Induction of Class II (Ia) Alloantigen Expression on Corneal Endothelium In Vivo and In Vitro

John J. Donnelly,* Weiye Li,* John H. Rockey,* and Robert A. Prendergast†

Class II (DR or Ia) alloantigens are potent inducers of cell-mediated immune responses. However, the expression of Class II alloantigens on corneal cells, except for Langerhans cells within the limbal epithelium, has not previously been described. We have induced Ia expression on cultured rabbit corneal endothelial cells with human immune interferon, and have demonstrated numerous Ia-positive cells in the endothelium during primary immunogenic uveitis in vivo. Cultured endothelial cells which had been treated with immune interferon in vitro functioned as stimulator cells in a mixed leukocyte reaction. Induction of Class II alloantigen expression by lymphokines may potenti-ate corneal allograft rejection. Invest Ophthal mol Vis Sci 26:575–580, 1985

The rejection of corneal allografts by immunological mechanisms has been described both in humans and in many animal model studies.1 Although in humans Class I (e.g. HLA-A, B, C) alloantigens are present on corneal cells,2 Class II (HLA-DR or DR) alloantigens are absent from normal adult corneas3 except for the Langerhans cells of the corneal epithelium.4

Previously we have demonstrated the presence of a lymphokine activity in the aqueous humor of rabbits with primary and secondary uveitis, which induces the expression of Ia alloantigens on rabbit macrophages.5 The induction of HLA-DR alloantigen expression on human vascular endothelial cells by lymphokines (immune interferon) has been described recently.6 In the present study we demonstrate the induction of Ia alloantigen expression on rabbit corneal endothelium in vivo during primary immunogenic uveitis, and in vitro with purified human immune interferon (IFN-γ). Ia-bearing corneal endothelial cells were effective stimulators of mixed leukocyte reactions. Lymphokine-induced class II alloantigen expression on the corneal endothelium may predispose to or amplify a rejection episode.

Materials and Methods. Primary culture of corneal endothelium: Corneas were removed aseptically from normal eyes of NZW rabbits and placed endothelial side upward in a machined aluminum block. The endothelial surfaces were irrigated with Ca++-Mg++-free Puck’s saline, and digested with 0.25% trypsin (Difco, Detroit, MI), 0.2% collagenase (Worthington, Freehold, NJ), and 0.25% trypsin sequentially. The corneas were incubated at 37°C for a total of 2–2.5 hours. Endothelial cells were carefully washed out with a fire-polished pipette to avoid damage to Descemet’s membrane. Free endothelial cells were washed in Dulbecco’s Modified Eagle’s Medium (DMEM) with 20% bovine serum (FBS) and plated in gelatin-coated 24-well plates. All experiments were carried out using cells which had undergone two or three passages after primary culture.

Induction of Ia antigens: Endothelial cells which had been subcultured one or two times after initiation were plated in gelatin-coated 24-well or 96-well plates and allowed to grow to sub-confluence (2–4 days). The growth medium was aspirated, the wells were washed with Hanks’ Balanced Salt Solution (HBSS), and fresh growth medium consisting of DMEM with 20% Nu-serum (Collaborative Research, Inc., Lexington, MA), with or without Human Immune Interferon (IFN-γ, Interferon Sciences, Inc., New Brunswick, NJ) at 500, 1,000 or 2,000 units/ml, was added. Nu-serum was used because it supports endothelial cell growth with a reduced percentage of FBS. Perinatal serum may contain α-fetoprotein which inhibits the induction of Ia expression on macrophages.7 Four days after the addition of IFN-γ, the endothelial cells were trypsinized and stained in suspension for Ia antigen. In selected experiments cultured cells were allowed to remain in the wells and were used as stimulator cells in a mixed lymphocyte reaction (MLR).

