The Distribution of HLA Antigens on Human Corneal Tissue

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Frozen sections of human corneas, as well as cultured cells derived from the epithelial, stromal, and endothelial layers, were examined for class I (HLA-A, B, C) and class II (HLA-DR) histocompatibility antigens using mouse monoclonal antibodies in an indirect immunofluorescence assay. On frozen sections, class I antigens were readily detected on corneal epithelium and keratocytes. Class I antigens were not detected on endothelial cells on frozen sections of adult corneas, but were identified on endothelial cells from some individuals less than 2 years of age. Class II antigens were not detected on corneal epithelial, stromal or endothelial cells on frozen sections. However, HLA-DR-positive dendritic cells were seen in corneal epithelium and were more numerous near the limbus. HLA-DR was expressed by cuboidal cells in the basal layer of conjunctival epithelium from several infants. Cultured cells derived from corneal epithelium, stroma, and endothelium consistently expressed class I but not class II antigens.


Modern surgical techniques and advances in corneal preservation have made primary failure uncommon, and graft rejection has become one of the leading causes of corneal transplant failure. Clinical studies suggest that the HLA system is involved in the rejection of corneal grafts and that matching for HLA or performing a pretransplant lymphocytotoxicity crossmatch may improve graft survival. Because HLA antigens are involved in both the afferent and efferent arcs of the immune response, knowledge of the distribution of these antigens on corneal tissue is important for understanding the rejection process.

We now report on the localization of class I (HLA-A, B, C) and class II (HLA-DR) antigens in human cornea using monoclonal antibodies in an indirect immunofluorescence assay.

Materials and Methods

Corneal Tissue

The corneal tissue used in this study was provided by the Georgia Lions Eye Bank (Atlanta, GA) and was normal by slit-lamp examination. The eyes were usually enucleated within 12 hr of death, washed with sterile saline and then antibiotic solution (polymyxin B, neomycin, and gramicidin). Each cornea was excised with a 3 mm scleral rim and placed in modified McCarey-Kaufman medium (M-K Medium, Aurora Biologicals, Buffalo, N.Y.) containing gentamicin (0.1 mg/ml) and stored at 4°C.

Antisera

Mouse monoclonal anti-HLA-A, B, C (ascites) was obtained commercially (IgG1, Cappel Laboratories, Cochranville, PA; IgG2α, clone W6/32 HLK, Pel-Freez, Rogers, AR). Monoclonal mouse anti-HLA-DR (nude mouse serum) also was purchased from Cappel Laboratories (IgG1). Controls included: (1) phosphate buffered saline containing 2% bovine serum albumin and 0.1% sodium azide (PBS-BSAA), (2) NS1 ascites control (Cappel Laboratories), (3) nude mouse serum control (Cappel Laboratories), (4) anti-HLA-A, B, C absorbed with pooled human platelets, (5) anti-HLA-DR absorbed with rat or human spleen lymphocytes, (6) OKT-4 (IgG2α), and (7) OKT-8 (IgG2) (Ortho Diagnostics, Inc.; Raritan, NJ). The ascites and nude mouse serum controls were produced in animals inoculated with the parent myeloma cell line used for production of the monoclonal antibody. All antisera were adjusted to 25 μg antibody protein/ml before use in the indirect immunofluorescence assay. Fluorescein-conjugated rabbit IgG anti-mouse IgG (FITC-IgG), heavy and light chain-specific (Cappel Laboratories), was used at a dilution of 1:160.
Cell Cultures

Corneal buttons were dissected into three layers as described by Stocker et al. The removal of endothelium-Descemet's membrane was facilitated by injection of the stroma with sterile saline prior to dissection. Explants of epithelium, stroma, and endothelium-Descemet's membrane were placed in 25 cm² culture flasks containing Eagle's minimum essential medium supplemented with 20% (v/v) fetal bovine serum (Hy clone, Sterile Systems, Inc.; Logan, UT, or M.A. Bioproducts; Walkersville, MD), 5% (v/v) calf serum, 2% essential amino acids, 1% nonessential amino acids, 2% glutamine, 7.5% NaHCO₃ (w/v), gentamicin (10 mg/ml), and amphotericin B (1.2 µg/ml). The flasks were left undisturbed until the explants attached.

Primary outgrowth from epithelial explants usually was established in 4–6 days. The epithelial explants then were removed prior to the outgrowth of stromal keratocytes. When primary epithelial cultures became confluent, subculture was performed using Dispase II (Boehringer Mannheim; Indianapolis, IN) as described by Green et al for dermal epithelium. Briefly, monolayers were washed with PBS (pH 7.4) and then incubated with Dispase II (1.2 units/ml in PBS) for 1 hr at 37°C. The detached epithelial sheet was cut in half, washed, and placed in a new 25-cm² flask containing complete culture medium. For the preparation of cell suspensions, epithelial sheets were treated with 0.25% trypsin (BactoTrypsin, Difco Laboratories; Detroit, MI) at 37°C.

