Development of organised conjunctival leucocyte aggregates after corneal transplantation in rats

S Banerjee, F C Figueiredo, D L Easty, A D Dick, S M Nicholls

Aim: To investigate the development of lymphoid aggregates in the conjunctiva after corneal transplantation in rats.

Methods: LEW or PVG strain corneas were transplanted orthotopically to PVG rats. Cornea and conjunctiva were examined clinically for up to 42 days. Eyes were removed with attached conjunctiva on days 10 and 15 after transplantation (before and during rejection), together with normal eyes, fixed, paraffin embedded, and examined immunohistochemically.

Results: Clinically, the temporal half of the upper palpebral conjunctiva of recipients of 10/19 allografts and 1/10 isografts developed pronounced swelling, correlating with inflammation and rejection. Histologically, the swelling comprised leucocytic aggregates with an altered overlying epithelium. Aggregates contained granulocytes, macrophages, and cells expressing major histocompatibility complex (MHC) class II, CD4, and CD8, all more numerous in allograft associated conjunctiva. Class II+ cells were more abundant at the surface, whereas macrophages and T cells were more numerous in the deeper stroma. There were few B cells. There was greater CD54 expression by vascular endothelium in allograft associated aggregates. Cells expressing TNFα and IFNγ but not IL1β were present in stromal and superficial areas.

Conclusions: Corneal transplantation in rats induces the development of organised conjunctival leucocytic aggregates in a fixed location that are significantly more pronounced in recipients of allografts compared with isografts and show characteristics of a Th1 type immune response. These aggregates have characteristics of conjunctiva associated lymphoid tissue and may be sites of presentation of graft antigens and lymphocyte proliferation at the ocular surface.

Materials and methods

Animals
Specific pathogen free, 8–10 week old female PVG (RT1b) and LEW (RT1l) inbred rats (Harlan, Bicester, UK) were used for corneal transplantation. All animals were cared for in accordance with the ARVO statement for the use of animals in ophthalmic and vision research.

Corneal transplantation and clinical examination
Penetrating keratoplasties were performed as previously described.21 PVG strain recipients were anaesthetised by separate intramuscular injection of Hypnorm (0.7 ml/kg;
overnight at 4˚C. The eyes with their adnexae were bisected (PLP, 0.25% paraformaldehyde) and further fixed in PLP were then injected with periodate-lysine-paraformaldehyde (National Diagnostics, Hull, UK). They were infiltrated under vacuum with low temperature paraffin wax at 54 ˚C and embedded, with the two halves of the eye central side sagitally, washed in phosphate buffered saline (PBS) and received Lewis or PVG strain grafts. Donor corneas were excised using a 3.5 mm trephine. The recipient graft bed was created using a 3 mm trephine. Corneas were sutured with 12 interrupted 11/0 Nylon sutures. Animals received 1% topical chloromycetin ointment (Forley Ltd, Ireland) immediately after surgery and on alternate day thereafter with 1% atropine sulphate eye drops (Schering-Plough, Welwyn Garden City, UK) for 2 weeks. Corneal appearance was scored by slit lamp biomicroscopy on alternate days. Corneal allograft rejection was defined previously as described. By evicting the eyelid, conjunctivae on the grafted and normal side was scored for swelling, hyperaemia, loss of pigmentation, and size of the area involved. Changes were scored as detailed in Table 1.

Tissue fixation, embedding, and sectioning

On days 10 and 15 after transplantation animals were killed and eyes were enucleated, conserving the bulbar and palpebral conjunctiva as far as the lid margin. Tissues were fixed and processed as described previously. Briefly, globes were then injected with periodate-lysine-parafomaldehyde (PLP, 0.25% paraformaldehyde) and further fixed in PLP overnight at 4˚C. The eyes with their adnexae were bisected sagitally, washed in phosphate buffered saline (PBS) and then rapidly dehydrated in graded ethanol and Histoclear (National Diagnostics, Hull, UK). They were infiltrated under vacuum with low temperature paraffin wax at 54˚C and embedded, with the two halves of the eye central side downwards in the same block, so that sections of cornea with attached conjunctiva were obtained from the centre outwards. Sections of 6 µm were cut, placed three per slide, dried at 37˚C overnight, and stored at −20˚C.