Peritoneal macrophages were obtained from NZW rabbits (not endothelial cell donors) injected intraperitoneally with 5% thioglycollate, as previously described.5 The macrophages were cultured in RPMI 1640 medium with 10% Nu-serum and 500, 1,000 or 2,000 units/ml of IFN-γ.5

Staining of cells for Ia antigens: The anti-Ia monoclonal antibody used in these studies was a gift of Drs. S. Lobel and K. Knight. The antibody recognizes a public specificity of rabbit class II alloantigens common to both the Ia1 and Ia2 haplotypes.8 The cell suspensions were pelleted and resuspended in 20 μl of monoclonal anti-Ia (1 mg/ml) on ice for 30 min. The cells then were washed once in 0.01 M phosphate-buffered saline, pH 7.4 (PBS), pelleted and resuspended in 20 μl of fluorescein-conjugated goat anti-mouse IgG (9 mg/ml) (Tago, Inc., Burlingame, CA) on ice for 30 min. The stained cells were washed in PBS, fixed in PBS with 1% paraformaldehyde, and examined with a Zeiss epi-fluorescence microscope.
Monolayer cultures of rabbit macrophages were stained as previously described.5

Factor VIII antigen staining: Corneal endothelium grown on glass coverslips was fixed in 100% methanol for 5 min at −20°C. The coverslips were washed in PBS, and stained for 1 hour at 37°C with a 1:40 dilution of goat anti-human Coagulation Factor VIII (Antihemophilia A Factor) (Miles Laboratories, Elk-
Primary immunogenic uveitis was induced in 4 groups of 3 outbred NZW rabbits by intravitreal injection of 2 mg of ovalbumin in the right eyes. At the peak of uveitis (usually 8 days after injection), the animals were sacrificed and the corneas were removed. The corneas were placed endothelial side up in aluminum block holders, extensively irrigated with PBS, and digested with 0.2% trypsin for 2 hours at 37°C. Trypsinization of isolated peripheral blood leukocytes under identical conditions did not alter the % Ia⁺ cells. Cells harvested from left (control) or right (inflamed) eyes of individual animals were pooled respectively and stained in suspension for Ia alloantigens.

Mixed lymphocyte reactions: Peripheral blood mononuclear cells (PBL) were isolated from NZW outbred rabbits by Ficoll-Hypaque density gradient sedimentation. Stimulator cells (endothelial cells incubated with or without IFN-γ, and isolated PBL) were irradiated with 2,700 R of γ-radiation from a 137Cs source. Responder cells at 1.5 × 10⁵ cells (3 day cultures) or 1 × 10⁵ cells (4 day cultures) per culture were added to an equal number of irradiated lymphocytes or to a monolayer of irradiated and extensively washed endothelial cells (approximately 10⁶ cells per culture) in 96-well plates. Anti-Ia monoclonal antibody (10 µg/ml) was added to selected cultures. This antibody inhibits lymphocyte proliferation in response to foreign Ia alloantigens in a mixed lymphocyte reaction. Cultures were pulsed with 1 µCi of [³H]thymidine after 48 or 72 hours and harvested 18 hours later.

All studies were carried out in accordance with the ARVO Resolution on the Use of Animals in Research.

Results. Corneal endothelial cells trypsinized from normal corneas and stained immediately in suspension with anti-Ia were 5-7% positive for Ia alloantigen. The contralateral eyes which had been injected with ovalbumin and had immunogenic uveitis yielded corneal endothelial cells which were 64% positive for Ia. Representative positive and negative cells are shown in Fig. 1. In some instances sheets of cells were observed which had detached from Descemet's membrane but which retained the hexagonal arrangement of corneal endothelium. These sheets contained cells positive for Ia antigen only if they were prepared from eyes with primary uveitis (Fig. 1).

The induction of Ia alloantigen expression in vitro on peritoneal macrophages and cultured corneal endothelium by human IFN-γ is shown in Table 1. Incubation with IFN-γ produced a 3-4 fold increase in Ia-positive endothelial cells and a 2-3 fold increase in Ia-positive macrophages. Representative Ia⁺ cultured endothelial cells are shown in Fig. 2. Staining...
**Fig. 2.** A, B, Cultured rabbit corneal endothelial cells, induced with IFN-γ for 4 days in culture, and stained in suspension with anti-la. Strong membrane staining is present (×1,005).

for human Factor VIII antigen of cultured corneal endothelial cells prepared in parallel with those shown in Table 1 demonstrated that 100% of the cells were positive for this antigen (Fig. 2).

