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The influence of cervical and thoracic lymphadenectomy on corneal allograft rejection in inbred rats

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ABSTRACT

Aim To investigate the site of alloantigen presentation in the rat following orthotopic corneal transplantation.

Methods Adult inbred Fischer 344 rats received penetrating corneal allografts from inbred Wistar-Furth donors (n=17), without lymphadenectomy. A second group (n=8) underwent bilateral removal of superficial cervical and facial lymph nodes on day -7 with respect to transplantation. A third group (n=9) underwent bilateral removal of superficial cervical, facial, internal jugular and posterior cervical nodes. Graft survival was assessed by corneal clarity and rejection was confirmed histologically.

Results All allografts underwent rejection. The median time to rejection for unmodified allografts was day 15, compared with day 14.5 for minimally lymphadenectomised recipients and day 18 for more extensively lymphadenectomised recipients (p>0.05, all comparisons). The median day to rejection for the combined group of lymphadenectomised rats was day 17 (p>0.05 compared with unmodified grafts). The rejection process was similar in all recipients.

Conclusions Removal of multiple lymph nodes in the neck and thorax did not significantly influence the incidence, tempo or nature of the corneal allograft response. Sensitisation and clonal expansion of corneal alloantigen-reactive cells cannot occur only in superficial cervical, facial, internal jugular and posterior cervical lymph nodes in the rat.
INTRODUCTION

Corneal allograft rejection is a significant clinical problem worldwide[1] and the approaches to reducing acute rejection that have improved outcomes for vascularized organ grafts, such as living-related donation and better systemic immunosuppression, are either inappropriate or have been much less useful for corneal transplantation. A number of innovative experimental therapies are under development to reduce the incidence of corneal graft rejection, but their applicability is limited by continuing uncertainty as to where presentation of cornea-derived alloantigen to host T lymphocytes occurs, and whether such anatomic locations are species-specific or can be generalised across species. The relevance of this to clinical practice is that it is not clear whether novel treatments to reduce the incidence or severity of corneal allograft rejection are best directed to the eye itself, to the immediate ocular environs, to regional sites, or systemically.

Options for the physical location of presentation of cornea-derived alloantigen include the cornea itself, surrounding tissues including the conjunctiva and ocular environs, locoregional lymph nodes, or more distant secondary lymphoid tissue. In the mouse, the evidence supports the tracking of ocular-derived antigen ocular via the uveoscleral pathway[2] to ipsilateral superficial cervical or submandibular lymph nodes. Cells released from murine corneal allografts appear later in cervical lymph nodes[3] and moreover, bilateral lymphadenectomy of the cervical[4, 5] or submandibular lymph nodes[6, 7] prior to corneal transplantation has been shown to prolong corneal allograft survival. We investigated whether a similar drainage pathway operates in the rat, a commonly-used experimental model for assessing early efficacy of new interventions to reduce allograft rejection.

MATERIALS AND METHODS

Experimental animals
Approval for experimentation was obtained from the institutional Animal Welfare Committee. Adult (12 week) male inbred Fischer 344 (F344, RTI) and Wistar Furth (WF, RTI) rats were bred within our facility. Rats were housed at 21°C in 50-55% humidity under a 12 hour light/12 hour dark cycle, and were allowed access ad libitum to water and dry rations (Ridley Agriproducts, SA, Australia). Genetic integrity of inbred strains was maintained by lineage records and tested yearly by allozyme electrophoresis.

Orthotopic corneal transplantation in the rat

Recipient F344 rats received penetrating 3 mm diameter corneal allografts from WF donors under general anaesthesia, as described previously.[8] The WF to F344 strain combination represents multiple major and minor histocompatibility barrier differences. Contemporaneous isografts (F344 donors, F344 recipients) were performed as controls. Donor corneas were secured with eight interrupted 10-0 nylon sutures. Thereafter, recipients were examined daily under the operating microscope and grafted eyes were scored for corneal clarity, neovascularization and ocular inflammation on a 0-4 numerical scale in 0.1 increments as described elsewhere.[9] Grafts were deemed to have failed once graft clarity reached a score of ≥2.0. After graft failure or after 60 days with a surviving graft, rats were euthanised by isoflurane overdose. Eyes were removed and processed for conventional histology.