Immunohistology

Immunoperoxidase staining was performed as previously described using the antibodies listed in Table 2. To identify cytokine expressing cells, an immunofluorescence double staining technique was used. For this, tissue sections were blocked with 4% normal serum of the host species of the first secondary antibody for 1 hour in a moist chamber. Then sections were incubated overnight at 4˚C with the first primary antibody (cell surface marker) or isotype control antibody, followed by incubation at room temperature for 1 hour with FITC conjugated secondary antibody (diluted 1:50) (Jackson ImmunoResearch, PA, USA). Intervening washes were done with PBS, 0.1% saponin. Sections were then blocked with 4% normal serum of the host of the second secondary antibody and incubated overnight with anti-cytokine antibody or isotype control antibody, followed by biotinylated secondary antibody diluted 1:100 (Jackson ImmunoResearch) for 1 hour. Both secondary antibodies were purchased pre-absorbed against immunoglobulins of the host species of the other primary and secondary antibodies and against rat immunoglobulin. Finally, sections were incubated with TRITC conjugated streptavidin diluted 1:200 (Jackson ImmunoResearch, PA, USA) and mounted in Vectashield (Vector, Burlingame, CA, USA). Positive control sections of spleen were included in each staining run.

Quantification of cells

Sections stained by the immunoperoxidase method were examined and stained cells were quantified at ×400 magnification using a Leica Leitz DMRB upright light microscope and an image analysis system (Quantimet; Leica, Cambridge, UK). Stained cells from eyes with lymphoid aggregates were counted in the deeper stromal area and in the superficial area of the aggregates where epithelium was absent (Fig 1). Immunofluorescent stained tissue was examined either on a Leica DMIRBE inverted epifluorescence microscope, confocal images being obtained using a TCS NT confocal laser scanning unit, or with Improvision Openlab software with digital controlled CCD camera. The confocal system was equipped with an argon krypton laser (488, 568, 647 nm lines) and argon ultraviolet laser (351 and 364 nm), providing three channels for simultaneous detection of fluorescence/reflectance.

The difference between allograft and isograft conjunctiva was assessed by comparing the incidence of aggregates with a score of 3 (the score which correlates with histologically identifiable aggregate) and above at day 10 and 15 after transplantation. A Fisher’s exact test was used. SPSS for Windows software was used for statistical analysis. A p value of <0.05 was considered significant.

Experimental protocol

We previously established that graft rejection in this model occurred in all animals between days 10 and 15 after transplantation (median day 13). A first group of animals was examined clinically before transplantation and on days 3, 6, 10, and 15 after transplantation and scored the development of leucocytic aggregates in relation to rejection. A second group was examined on days 18, 21, 25, 30, and 42 to determine whether these aggregates persisted. A further group of animals was killed after transplantation when conjunctival swelling was found to be clinically most prominent—that is, on days 10 (early stage of rejection) and 15 (rejection under way in all animals). The groups of animals used for immunostaining are shown in Table 3. Conjunctivae of unoperated rats were also examined. Every 13th slide was stained with haematoxylin and eosin (H&E) to verify the location of the aggregates in relation to the cornea. Intervening sections were then stained in sequence with the appropriate monoclonal antibody or negative control antibody and cell infiltrate was quantified in specimens with the leucocytic aggregates. We were unsuccessful in assessing expression of IL-2, IL-4, IL-10, and TGFβ in control tissue expected to contain these cytokines and therefore concluded that the antibodies available were not suitable for immunohistology. NK cell numbers were estimated as a percentage of infiltrating cells.

<table>
<thead>
<tr>
<th>Score</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjunctival swelling</td>
<td>Mild</td>
<td>Moderate with diffuse margin</td>
<td>Moderate with discrete margin</td>
<td>Severe with overhanging edge</td>
</tr>
<tr>
<td>Pigmentation over swelling</td>
<td>Loss of some pigment</td>
<td>Loss of some pigment</td>
<td>Depigmented</td>
<td>Depigmented</td>
</tr>
<tr>
<td>Hyperaemia</td>
<td>None</td>
<td>Generalised</td>
<td>Localised petechiae</td>
<td>Localised patchy, haemorrhagic appearance</td>
</tr>
</tbody>
</table>

*Localised in temporal area of upper lid.

Table 1 Scoring of clinical conjunctival changes
RESULTS
Morphology of conjunctiva in unoperated and transplanted eyes
Clinical examination of conjunctiva of unoperated animals (n = 6) showed normal blood vessels, scattered pigment and a uniform surface without swelling (Fig 2A). Histologically the epithelium was intact with melanocytes and without lymphoid aggregates. Conjunctival blood vessels exhibited a flattened endothelium and were most prominent adjacent to the limbus. Immunohistochemical staining showed a few scattered CD4+ and CD8α+ T cells, class IIα cells, and ED2+ macrophages (Fig 2B) and occasional granulocytes. CD4+ cells were both dendritic and round. Intraepithelial CD8α+ cells were seen (Fig 2C).

