Cell subpopulations in failed human corneal grafts

Lucia Kuffová, Vladimír Holáň, Lynne Lumsden, John V Forrester, Martin Filipč

Abstract

Background/aims—Inflammatory cells and antigen presenting cells (APC) are not present under normal circumstances in the centre of the healthy cornea. The purpose of this study was to investigate and phenotype the inflammatory cell populations, particularly with reference to T cell subpopulations and macrophages, and to localise dendritic cells (DC) and other MHC class II positive cells in three groups of grafted corneas: rejected non-inflamed, rejected inflamed grafts, and control dystrophic explants.

Methods—15 corneal buttons removed during keratoplasty from non-inflamed “quiet” previously grafted corneas, five inflamed corneas requiring urgent re-grafting for “graft melting” (in “high risk” corneas), and 10 control dystrophic opaque corneas explanted during their first graft procedure were examined. Cryosections of corneas were immunostained with a panel of monoclonal antibodies (mAb) against CD3, CD4, CD8, CD14, CD25, CD68, HLA-DR, and HLA-DP molecules using the StreptABC method. DC were detected by dual immunostaining as CD1a+ and MHC class II+ and CD19-. Cell densities in immunostained tissue sections were evaluated using a scale from 0 to +4.

Results—Immunostaining in control dystrophic corneas was negative for all antibodies. A moderate to high density of CD8+, CD14+, and CD68+ cells was observed in the majority of rejected non-inflamed as well as in rejected inflamed corneal buttons. Strong positivity for HLA-DR and HLA-DR molecules in the epithelium, stroma, and endothelium was also demonstrated. Weak positivity for CD4 and CD25 was observed in six of 15 and 11 of 15 rejected corneas, respectively. The presence of dendritic cells in the basal layer of the epithelium and in the stroma was observed in 50% of the grafts.

Conclusions—A high frequency of macrophages, the presence of DC in the explants, and strong expression of HLA-DR and HLA-DR molecules on resident cells are characteristics of rejected corneal allografts, whether actively inflamed or not. The presence of DC in the stroma of the grafted cornea suggests that they may be mainly responsible for T cell activation and graft rejection since DC are known to be a 100-fold more potent than macrophages as APC.

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and only occasional CD1a (Leu-6) positive cells were found in the corneal epithelium. Larkin et al made similar observations regarding the T cell and macrophage infiltration, and also investigated the frequency of other cells such as natural killer (NK) cells, but made no reference to dendritic cells. Most of these studies have been performed with the aim of identifying tissue damaging cytotoxic cells. However, the nature of initiating APC involved in corneal graft rejection is still unclear.

Graft rejection generally is considered to occur by both direct and indirect mechanisms. Direct mechanisms are dependent on there being a sufficient population of donor APC and, apart from one report, the central cornea is generally considered to be devoid of APC. Rejection, therefore, most likely occurs via the indirect mechanism in which host APC present alloantigen to T cells. In corneal grafts the cell which presents the antigen is not known. In only two studies to date were occasional CD1a positive cells detected in the central epithelium of rejected corneal allografts. CD1a is expressed on LC, a subset of B cells, and cortical thymocytes. Accordingly, it has been suggested that macrophages or even aberrantly MHC class II expressing corneal cells such as the corneal epithelium, endothelium, or keratocytes might act as APC in the rejecting graft.

We have re-examined this question by studying the immunohistochemical staining of the cellular infiltrate in rejecting grafts using dual immunofluorescence to determine whether cells expressing CD1a+, MHC class II+, but negative for CD19, occurred within the rejecting graft and were therefore in a position to act as profession antigen presenting dendritic cells.

### Materials and methods

#### PATIENTS

A total of 30 diseased corneas from patients undergoing penetrating keratoplasty were obtained at the 2nd Department of Ophthalmology, Charles University, Prague, after informed consent was given. Investigations were performed according to the guidelines of the Declaration of Helsinki. The clinical details of the corneal recipients (a total of 15 females between the ages of 26–76 years with a median of 53.5 years and 15 males between the ages of 13–74 years with a median of 36.7 years) are given in Tables 1, 2, and 3.