The ability of IFN-γ-treated la-bearing corneal endothelial cells to serve as stimulator cells in the mixed leukocyte reaction (MLR) is shown in Table 2. Incubation of PBL with irradiated allogeneic lymphocytes produced approximately a 5-fold increase in [3H]thymidine uptake compared to non-irradiated PBL alone. Incubation of PBL with irradiated IFN-γ-treated endothelial cells resulted in 4-5-fold greater [3H]thymidine incorporation than PBL plus non-treated endothelial cells or PBL alone (Table 2).

In a separate experiment, anti-la monoclonal antibody was added to cultures containing lymphocytes and IFN-γ-treated corneal endothelial cells. Lymphocytes plus IFN-γ-treated endothelium, and lymphocytes plus non-IFN-γ-treated endothelium were used as controls. The lymphocyte proliferation induced by the IFN-γ-treated endothelial cells was reduced to control levels by the addition of the anti-la. Tritiated-thymidine incorporation was 6,975 ± 2,423 (SD) cpm (n = 5) for lymphocytes plus IFN-γ-treated endothelial cells, 3,222 ± 1,214 cpm (n = 5) for lymphocytes plus IFN-γ-treated endothelial cells plus anti-la, and 3,310 ± 603 cpm (n = 5) for lymphocytes plus non-IFN-γ-treated endothelial cells. Proliferation was reduced significantly in cultures of lymphocytes plus IFN-γ-treated endothelial cells and anti-la compared to cultures of lymphocytes plus IFN-γ-treated endothelial cells (P < 0.025, Student t-test), but was not significantly different from cultures of lymphocytes plus non-IFN-γ-treated endothelial cells (P > 0.05).

**Discussion.** The presence of a high number of la+ cells in trypsinized corneal endothelial preparations from eyes with primary immunogenic uveitis indicates that the endothelium may have been induced to express la alloantigens. A likely cause of this is the la-inducing activity known to be present in the aqueous humor of these eyes. It is unlikely that inflammatory cells adherent to the endothelial surface would produce such a dramatic shift in the number of la+ cells since the corneas had been extensively irrigated with PBS prior to digestion with trypsin. An alternative explanation is that la alloantigens are passively acquired from the surrounding tissue fluids. This may occur but the ability of cultured corneal endothelium to be induced for la expression by IFN-γ argues in favor of a significant role for la biosynthesis. Further studies employing alloantisera and grafted corneas in vivo, and endogenous labeling of
cellular products in vitro, will help to resolve this question.

The ability of IFN-γ treated cultured corneal endothelial cells to serve as stimulator cells in a mixed leukocyte reaction demonstrates that the la alloantigens expressed can be recognized and responded to by T-cells of an appropriate specificity. This indicates that la-bearing endothelial cells in a corneal allograft could excite a localized cell-mediated response in the anterior chamber provided the appropriate T-cells and accessory cells gain access to the graft. Thus lymphokine production in an eye with a corneal allograft could lead to expression of Class II alloantigens on the donor endothelium, thereby contributing

### Table 1. la-positive cells after incubation with IFN-γ

<table>
<thead>
<tr>
<th>IFN-γ (units/ml)</th>
<th>Peritoneal macrophages*</th>
<th>Corneal endothelium†</th>
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<tbody>
<tr>
<td>0</td>
<td>14 ± 4.0</td>
<td>5 ± 0.4</td>
</tr>
<tr>
<td>500</td>
<td>30 ± 2.9‡</td>
<td>20 ± 4.0‡</td>
</tr>
<tr>
<td>1000</td>
<td>31 ± 9.2</td>
<td>15 ± 0.3</td>
</tr>
<tr>
<td>2000</td>
<td>38 ± 5.9</td>
<td>17 ± 1.2</td>
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</tbody>
</table>

* Percent la positive cells adherent to glass coverslips. Based on at least 100 cells counted in each of 4 replicates.
† Percent la positive cells stained in suspension after trypan blue exclusion. Based on at least 500 cells counted per sample.
‡ Two-tailed P < 0.01 by the Student t-test for two means, comparing IFN-γ-treated and nontreated cells.