Clinical examination 3 days post-transplantation, revealed swelling and hyperaemia of the upper temporal palpebral conjunctiva. This increased until day 10 to form a discrete oval swelling lacking pigment with numerous capillaries (Fig 2D), which then subsided in most isografts by day 15 (Fig 3), but persisted until day 25 in some allografts, after which the only residual change was lack of pigment. Clinical conjunctival scores were compared in all rats examined on days 10 and 15. These scores were 3 or greater in conjunctivas of 10/19 allografts recipients and 1/10 isograft recipients on days 10 and 15. These scores were compared in all rats examined on day 15 after transplantation (p = 0.03), but there was no significant difference on day 10 (p = 0.1). In four allograft and four isograft recipients we performed a second corneal transplant 42 days after the first transplant. Conjunctival swelling developed more rapidly (within 3 days) and with clinical scores greater than 3 only in animals (two allografts and one isograft) where swelling of at least score 3 was induced by the first transplantation.

Histologically, conjunctivas of transplanted eyes had a diffuse mixed cell infiltrate. Fully developed leucocytic aggregates, corresponding to a clinical score of 3, were identified by the following features: subepithelial leucocytes in a dense follicle-like arrangement in association with a thickened, invaginated epithelium overlying its periphery, while the epithelium overlying the area of greatest infiltration was often thin or absent (Fig 4A), and absence of goblet cells. Underlying the leucocytic aggregate, the vascular endothelium was occasionally cuboidal, resembling HEVs of lymph nodes (Fig 4B). CD54 (ICAM-1) was heavily expressed on the endothelium of capillaries associated with aggregates of allografts (Fig 4C), to a lesser extent with isografts, but not on endothelium elsewhere in the conjunctiva. CD54 was expressed in the basal layers of the conjunctival epithelium (Fig 4C) and more strongly in the epithelium adjacent to the aggregates.

Changes in conjunctival epithelium associated with lymphoid aggregates
Three anti-cytokeratin antibodies26 27 were used to characterise the epithelium associated with the aggregate, particularly whether the palisade-like cells observed on the surface (Fig 4D) were altered epithelial cells. This would confirm, firstly, the observed increase in the thickness of epithelium in the periphery of lymphoid aggregates and, secondly, absence of epithelium over the areas of greatest infiltration. No expression of either cytokeratin 4 (specific for parabasal and basal cells of non-keratinised stratified squamous epithelium) or 14 (non-keratinised stratified squamous epithelium) was observed. The pan-cytokeratin antibody confirmed these findings, except that there were a few scattered cells in the substance of the lymphoid aggregate (Fig 4D), which are likely to represent invaginated epithelium. The lack of MHC class I expression also supported the absence of epithelium (Fig 4E) above the leucocytic infiltration.

Composition of infiltrating cells in conjunctiva after transplantation
The infiltrate in areas outside the aggregate towards the limbus consisted predominantly of macrophages and MHC
class II⁺ cells, and was more dense in the upper lid (Fig 4F). Aggregates were identified by histology in conjunctivas of 2/12 isograft recipients (two on day 10 and one on day 15) and 8/15 allograft recipients (four on day 10 and four on day 15).

All cell types were more abundant in aggregates associated with allografts compared to isografts (Fig 5). Within these aggregates, the cells exposed to the tear film were palisade-like (arrowheads in Fig 4D and G), whereas deeper cells were frequently arranged in a circular follicle-like pattern (Fig 4H). MHC class II⁺ cells were predominant in the superficial areas—that is, area “a” in Fig 1 (Fig 6A), whereas in the stroma (area “b” in Fig 1), T cells (CD4⁺, Fig 6B and CD8⁺, Fig 6D) were more numerous. There was a higher proportion of T cells relative to macrophages and granulocytes in the allografts in comparison with isograft associated aggregates (Fig 5A and B). CD4⁺ cells co-localised with CD25 (compare Figs 6B and C). CD8⁺ cells were mainly in stroma and ED2⁺ cells were arranged in the stroma in a circular pattern (Fig 4H). Granulocytes represented the majority cell infiltrate and were distributed randomly throughout (Fig 6E). There were a few B cells located in the deeper stromal areas. We estimated NK cells to comprise up to 10% of the cells in the lymphoid aggregate, located mainly in the stroma (Fig 4G).

