The Role of Class II Antigen-Expressing Cells in Corneal Allograft Immunity

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The goal of this study was to investigate the relationship between the presence of Class II antigen-expressing cells (Class II⁺) cells in the cornea and the generation of allograft immunity. Wistar/Furth (W/F) rat Class II⁺ cells were injected in various numbers (0.01, 0.1, 1.0, 5.0, 10.0, and 20.0 × 10⁶) into the corneal stroma of Fischer 344 (F344) rats. For comparison, the same numbers of W/F Class II⁺ cells were injected directly into the peritoneal cavity of F344 rats. Also, W/F cells were injected into the corneas of F344 rats and the corneas were "grafted" intraperitoneally in F344 hosts. The results showed that up to 20 × 10⁶ class II⁺ cells injected in situ into the corneal stroma did not elicit a serum cytotoxic antibody response or a splenic or blood cytotoxic T-cell response against donor Class II antigens. In contrast, systemic immune responses were elicited by both direct intraperitoneal injection of large numbers (10 or 20 × 10⁶) of allogeneic Class II⁺ cells and by intraperitoneal grafting of syngeneic corneas carrying similar numbers of allogeneic Class II⁺ cells. F344 recipients of a syngeneic cornea containing 10 or 20 × 10⁶ W/F Class II⁺ cells or a suspension of W/F cells exhibited an accelerated rejection of W/F skin allografts (13.3 versus 9.0 days, first- versus second-set rejection). These results indicate that the number of Class II⁺ cells required to elicit a systemic immune response is larger than the number of Class II⁺ cells present in the normal cornea. Invest Ophthalmol Vis Sci 31:2254–2260, 1990

The cells of the normal cornea consist almost exclusively of epithelial cells, stromal keratocytes, and endothelial cells, with relatively few immune accessory cells expressing Class II antigens.¹⁻⁶ The Class II antigen-expressing (Class II⁺) Langerhans cells present in the normal cornea are distributed in a gradient from the peripheral cornea and conjunctiva, where the largest numbers are found, to the central cornea, where there are few or none.³⁻⁶⁻⁸ Both resident and infiltrating Class II⁺ cells are present in larger numbers in corneas subjected to various stresses;¹⁻¹⁰ their distribution changes markedly in various corneal disease states, including corneal inflammatory reactions,⁷⁻⁹ corneal infections,⁶¹¹ and the corneal allograft immune response.⁶⁻⁷⁻¹⁰

Several investigators proposed that Class II⁺ cells of donor origin contribute to the immunogenicity of corneal allografts.²⁻⁵⁻⁶⁻¹₀⁻¹²⁻¹³ Although it has been shown that both Class I and II histocompatibility antigens expressed on corneal cells may stimulate an allograft reaction,⁵⁻⁷ it has been suggested that cells expressing Class II antigens are more efficient in this capacity.⁷⁻⁹⁻¹⁰

Cells in various tissues undergoing immunologic attack are induced to express Class II antigens that they normally do not express.¹⁴⁻²² Young and McMillan²³ showed that corneal stromal keratocytes in culture can be induced to express Class II histocompatibility antigens when exposed to the lymphokine, gamma interferon. These investigators speculated that the induced expression of Class II histocompatibility antigens could be an important early event in the initiation of the corneal allograft immune reaction.²³

Our purpose was to determine the relationship between the presence of Class II⁺ cells in the cornea and the stimulation of a host antigraft reaction. These results demonstrate that the presence of large numbers of allogeneic Class II⁺ cells in the cornea does not elicit a systemic immune reaction; this suggests that the immunity engendered by Class II⁺ allogeneic cells in the cornea is a local rather than a systemic phenomenon. The role of donor Class II⁺ cells in eliciting immune rejection of corneal grafts in the human eye requires further study.
Materials and Methods

Animals

Female rats of the inbred strains, Wistar/Furth (W/F, Rtl\textsuperscript{1}t) and Fischer 344 (F344, Rtl\textsuperscript{1}v), weighing approximately 120 g, were used (Harlan Sprague Dawley, Indianapolis, IN). All animals were maintained and handled according to the ARVO Resolution on the Use of Animals in Research.