Lymphadenectomy in the rat

In some rat recipients of corneal allografts, partial bilateral lymphadenectomy was performed 7 days prior to orthotopic corneal transplantation. A 2 cm midline incision was made through the thoracic skin of anaesthetised rats and the skin reflected to allow visualization of lymph nodes in the neck and thorax. In the first such group, the superficial cervical and facial nodes (identified according to Tilney[10]) were removed bilaterally. The wound was closed with 4-5 disposable tissue staples. In the second such group, the superficial cervical, facial, internal
jugular and posterior cervical nodes were removed bilaterally. The fat pads in which lymph nodes were embedded were carefully dissected, and lymph nodes were counted as they were removed. They were then placed in buffered formalin for confirmatory histology.

**Histology**

Tissue samples were fixed in buffered formalin for at least 24 hours, before being dehydrated and embedded in paraffin wax. Sections (5 µm thick) were cut at the microtome and mounted on chrome alum-subbed slides prior to staining in haematoxylin and eosin and mounting in DePex mounting medium (BDH Laboratory Supplies, Poole, UK). Excised lymph nodes were subsequently examined at the light microscope to confirm lymphoid architecture and that the capsule was intact.

**Statistical analysis**

Corneal graft survival amongst experimental groups, measured as post-graft day of rejection, was compared using the Kruskal Wallis test (three groups) or Mann-Whitney U test (two groups), corrected for ties, in SPSS statistical software (SPSS Inc., Chicago, IL, USA), with p<0.05 considered significant.

**RESULTS**

All allografts underwent rejection, irrespective of lymph node status (Table 1). The median time to rejection for unmodified allografts (n=17) was day 15, compared with day 14.5 for the group (n=8) in which bilateral superficial cervical and facial lymph nodes had been removed 7 days prior to corneal transplantation, p>0.05, and day 18 for the group (n=9) in which bilateral superficial cervical, facial, internal jugular and posterior cervical lymph nodes had been removed 7 days prior to transplantation, p>0.05 compared with unmodified allografts (Table 1). The median day to rejection for the combined groups receiving lymphadenectomy
(n=17) was day 17, p>0.05 compared with unmodified grafts. Partial bilateral lymphadenectomy was well tolerated: one rat of a total of 17 developed minor lymphoedema in the neck at 24 hours after surgery, which completely resolved over the following 4 days without treatment. All unmodified isografts survived to greater than 60 days post-graft, indicative both of genetic homogeneity of the F344 strain and the technical success of the corneal transplant surgery.

**Table 1** Corneal graft survival following partial lymphadenectomy

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Number of rats</th>
<th>Graft</th>
<th>Day to rejection</th>
<th>Median day to rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3</td>
<td>isograft</td>
<td>&gt;60, &gt;60, &gt;60</td>
<td>&gt;60</td>
</tr>
<tr>
<td>None</td>
<td>17</td>
<td>allograft</td>
<td>11, 12, 12, 13, 13, 14, 14, 14, 15, 15, 15, 16, 18, 19, 19, 20, 20</td>
<td></td>
</tr>
<tr>
<td>Lymphadenectomy</td>
<td>8</td>
<td>allograft</td>
<td>12, 14, 14, 14, 15, 18, 21, 27</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.5</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Lymphadenectomy</td>
<td>9</td>
<td>allograft</td>
<td>15, 15, 16, 17, 18, 19, 19, 22, 25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>(superficial cervical, facial lymph nodes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(superficial cervical, facial, internal jugular, posterior cervical lymph nodes)</td>
<td></td>
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</tbody>
</table>
The rejection process was similar both clinically and histologically in every recipient rat, irrespective of lymph node status. There was no difference in the day post-operatively at which recipient blood vessels first crossed the graft-host junction (data not shown). Histology of clinically rejected corneal allografts from unmodified rats or rats that had undergone lymphadenectomy showed the expected pattern of stromal oedema, mononuclear cell infiltration into the graft and anterior chamber, and corneal endothelial cell loss. No difference was apparent amongst the groups. Histology of haematoxylin and eosin-stained sections confirmed that all lymph nodes removed during bilateral lymphadenectomy were secondary lymphoid tissue, as assessed by morphology and cytology.