Cytokine expression
Leucocytic aggregates were found in 4/11 conjunctivas examined for cytokine expression, all of which were allografts. In these aggregates, there was no detectable IL1𝛽 despite consistent expression in positive control spleen derived from the same animals and processed simultaneously. IFNγ and TNFα were strongly expressed in all four aggregates. IFNγ expression was focal, but present in both the superficial and deeper follicular areas (Fig 6F), whereas TNFα (Fig 6G) was in general more diffusely distributed. Double immuno-fluorescence staining revealed that CD8⁺ and ED2⁺ cells expressed both IFNγ and TNFα (Fig 6H, I, and J).

**DISCUSSION**
We were not able to demonstrate organised lymphoid aggregates in normal rat conjunctiva, confirming previous work.¹ The normal (unoperated) rat conjunctiva had a diffuse distribution of leucocytes, mostly macrophages, but also a few scattered MHC class II⁺ dendritic cells, CD4⁺ and CD8⁺ T cells (both intraepithelially and within stroma) and granulocytes, resembling the resident leucocyte population in healthy human conjunctiva.² However, there were differences in that cells were less numerous compared to numbers in humans and there were no follicular aggregates containing B cells, as seen in humans.¹

The organised lymphoid tissue induced by transplantation was more pronounced in recipients of allografts than isografts, displayed the characteristic of a transient Th1 response, and a structure that might facilitate antigen presentation across the ocular surface. Expression within the conjunctiva of IFNγ and TNFα by ED2⁺ macrophages and CD8⁺ T cells supports classic activation of macrophages¹⁰ ¹¹ and CD8⁺ T cells and represents a strong pro-inflammatory milieu. Lymphoid hyperplasia has also been observed in the conjunctiva of rabbits during corneal allograft and xenograft rejection.¹² ¹³ It can be argued, at least in rats, that it conforms to a more traditional concept of CALT as a tissue associated with T cell immunity¹² and lacking features considered to be definitive of organised MALT, such as B cell follicles or IgA production and the functional capacity to induce or promote tolerance.

Clinical signs of conjunctival changes were seen as early as 3 days post-transplantation, and were similar in isografts and allografts until day 6, implying that aggregate development was initiated by the non-specific injury of the surgery. The fact that there was equal operative trauma around the entire cornea but the aggregates always arose in the upper temporal palpebral conjunctiva, suggests that this location may be structurally predetermined. By the time rejection was under way (day 15), aggregate development was significantly

<table>
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<th>Table 3</th>
<th>Eyes examined for immunohistology and incidence of histologically identified leucocytic aggregates</th>
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<tbody>
<tr>
<td>Type of staining performed</td>
<td>Day 10⁺</td>
</tr>
<tr>
<td>Leucocytes, MHC, adhesion molecules</td>
<td>3/4</td>
</tr>
<tr>
<td>Cytokines</td>
<td>1/2</td>
</tr>
<tr>
<td>Cytokeratins, NK cells double stain cytokine/cell phenotype</td>
<td>Not done</td>
</tr>
</tbody>
</table>

*After transplantation.
†Denominator indicates number of eyes examined.

Figure 2  (A) Appearance of normal conjunctiva of the temporal half of the upper lid (lid everted with forceps) without clinically evident leucocytic aggregate. Note absence of oedema and normal vasculature (original magnification x15). (B) ED2⁺ macrophage (arrowheads) and (C) CD8⁺ cells in normal conjunctiva. (D) Clinical appearance of aggregate on day 10 after transplantation showing hyperaemia, overhanging edge (arrow), and swelling, clinical score 4 (original magnification x10).
greater in allografts than in isografts. This, together with the
greater cell infiltrate and numbers of activated cells in
allograft associated aggregates, implies that maximal de-
velopment was alloantigen dependent. Such a notion is further
supported by similarities in the nature of the infiltrate in
conjunctiva and allograft cornea, including a granulocyte
infiltration and lack of B cells. Indicators of alloactivation
included a higher proportion of T cells and CD25, CD2, CD18,
and CD54 expressing cells in allograft associated aggregates,
overall with a Th1-like (IFNγ and TNFα) cytokine profile.
Strong CD54 expression on vascular endothelium within the
lymphoid aggregates and morphological changes in some
blood vessels consistent with development of HEV suggest
that they are sites of extravasation and proliferation of T cells.
The thinning and loss of epithelium and concentration of
CD2 and MHC class II DCs at the surface of the aggregates,
where they might gain access to antigen shed from the
epithelium further support this. In the deeper areas, MHC
class II DCs were interspersed with CD4, CD8, and CD25 cells,
as would be expected in conditions of active antigen
presentation and T cell proliferation. Concentration of T cells
in a “trail” between the aggregates and the limbus is
suggestive of T cell trafficking between this area and the
cornea. Whether the aggregates are a site of antigen
presentation to naive T cells or whether such presentation
occurs only in regional draining lymph nodes remains

Figure 3 Clinical scores of conjunctiva in allografts (▲, n=7) and
isografts (●, n=6) before and during rejection. Broken line indicates
score equivalent to histological identifiable lymphoid aggregate.