Cell Suspensions

The W/F and F344 rats were killed by sodium pentobarbital euthanasia. The spleens were removed using sterile technique, prepared as single cell suspensions, and freed of erythrocytes and dead cells on Lympho-paque gradients (Accurate Chemical and Scientific, Westbury, NY). Total cell number and cell viability were determined by hemocytometer count using the vital dye, trypan blue.

Cell Fractionation

Two micrograms of mouse monoclonal, rat anti-Class II antibody were incubated with 1 mg of magnetic immunobeads (Dynabeads M-450; Robbins, Mountainview, CA) for 4 hr at room temperature with gentle rocking. After incubation the labeled beads were suspended in 10 ml of RPMI-1640 (Grand Island Biological, Grand Island, NY), containing 0.3% bovine serum albumin (Sigma, St. Louis, MO) (RPMI/BSA), and concentrated using a Dynal Magnetic Particle Concentrator (Robbins). The supernatant was discarded, and the labeled beads were resuspended in an additional 10 ml of RPMI/BSA and again collected with the particle concentrator. Washing and concentration of the beads were repeated two additional times.

Ten microliters of the suspension containing approximately \(4 \times 10^6\) antibody-coated beads was adequate for the isolation of \(5 \times 10^6\) Class II\textsuperscript{+} cells. In practice, 0.5 ml of antibody-coated beads was incubated with \(1 \times 10^8\) rat spleen cells (SC) for 30 min at 4°C on a rocking platform. The RMPI/BSA was added to a final volume of 10 ml, the beads with attached cells were collected in the magnetic particle concentrator, and the supernatant removed. The washing and concentration procedure was repeated three additional times.

The bead-cell complexes were incubated at 37°C for 8 hr, during which time the cells separated from the beads. At the end of the incubation, the detached beads were collected on the magnetic particle concentrator and the cell suspension collected in the supernatant. Cell number and viability were determined by hemocytometer count. The viability of cells purified by this method was uniformly greater than 97%. The cell suspensions were held at 4°C for use in the experimental protocol.

The presence of Class II histocompatibility antigens on the cells was confirmed by immunoperoxidase staining as previously reported. At least 93% of the immunobead-purified cells were Class II\textsuperscript{+}.

Cell-Mediated Cytotoxicity

Peripheral blood lymphocytes (PBLs), peritoneal exudate lymphocytes (PELs), and SCs were used as the sources of cytotoxic effector cells. Animals that had been injected with allogeneic Class II\textsuperscript{+} cells were killed at the times indicated in the experimental design. To obtain PBLs, 5–7 ml of whole blood was collected in heparinized syringes. For PELs, the peritoneal cavity was lavaged with 10 ml of RPMI/BSA. For SCs, the spleen was removed with sterile dissecting instruments and reduced to a cell suspension.

The cell suspensions obtained from blood, peritoneal exudates, and spleens were fractionated over Lympho-paque gradients. Next, each cell suspension was separately enriched for T-cells by nylon-wool fractionation. Approximately 0.5 g of scrubbed nylon-wool (type 200L; Dupont, Wilmington, DE) was packed in a 10-ml syringe and equilibrated with RPMI/BSA at 37°C for 1 hr. The mononuclear cells obtained from the peritoneal washout, peripheral blood gradient, or approximately one third of a total spleen cell suspension (30 \( \times \) 10\textsuperscript{6} cells) were added to 2 ml of RPMI/BSA and pipetted onto each column. After 1 hr at 37°C the nonadherent T-lymphocytes were eluted from the nylon column with 50 ml of RPMI/BSA. The T-cells were washed, counted, and resuspended in complete tissue culture medium consisting of RPMI-1640 supplemented with 10% fetal bovine serum (Grand Island Biological).