DISCUSSION
The bilateral removal of some or all of the superficial cervical (sometimes called submandibular), facial, internal jugular and posterior cervical (sometimes called deep cervical) lymph nodes did not influence the incidence, tempo, or clinical or histological appearance of corneal allograft rejection in the WF to F344 inbred rat strain combination. This finding was unexpected, given a wealth of evidence that removal of the analogous lymph nodes in the mouse will prolong corneal allograft survival significantly, and in some instances indefinitely,[4] in both weak[4, 5] and strong strain combinations,[6, 7] although not necessarily when only ipsilateral nodes are removed,[11] nor when recipients are presensitised with donor-specific antigen.[7] Furthermore, there is at least one report that ipsilateral or bilateral excision of multiple lymph nodes in the neck and thorax of Sprague-Dawley rats prolonged Wistar strain corneal allograft survival significantly, although all animals rejected their grafts in time.[12] Comparison of the nomenclature of lymph nodes in the rat as described by Tilney,[10] with that of the mouse as described by Van den Broeck et al.[13] indicates that the nomenclature in the two species does not coincide. In the rat, the nodes in the head, neck and upper thorax are the superficial cervical nodes, the facial nodes,
and the internal jugular and posterior cervical nodes. In the mouse, the nodes that correspond anatomically are the mandibular and accessory mandibular lymph nodes, the superficial parotid nodes and the cranial deep cervical nodes. Our results do however indicate that the influence of partial lymphadenectomy on corneal allograft survival cannot be generalised across species.

Where might cornea-derived alloantigen be presented to T cells in our rat model? The location of secondary lymphoid tissue in rats is consistent amongst different rat strains and individual animals, although occasional variation in the size and number of lymph nodes does occur, and may be dependent in part upon the age of the animal.[10] The superficial cervical, facial and internal jugular groups of peripheral lymph nodes drain the rat head and neck, but Tilney notes that rarely, a tiny posterior auricular lymph node drains the rat ear, although generally lymphatics that parallel the auricular vein empty into the posterior facial channel.[10] This is of some interest because of the well-established clinical association between viral keratoconjunctivitis and concomitant enlargement of the ipsilateral preauricular lymph node in humans, and the evidence that the preauricular node drains the human eyelid and conjunctiva,[14] and the ciliary body.[15]

We cannot be certain that cornea-derived antigen reaches secondary lymphoid tissue after uptake by dendritic cells in the cornea itself. Some alloantigen may be released into the anterior chamber during or following surgery and be taken up by macrophages elsewhere in the anterior segment, for example, in the iris as is the case for soluble antigen.[16] Certainly, soluble antigen introduced into the anterior chamber of the eye distributes widely into secondary lymphoid tissue via the venous circulation.[17] We can, however, be reasonably confident that the major site of presentation is not in the nodes that we removed.

We did not perform splenectomy. However, Bourne et al examined the effects of splenectomy on penetrating corneal graft survival in the outbred rabbit.[18] They found no significant effect of splenectomy on the incidence or tempo of rejection of orthotopic corneal
allografts in splenectomised recipients, compared with normal recipients. In contrast, splenectomy in the mouse reduces the prolongation of corneal allograft survival that is induced by so-called ACAID.[19] In essence, the available evidence from the rabbit and mouse suggests that the spleen is either without influence on corneal allograft survival, or else is somewhat tolerogenic.

The conjunctiva itself contains conjunctiva-associated lymphoid tissue.[20, 21] As early as three days after surgery, rats bearing corneal grafts develop organised lymphoid aggregates within the conjunctiva in which sensitization may occur,[22] although there has been at least one report that foreign antigen applied directly to the conjunctiva leads to T cell anergy.[23] Local antigen presentation within the ocular environs, if not within the conjunctiva, is suggested by the findings[24, 25] that expression of recombinant cytokines by corneal endothelium can prolong ovine corneal graft survival: the physical half-life of these molecules is probably too short to explain modulation of afferent pathways very far outside the eye.

In conclusion, the effects of lymphadenectomy on corneal graft survival obtained in one species cannot necessarily be generalised to another, even those as closely related as the mouse and rat, and any temptation to pursue a similar approach in humans should be tempered by our unexpected finding. The adaptive immune response is marked by redundancy, a likely consequence of its importance to the survival of the individual. It would thus not be surprising to discover that sensitization to foreign corneal alloantigen occurs in multiple anatomical locations, as is apparently the case in the rat.

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Competing interests None declared.

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Contributor statement SLB and KK each performed some of the experimental work, and were both involved in analysis and interpretation of the data. HMB and DJC were involved in design of the experiments and the drafting and revision of the manuscript for important intellectual content. KAW was responsible for conception and design of the study, its interpretation, and the writing of the manuscript. All authors have approved the final version. KAW takes overall responsibility for this work.
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