

The fate of the endothelium during the rejection process could be followed to a certain extent through the intensity of hydrolase staining, particularly that of ALP and DPPIV; the latter is positive only in the corneal endothelium and aqueous outflow tissues of the normal Lewis anterior segment [9]. Previous authors have studied the survival of the donor endothelium using various markers, including sex chromatin [7] and tritiated thymidine [12, 28]. In the present study, weak or absent ALP or DPPIV activity during the end-stages of the rejection process indicated both morphological and functional disruption of the endothelium.

In conclusion, the alterations in corneal hydrolase activity were investigated in the Lewis rat following penetrating keratoplasty. Serial sectioning and subsequent immunocytochemistry allowed for the localization of hydrolases within the invading cellular infiltrate and capillaries during the rejection process. Knowledge of the localization of the various cellular components and their contribution to graft rejection in the rat penetrating keratoplasty model has relevance in understanding cellular processes which may be involved in graft failure in human corneal transplants.

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