#### PREPARATION OF TISSUES FOR IMMUNOHISTOCHEMISTRY AND IMMUNOFLOUORESCENCE

Samples of rejected and explanted corneas were removed, transferred to foil cups containing OCT medium (Miles Corp, Elkhart, IN, USA) and snap frozen in liquid nitrogen cooled isopentane. They were stored at −80°C until used.

#### IMMUNOHISTOCHEMISTRY

We used the StreptABC method as described previously. Cryostat sections (5–6 µm) of tissues were taken onto chromalum coated slides at −20°C and air dried. The samples were fixed for 10 minutes in acetone, rehydrated twice for 5 minutes in TRIS buffered saline (TBS), and incubated with the primary monoclonal antibody in a humid chamber for 1 hour at room temperature and then incubated overnight at 4°C with the respective secondary antibodies and streptavidin-peroxidase. The primary antibody used was CD1a (clone NCC 201, Immunotech, Marseille, France), MHC class II (clone Leu 3a, Becton Dickinson, USA) and CD19 (clone 4D4, Immunotech, Marseille, France). After washing, the slides were incubated for 10 minutes with 3,3′-diaminobenzidine tetrahydrochloride and 0.05% hydrogen peroxide. The sections were counterstained with chromalum and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA). Controls included omission of primary antibody and fluorescein-conjugated secondary antibody.

### Table 1

<table>
<thead>
<tr>
<th>No</th>
<th>Age</th>
<th>Sex</th>
<th>Reason for keratoplasty</th>
<th>Postoperative complication</th>
<th>Time after transplantation and result of the last medical examination</th>
<th>Postoperative treatment</th>
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<tbody>
<tr>
<td>1</td>
<td>43</td>
<td>F</td>
<td>keratoconus</td>
<td>no rejection</td>
<td>40 months/clear</td>
<td>topical steroids</td>
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<tr>
<td>2</td>
<td>19</td>
<td>M</td>
<td>keratoconus</td>
<td>no rejection</td>
<td>40 months/clear</td>
<td>topical steroids</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>M</td>
<td>keratoconus</td>
<td>no rejection</td>
<td>51 months/clear</td>
<td>topical steroids</td>
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<tr>
<td>4</td>
<td>33</td>
<td>M</td>
<td>keratoconus</td>
<td>no rejection</td>
<td>42 months/clear</td>
<td>topical steroids</td>
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<tr>
<td>5</td>
<td>53</td>
<td>F</td>
<td>PBK</td>
<td>no rejection</td>
<td>59 months/clear</td>
<td>topical steroids</td>
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<td>6</td>
<td>35</td>
<td>F</td>
<td>PBK</td>
<td>no rejection</td>
<td>41 months/clear</td>
<td>topical steroids</td>
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<tr>
<td>7</td>
<td>75</td>
<td>F</td>
<td>PBK</td>
<td>no rejection</td>
<td>60 months/clear</td>
<td>topical steroids</td>
</tr>
<tr>
<td>8</td>
<td>41</td>
<td>F</td>
<td>Fuchs endothelial dystrophy</td>
<td>no rejection</td>
<td>42 months/clear</td>
<td>topical steroids</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>F</td>
<td>Fuchs endothelial dystrophy</td>
<td>endoth. rejection</td>
<td>38 months/clear</td>
<td>topical steroids</td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>F</td>
<td>Fuchs endothelial dystrophy</td>
<td>no rejection</td>
<td>42 months/clear</td>
<td>topical steroids</td>
</tr>
</tbody>
</table>

PBK = pseudophakic bullous keratopathy.
Table 3  A list of keratoplasty patients with rejected inflamed grafts and their characteristics