Figure 4 (A) Location of leucocytic aggregate (arrow) in the palpebral conjunctiva (haematoxylin and eosin). Note goblet cells are absent in adjacent
epithelium. m = graft margin. (B) Capillary displaying HEV morphology. (C) CD54 expression on basal epithelium, infiltrating cells, and capillary
endothelium (inset). (D) Pancytokeratin staining showing lack of epithelium (note pallisading arrangement, arrowheads) overlying the aggregates but
occasional cells (arrow) in the stroma. (E) MHC class I staining is not evident in superficial aggregate. (F) Composite picture showing CD4 cells in
upper (UL) and lower (LL) conjunctiva of the same section. Infiltration is more dense in UL, between the aggregates (CA) and the cornea (CO), than in LL.
(G) NK cell are mainly in the stroma. Note pallisading (arrowheads) of cells in superficial layers. (H) ED2 macrophages in circular arrangement in the
aggregate defined by arrows. All unlabelled bars are 100 μm.
uncertain. Although both IFNγ and TNFα were present in the aggregate, we could not detect IL-1β expression, which is central to activation and migration of DC from the site of antigen capture. However, such expression may not be necessary as late as day 10 after transplantation when many other activating signals are likely to be present.

Unlike the gut and respiratory mucosa, the conjunctiva is not primarily an absorptive surface and lacks the characteristic M cells of the single layered epithelium of GALT and BALT. The loss of epithelium we observed overlying aggregates occurred only at this site and may simply have been a pathological consequence of the large numbers of infiltrating cells. Alternatively, it may be a specific adaptation to facilitate rapid antigen uptake and T cell activation in appropriate conditions in a species in which the normal conjunctiva contains few lymphocytes and/or in a mucosa where the epithelium is normally stratified, bears tight junctions in the apical layers and, is relatively impermeable. It is reminiscent of reticulosis of tonsils during acute tonsillitis and of the extreme thinning and loss of epithelial basement membrane observed in organised human CALT at the point of maximal leucocytic aggregation. It is suggested that breakdown of epithelial basement membrane occurs in MALT in areas of leucocytic infiltration to facilitate antigen transport. It may be argued that the loss of epithelium is an artefact of tissue processing. However, we believe it not to be the case because we have seen progressive thinning of the epithelium in consecutive sections.
and in many instances (for example, Fig 4A and H) the aggregate protruded above the surface of the adjoining epithelium and contained unusual (palisade-like) cells at the surface.

The general lack of B cells in rat conjunctiva is consistent with the evidence reviewed by Sullivan32 that the conjunctiva is not a component of the secretory immune system—that is, a site of local B cell proliferation for IgA production, which is not a component of the secretory immune system—that is, was primed during previous encounters with common environmental antigens, but cross react with antigens of the graft.41 Although the aggregates we observed resemble certain organised GALT structures composed mainly of T cells which develop after birth in response to antigenic load,29 and to CALT as traditionally described, containing mitotic lymphocytes, lymphatic channels and modified overlying epithelium.33 We cannot discount the possibility that they were initiated by mild microbial infection, although we emphasise that antibiotic ointment was regularly administered to the eye and there was no sign of endophthalmitis at any time. As we did not find aggregates in association with all allografts, they are clearly not essential for graft rejection or a major site of alloactivation. Comparable lymphoid hyperplasia in humans associated with rejection has not been reported, but in rabbits its development seems to reduce the success of immunosuppressive therapy.33 There have been no human studies to indicate whether the extent of pre-existing organised lymphoid tissue in the conjunctiva, irrespective of the original inflammatory stimulus, is a predisposing factor to graft rejection. Such a link is possible, particularly in view of the evidence that alloreactive T cells are of memory phenotype—that is, were primed during previous encounters with common environmental antigens, but cross react with antigens of the graft.41 Although the aggregates did not persist for long after a corneal transplant, in animals that had rejected a graft, further experiments using a strain combination of transplant, the aggregate protruded above the surface of the adjoining epithelium and contained unusual (palisade-like) cells at the surface.

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