### Table 2. Mixed lymphocyte reactions induced by corneal endothelial cells in 3-day cultures

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Counts per minute [µCi] thymidine ± SD</th>
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<tbody>
<tr>
<td>Lymphocytes alone</td>
<td>1,421 ± 434</td>
</tr>
<tr>
<td>Lymphocytes and endothelial cells*</td>
<td>1,348 ± 367</td>
</tr>
<tr>
<td>Lymphocytes and IFN-γ-treated endothelial cells*</td>
<td>5,978 ± 1,165†</td>
</tr>
<tr>
<td>Lymphocytes and allogeneic lymphocytes*</td>
<td>9,052 ± 1,288</td>
</tr>
<tr>
<td>Lymphocytes and allogeneic lymphocytes*</td>
<td>1,582 ± 1,049</td>
</tr>
<tr>
<td>and anti-la</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes and syngeneic lymphocytes*</td>
<td>5,745 ± 2,076</td>
</tr>
<tr>
<td>Lymphocytes and Con A (5 µg/ml)</td>
<td>6,572 ± 1,569</td>
</tr>
</tbody>
</table>

* Irradiated, 2,700 rads.
† Two-tailed P < 0.001 by the Student t-test for two means, comparing IFN-γ-treated and nontreated endothelial cells as stimulators.
to the alloimmunization of the recipient, and targeting the donor endothelium for cell-mediated immune destruction. The importance of Ia alloantigen induction to endothelial rejection of human corneal allografts merits further study.

Key words: Class II alloantigens, Ia antigens, corneal endothelium, immune interferon, rabbit

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5-Fluorouracil Toxicity to the Ocular Surface Epithelium

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The antimetabolite, 5-fluorouracil (5-FU), has been used to control proliferation of retinal pigment epithelial cells and fibrocytes, and is currently the subject of a multicenter clinical trial of its value in the control of scarring after glaucoma operations. To evaluate possible ocular surface toxicity, the effect of 5-FU on the mitotic rate and differentiation of the ocular surface epithelium in rabbits was measured. 5-FU was instilled into eyes with 10-mm diameter central epithelial wounds and into nonwounded eyes at a dose of 9 mg per day for 4 days. Saline treated control wounded eyes healed within 4 days (n = 5) while 40% (4 of 10) of the 5-FU treated wounded eyes had defects at 4 days. The normal mitotic rate of the corneal epithelium was 1.0 ± 0.3 (n = 4) tritiated thymidine labeled cells per 100 basal corneal epithelial cells after 2.5 hr incubation. Saline treated control wounded eyes had an increased mitotic rate, 7.1 ± 1.3 (n = 5) labeled cells per 100 basal corneal epithelial cells after 2.5 hr incubation. Topical 5-FU decreased both of those rates to about 1% of normal. The normal conjunctival epithelial mitotic rate was 1.8 ± 0.4 (n = 4) labeled cells per 60 basal cells after per 2.5 hr incubation. This rate was the same in wounded eyes, but was decreased in eyes treated with 5-FU. Thus, 5-FU (9 mg/day topically) has serious toxic effects to ocular surface epithelium which must be carefully considered if this drug is to be used clinically. Invest Ophthalmol Vis Sci 26:580–583, 1985

The antimetabolite, 5-fluourouracil (5-FU), is a pyrimidine analog which has been shown to block mitosis of retinal pigment epithelial cells and of fibrocytes in vitro and in vivo. It has also been used experimentally to prevent fibrocyte proliferation and scarring of the filtering bleb in glaucoma surgery,1,4 and is now the subject of a multicenter clinical trial to evaluate its effectiveness in controlling scarring of filtration blebs. In some studies, there appeared to be some toxicity of 5-FU to the ocular surface epithelium in the form of persistent epithelial defects. The purpose of this article is to evaluate these potential toxic effects of 5-FU. To do so, topical 5-FU was applied to normal and wounded rabbit epithelium and the effect of the drug on the appearance and mitotic rate of the epithelium was studied.

Materials and Methods. Animals: All investigations involving animals that are described in this manuscript...