<table>
<thead>
<tr>
<th>No</th>
<th>Age</th>
<th>Sex</th>
<th>Reason for keratoplasty</th>
<th>Postoperative complications</th>
<th>Rejection episode</th>
<th>Time after transplantation and result of the last medical examination</th>
<th>Postoperative treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>71</td>
<td>M</td>
<td>graft melting</td>
<td>no rejection</td>
<td>—</td>
<td>16 months/graf failure</td>
<td>topical steroids</td>
</tr>
<tr>
<td>27</td>
<td>26</td>
<td>F</td>
<td>graft melting</td>
<td>graft failure</td>
<td>1 month</td>
<td>retransplantation</td>
<td>topical steroids, systemic steroids, systemic cyclosporine</td>
</tr>
<tr>
<td>28</td>
<td>60</td>
<td>M</td>
<td>graft melting</td>
<td>graft failure</td>
<td>10 months</td>
<td>retransplantation</td>
<td>topical steroids</td>
</tr>
<tr>
<td>29</td>
<td>26</td>
<td>F</td>
<td>graft melting</td>
<td>graft melting</td>
<td>1 month</td>
<td>retransplantation</td>
<td>topical steroids, systemic cyclosporine</td>
</tr>
<tr>
<td>30</td>
<td>66</td>
<td>F</td>
<td>spontaneous perforation</td>
<td>stromal rejection</td>
<td>1 month</td>
<td>retransplantation</td>
<td>topical steroids, systemic cyclosporine</td>
</tr>
</tbody>
</table>

Figure 1(A) Corneal specimen obtained from a patient with keratoconus stained mAb anti-CD68 (original magnification ×400). No staining was observed. (B) Strongly positive MHC class II cells (HLA-DR) in the deep layer of the corneal epithelium and in the stroma from a rejected cornea clinically showing no inflammation at the time of surgery (original magnification ×400). (C) MHC class II (HLA-DR) positive cells in the superficial layers of the epithelium from same specimen as (B) (original magnification ×20). (D) Corneal explant obtained from patient after keratoplasty “a cloud” (in severe inflammation at time of surgery). Specimen shows strong staining for macrophages (mAb anti-CD68) especially around the stroma (arrow) (original magnification ×400). (E) Patchy MHC class II (HLA-DR) positive staining of endothelial cells from same specimen as (D) (original magnification ×400). (F) Intensive staining for MHC class II (HLA-DR) on dendritic-like cells in stroma of a corneal specimen from patient with epithelial wound healing problems and no inflammation at the time of corneal transplantation (original magnification ×600). Dual immunofluorescence staining of corneal stromal cells with MHC class II (HLA-DR) and CD1a. (G) Widespread MHC class II staining of many cells in corneal stroma. Arrows show dendrites-like cells in corneal stroma. (H) CD1a staining of individual cells from same region as (G). (I) Dual overlay showing co-expression of MHC Class II and CD1a on stromal dendritic cells, same field as (G) and (H) (original magnification ×600).
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Table 5 Analysis of antibody staining in grafted corneas

<table>
<thead>
<tr>
<th>Grade of antibody staining</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD14</th>
<th>CD68</th>
<th>CD25</th>
<th>HLA-DR</th>
<th>HLA-DP</th>
<th>CD1a/MHC class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (n=10)</td>
<td>0*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 2 (n=15)</td>
<td>0.67 (1.17)</td>
<td>0.37 (0.61)</td>
<td>0.87 (0.83)</td>
<td>2.14 (1.70)</td>
<td>2 (1.24)</td>
<td>0.47 (0.51)</td>
<td>2.8 (1.33)</td>
<td>2.27 (1.33)</td>
<td>1.34 (1.16)</td>
</tr>
<tr>
<td>Group 3 (n=5)</td>
<td>2 (0.63)</td>
<td>0.80 (0.74)</td>
<td>1.8 (0.74)</td>
<td>3 (0.89)</td>
<td>3.8 (0.89)</td>
<td>0.8 (0.4)</td>
<td>3.4 (0.8)</td>
<td>3.2 (0.74)</td>
<td>1 (1.09)</td>
</tr>
</tbody>
</table>