Class II\textsuperscript{+} cells used as \(^{31}\text{Cr}\)-labeled target cells were obtained from the spleens of W/F strain rats and putor. Washing and concentration of the beads were suspended in culture medium containing 100 \( \mu \text{Ci}\) of Na\(^{51}\text{CrO}_4\) (ICN Radiochemicals, Irvine, CA) at a concentration of \(1 \times 10^7\) cells/ml and incubated at 37°C for 60 min. The cells were washed three times in culture medium, incubated for an additional 30 min at 37°C, and washed once more. The effector and target cells were mixed together in round-bottom microcytotoxicity plates (Falcon Labware; Becton Dickinson, Lincoln Park, NJ) in ratios of 1:1, 10:1, 20:1, 40:1, 80:1, and 160:1.

Groups of eight replicate wells were established for each mixture of cells. The plates were incubated for 5 hr at 37°C, then centrifuged at 250 \( \times \) g for 5 min. Fifty-microliter aliquots of the supernatant from each
well were collected individually in 12 x 75-mm plastic test tubes (Falcon; Becton Dickinson) and the amount of radioactivity determined in a gamma counter. Control wells for determining the amount of spontaneous ⁵¹Cr release contained target cells alone. The total releasable ⁵¹Cr was determined by adding 50 µl of a 1% solution of Triton X-100 to wells containing ⁵¹Cr-labeled cells. The percentage of specific cytotoxicity was calculated according to the formula:

\[
\text{Percent Specific Cytotoxicity} = \frac{\text{Experimental CPM} - \text{Background Release CPM}}{\text{Release CPM} - \text{Background Release CPM}} \times 100
\]

**Serum Alloantibody Assay**

Endogenous complement in sera obtained from F344 strain recipients was inactivated by heating at 56°C for 30 min. The anti-Class I antibody was removed from the sera by repeated absorption with W/F strain erythrocytes. Removal of the anti-Class I antibody was assured by testing the absorbed serum in hemagglutination assay as previously reported.²⁵ Spleen cells from a W/F donor were gradient purified on Lympho-paque and the Class II⁺ cells enriched using the magnetic immunobead technique. The purified cells were suspended at a concentration of 5 x 10⁶ cells/ml in RPMI/BSA and dispensed in 25-µl volumes into the wells of round-bottom microtiter plates containing serial twofold dilutions of serum samples obtained from F344 donors. Next, 25 µl of a 1:10 dilution of Low Tox M rabbit complement (Accurate Chemical and Scientific) was added to all wells, and the plates were incubated for 45 min at 3⁷°C. The percentage of cells killed was determined by hemocytometer chamber counts using trypan blue as the indicator. Control sera obtained from nonimmune F344 donors and control wells containing only the complement were done with each assay.

**Skin Grafting**

The technique of skin grafting rats originally reported by Krapienis²⁶ and used in previous studies²⁵ was employed. Rats of the W/F and F344 strains were anesthetized with an intramuscular injection of Ketaset 60 mg/kg (ketamine hydrochloride; Bristol, Syracuse, NY) and Rompun 0.6 mg/kg (xylazine; Butler, Columbus, OH). The W/F skin was obtained from the dorsal basal region of the tail and grafted onto the dorsal cervical region of the F344 recipients. The grafts were inspected daily after removal of the gauze covering. Complete rejection of the skin graft was recorded as the day on which the graft was dry to the touch, discolored, and thought to be inviable.²⁵²⁶

**Experimental Design**

To test the capacity of Class II⁺ cells to elicit humoral and cellular immune responses after introduction into allogenic recipients, several experimental groups were studied. The W/F Class II⁺ cells were injected into the corneas or peritoneal cavities of F344 rats. For corneal injection, six groups of 12 F344 rats each were anesthetized and given injections of 0.01, 0.1, 1.0, 5.0, 10.0, or 20.0 x 10⁶ purified W/F strain Class II⁺ cells into the corneal stroma using a previously described technique.²⁴ Two other groups of F344 rats received equivalent injections of W/F Class II⁺ cells as a suspension or as corneal implants into which W/F cells had first been injected. This latter group was included to determine the effect of the corneal milieu on the immunogenicity of cells. The technique of implanting corneas into the peritoneal cavity was done as previously described.²⁵ Negative-control F344 rats received 1.0-ml volumes of RPMI-1640.