Primary cultures of stromal keratocytes were dispersed by treatment with 0.25% trypsin at 37°C and subcultured into medium containing 10% fetal bovine serum. Cultures of endothelium were established and maintained as described by Yue and Baum. Primary cultures were spread by treatment with 0.25% trypsin-verase at 37°C. Cell suspensions were prepared as described above, resuspended in complete medium at 1.0 × 10⁶ cells/ml and allowed to recover for 1 hr at room temperature before use in the indirect immunofluorescence assay.

Preparation of Frozen Tissue

Segments of corneal tissue were frozen in OCT freezing compound (Lab-Tek Products; Naperville, IL) by rapid immersion into liquid nitrogen. They were stored at −70°C and tested within 1 week.

Indirect Immunofluorescence Assay

Corneal sections of 6 µm were prepared, allowed to air-dry for 30 min, and washed with PBS-BSAA. The excess buffer was blotted away and 40 µl of antiserum or control reagent was added. Slides were placed in a moist chamber and incubated for 30 min at room temperature. The antiserum was removed and the sections were washed twice in PBS-BSAA. Forty microliters of FITC-IgG was added. The slides were then incubated in a moist chamber at 4°C for 30 min. Excess antibody was removed and the slides extensively washed with PBS-BSAA. Sections were mounted in 20% glycerol-PBS (pH 7.4) and examined using an Olympus BHT microscope equipped with a BH-RFA reflected light fluorescence attachment, DM500 dichroic mirror, mercury HBO 100W burner, 0515 barrier filter, 490 (B) exciter filter, and EY455 filter. Fluorescence was scored blindly by at least two observers using a scale of 0–4+. Cells that gave a fluorescent reaction with anti-HLA-DR and appeared dendritic in morphology were assumed to be Langerhans cells.

Suspensions of cultured cells were washed once in PBS-BSAA, and the concentration adjusted to 1.0 × 10⁵ cells/ml. One milliliter of the cell suspension was transferred to a microcentrifuge tube and centrifuged at 3,000 × g for 2.5 min. The supernatant was removed and 25 µl of antiserum or control reagent was added to the cell buttons. The cells were resuspended and incubated for 30 min at room temperature. Cells were washed twice with PBS-BSAA and 25 µl of FITC-IgG was added. The cells were then incubated for 30 min at 4°C. After extensive washing in PBS-BSAA, the cells were resuspended in 20 µl of 20% glycerol-PBS and mounted on a glass slide beneath a coverslip. One-hundred cells from both test and control assays were examined for each cell culture. At least five cultures of each cell type were examined. Fluorescence was scored as previously described.

Results

Frozen Sections

Frozen sections of corneas from individuals ranging in age from 1 day–94 years were examined for class I (HLA-A, B, C) and class II (HLA-DR) antigens using mouse monoclonal antibodies in an indirect immunofluorescence assay (Table 1).

Corneal epithelium (Fig. 1A) and conjunctival epithelium (not shown) consistently showed bright fluorescence with anti-HLA-A, B, C (Table 1). Interlamellar fluorescence was observed in the stroma (Fig. 1B), and the fluorescent reaction correlated with the location of keratocytes on serial sections stained with hematoxylin and eosin.

Endothelium from donors over 2 years of age did not react with anti-HLA-A, B, C. However, the endothelium from 9/14 donors less than 2 years of age expressed HLA-A, B, C (Fig. 2, Table 1). The intensity of fluorescence of endothelium was much less than that of epithelium but was clearly positive when com-
pared with controls. There was no correlation between the demonstration of HLA-A, B, C and time from death to enucleation, time from enucleation to freezing, or time from freezing to testing. Isolated HLA-DR-positive dendritic cells were observed frequently in peripheral corneal epithelium (Table 1) and perilimbal conjunctival epithelium (39/40 specimens). Rarely, isolated HLA-DR-positive dendritic cells were present in central corneal epithelium. Unexpectedly, cuboidal cells in basal conjunctival epithelium in 6/15 specimens from individuals less than 5 years of age were HLA-DR-positive (Fig. 3).

Cultured Cells

Primary and secondary cultures of epithelial, stromal, and endothelial cells were established according to the method of Yue and Baum. The morphologic characteristics and patterns of growth of these cell cultures were similar to those previously described for each cell type (Figs. 4A, 5A, 6A). As can be seen (Figs. 4B, 5B, 6B), cells derived from all three layers were HLA-A, B, C-positive. All cultured cells were HLA-DR negative. One endothelial cell culture ceased to express HLA-A, B, C after entering phase III, in spite of good (90%) viability.

Discussion

This study clearly demonstrates that class I (HLA-A, B, C) histocompatibility antigens are expressed on corneal epithelium and keratocytes. Class I antigens also were demonstrated on endothelium of corneas from some donors less than 2 years of age but not on corneal endothelium from older donors. Recently, we have reproduced these observations using an indirect immunoperoxidase assay. Platelet absorbed anti-HLA-A, B, C was included as a control confirming the specificity of the reaction (unpublished results). These findings suggest that the expression of class I antigens on corneal endothelium decreases with age. They are consistent with those of Sang et al (personal communication), who found class I antigens on fetal but not adult corneal endothelium, and Fujikawa et al who did not detect HLA-A, B, C on endothelium of specimens from individuals 16–40 years of age.