p Value: 0.005† 0.128 0.034† 0.101 0.05† 0.101 0.198 0.046† 0.309

*Grades 0–4 based on actual cell counts in tissue sections (see Methods). Scores represent mean grade (SD) with n = number of samples as indicated.
†Significance of differences between group 2 (clinically no inflammation) and group 3 (clinically inflamed) patients. Statistics were not applied to data combining groups 1 and 2 and groups 1 and 3 since no inflammatory cells were observed in the dystrophic corneas and they are clearly different (score zero).

Discussion

Immunological rejection is the most important cause of graft failure in corneal transplantation. Since there are only limited data on cells which might mediate the immune response particularly antigen presentation in the eye, we measured the expression of HLA-DR and HLA-DP molecules and looked for the presence of DC and macrophages in the corneal buttons from rejected and melted corneal grafts and from control corneas. In this report, for the first time dual immunofluorescent staining has been used for detection of DC using positivity with MHC class II and CD1a and negativity with CD19 antigens as criteria. In a previous paper, low numbers of CD1a+ cells were observed in the corneal epithelium, but not in the corneal stroma. We observed a population of cells which were MHC class II+, CD1a+, CD19−, had a dendritic morphology in corneal epithelial layer but also in the corneal stroma, and which we believe to be definitive dendritic APC. The source of these cells may be from migrating limbal LC, which have become activated, or from conjunctival stromal DC, which have been recruited from bone marrow.

In the group of 15 corneal buttons examined after rejection (from eyes with no inflammation), a significantly less intense infiltration of CD3, CD4, and CD8 positive cells was found in comparison with explants with severe inflammation. However, prominent expression of HLA-DR and HLA-DP antigens on tissue resident cells was seen in a majority of rejected grafts. It is possible that the expression of class II MHC molecules on the non-professional APC might be induced by cytokines produced by inflammatory cells, as has been proposed, and we have observed extensive MHC class II expression on epithelial, stromal, and even on endothelial cells. However, since antigen presentation requires not only MHC class II antigen upregulation but also expression of co-stimulatory molecules, it is unlikely that MHC class II antigen alone on tissue cells is responsible for alloantigen presentation to cytotoxic T cells.

We suggested therefore that professional DC are responsible for initiating graft rejection. The number of CD1a/MHC class II double positive cells was not significantly higher in a group with severe inflammation at the time of surgery than in the group with no inflammation. The number of macrophages, which were also regularly observed in rejected corneas, was variable. We have found significantly increased numbers of CD68+ cells in the severely
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Inflamed corneas. These cells may also act locally as APC within the corneal graft and thus perpetuate the rejection reaction, but macrophages are 100 times less potent as APC than DC. In addition, it is likely that the influx of macrophages may be secondary to the release of a variety of cytokines and chemokines produced by the graft infiltrating lymphocytes. One of these cytokines, migration inhibitory factor (MIF), is produced by many cells including corneal cells.

The most important clinical question is the long term acceptance of the corneal graft. From previous studies reduced corneal graft survival time was observed in recipients with grafts containing high numbers of APC. It was shown that transplantation of syngeneic grafts evokes a focal inflammatory reaction in the vicinity of the sutures and in the wound. Allogeneic grafts induce diffuse inflammation with CD8+ and CD4+ lymphocytes and macrophages. The ratio of CD8+ to CD4+ lymphocytes changes with time after grafting, being higher immediately after grafting.

Taken together, the results of this study support the view that early non-specific inflammation such as occurs in association with surface wound healing problems, previous corneal graft “melting”, and even sutures in the cornea may lead to amplification of the inflammatory response and may trigger an immune response in the eye mediated by CD1a+ and MHC class II+ professional APC. This then leads to chemotraction of antigen specific and non-antigen specific T cell populations with further tissue damaging macrophage infiltration and finally graft rejection.

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