Cultured corneal epithelial, stromal, and endothelial cells showed bright fluorescence with anti-HLA-A, B, C. These observations confirm the ability of cultured epithelial and stromal cells to express class I HLA antigens as originally reported by Newsome et al and of cultured endothelial cells to express class I HLA antigens as originally reported by Fujikawa et al. These studies of Newsome et al were performed with cultured cells using a cytotoxic plating inhibition test and human alloantisera. Although there was good overall agreement, the HLA specificities detected on cultured cells by Newsome et al were not always the same as those on peripheral blood lymphocytes. In some cases, cultured cells were found to express HLA antigens not present on peripheral blood lymphocytes, while in other cases, antigens present on peripheral blood lymphocytes were not detected on cultured cells. These findings suggest that antigen modulation or expression of neoantigens occurs when corneal cells are cultured. We have studied cultured stromal fibroblasts from two individuals with human HLA alloantisera using cytotoxicity and absorption techniques. All HLA-A locus antigens and one HLA-B locus antigen were detected in a complement-dependent cytotoxicity assay. Absorption studies were necessary to demonstrate the other three HLA-B locus antigens (unpublished observations). These observations indicate that cultured stromal cells express the same antigens as peripheral blood lymphocytes and argue against antigen modulation or neoantigen expression in vitro.

The presence of HLA-A, B, C in high density on corneal epithelial cells provides an explanation for the decreased rejection rates found with xenografts (reviewed in reference 12) and human allografts when epithelium was removed prior to transplantation. Class I antigens on keratocytes and endothelial cells could serve as targets for immunologic destruction, even if donor epithelium were removed at surgery or replaced by recipient epithelium. Our results suggest that proliferating cultured endothelial cells express more class

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### Table 1. Class I and class II histocompatibility antigens on frozen sections of human corneas

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Corneal epithelium</th>
<th>Corneal endothelium</th>
<th>Conjunctival epithelium</th>
<th>Corneal epithelial dendritic cells</th>
<th>Corneal endothelium</th>
<th>Basal conjunctival epithelium</th>
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<tr>
<td>0-5</td>
<td>21/21*</td>
<td>9/14†</td>
<td>21/21</td>
<td>10/15</td>
<td>0/7</td>
<td>6/15</td>
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<tr>
<td>6-25</td>
<td>9/9</td>
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<td>9/9</td>
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<td>8/8</td>
<td>5/8</td>
<td>0/5</td>
<td>0/8</td>
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<td>0/4</td>
<td>7/7</td>
<td>4/8</td>
<td>0/5</td>
<td>0/7</td>
</tr>
</tbody>
</table>

* Number of positive specimens/number of specimens examined. Endothelium was not always adequately preserved; therefore, the total number of specimens examined for endothelial antigens was reduced.
† All donors less than 2 years old.
I antigen than do endothelial cells in situ. Since adult human endothelial cells are now known to proliferate \textit{in vivo}, one might speculate that proliferation of grafted donor endothelial cells might occur during the postoperative healing process. Increased expression of HLA antigens on such proliferating cells conceivably could help explain the fact that most rejection episodes occur during the first postoperative year.

Our finding of large numbers of HLA-DR-positive cells in the basal conjunctival epithelial layer on several specimens from infants was unexpected. These cells were clearly cuboidal and did not possess the dendritic processes characteristic of Langerhans cells. Class II histocompatibility antigens have been demonstrated on epithelial cells from salivary gland, gut, lactating mammary gland, renal tubules, and colorectal cancer. Furthermore, graft-versus-host disease can induce the expression of class II antigens on epidermal and colonic epithelium in the rat. More recent evidence indicates that naturally occurring antigenic exposure can induce class II antigen expression by both epidermal and gut epithelial cells. Whether the DR-positive cuboidal cells observed in infant conjunctival epithelium are actually basal epithelial cells that transiently express DR, Langerhans-type cells with unusual morphology, precursors of Langerhans cells, or some other cell type remains to be elucidated.

The clinical significance of class I HLA antigens is supported by several reports of prolonged graft survival in HLA-A,B-matched corneal transplants. The finding that class I antigens are more easily demonstrated on endothelium from younger individuals raises the
Fig. 4. A, Cultured epithelial cells grown in monolayer (original magnification phase, ×200). B, Suspension of cultured epithelial cells showing fluorescence with anti-HLA-A,B,C (original magnification, ×400).

keratocytes showing positive fluorescence with anti-HLA-A,B,C (original magnification, ×400).

showing positive fluorescence with anti-HLA-A,B,C (original magnification, ×400).
possibility that rejection might be more common when younger donor tissue is used for transplantation, a hypothesis that awaits clinical investigation. Since HLA-DR antigens have not been detected on corneal cells other than epithelial dendritic cells, it will be of interest to determine whether tissue matching for DR-locus antigens improves graft survival.

Key words: antigens—immune response, cornea, cultured cells, endothelium, epithelium, histocompatibility determinants, HLA antigens, keratoplasty, stroma, transplantation

Acknowledgments

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References