The time course of the development of humoral and cellular immunity was determined by bleeding and killing two F344 recipients from each experimental and control group at 1, 2, 3, 4, and 5 weeks after grafting or injection. To determine whether the introduction of large numbers of Class II⁺ cells is capable of generating second-set graft rejection, four groups of F344 rats were grafted with W/F skin. One group consisted of eight F344 rats that had been injected with 20 x 10⁶ W/F Class II⁺ cells into the corneal stroma 5 weeks earlier. A second group involved eight F344 rats that had received a syngeneic cornea containing 20 x 10⁶ W/F Class II⁺ cells implanted intraperitoneally 5 weeks earlier. A third group included eight F344 rats that had received an intraperitoneal injection of 20 x 10⁶ W/F Class II⁺ cells 5 weeks earlier. The control group consisted of 12 normal F344 rats; this group was used to determine the median survival time (MST) of W/F skin on the allogeneic F344 recipients.

**Data Analysis**

Each experiment was repeated at least three times. The data in the ⁵¹Cr release assays and the serum alloantibody assays were reduced to means and standard deviations of the means, and results of the different experimental and control groups were compared for statistical significance by analysis of variance.
Results

Serum Alloantibody Response

None of the F344 rats that received W/F Class II\(^+\) cells injected into the corneal stroma developed a serum alloantibody response over the 5-week period of the study (Fig. 1A). In contrast, the animals that received an intraperitoneal injection of either 10 \(\times 10^6\) or 20 \(\times 10^6\) W/F strain Class II\(^+\) cells showed a serum alloantibody response measurable at both 1 and 2 weeks after injection (Fig. 1A). Lower cell numbers did not elicit an antibody response.

The F344 corneas injected with W/F Class II\(^+\) cells and implanted intraperitoneally in F344 rats also elicited an antibody response. The corneas containing the largest number of cells (20 \(\times 10^6\)) generated the greatest response, which became evident 14 days after transplantation and reached a peak at approximately 21 days (Fig. 1B). Corneas carrying 10 \(\times 10^6\) Class II\(^+\) cells produced a small, but measurable response; lower numbers of cells did not elicit a response above control levels (Fig. 1B).

Cell-Mediated Immune Response

The W/F Class II\(^+\) cells injected into the corneal stroma did not elicit a measurable cellular cytotoxic response in the blood, spleen, or peritoneal cavity of the F344 recipients over the 5 weeks of the study. The

Skin Allograft Rejection

The MST of W/F skin grafts on naive F344 recipients was 13.3 ± 1.6 days. The W/F skin grafts subjected to a second-set reaction became vascularized by day 5, underwent rapid discoloration, shrinkage,
Fig. 3. Cell-mediated cytotoxic responses of F344 rats "grafted" intraperitoneally with syngeneic corneas carrying 0.01, 0.1, 1.0, 5.0, 10.0, or 20.0 × 10^6 W/F Class II+ cells. The presence of cytotoxic T cells in the blood, spleen, and peritoneal cavity was assessed. No cytotoxic cells were found in the blood. Ten (circles) and 20 (squares) × 10^6 cells elicited positive peritoneal (A) or splenic (B) cytotoxic responses, whereas the response to lower cell numbers fell within the range (shaded area, 0-10%) of cytotoxicity obtained with peritoneal or spleen cells from nonimmune animals. The results obtained with 10 and 20 × 10^6 cells at 21 and 28 days after immunization were statistically significantly different from one another (P < 0.005). The results obtained with these cell concentrations at 21 and 28 days were statistically significantly different from the lower cell numbers tested.

and became dry scabs within 3-5 days thereafter. The W/F grafts which were rejected in 10 days or less were considered to have been subjected to a second-set response.

None of the F344 rats that received allogeneic Class II+ cells injected into the corneal stroma had a second-set skin allograft rejection (Table 1). In contrast, F344 recipients of intraperitoneally transplanted syngeneic corneas carrying either 10 × 10^6 or 20 × 10^6 allogeneic Class II+ cells rejected W/F strain skin allografts in an accelerated fashion (Table 1). Similarly, F344 animals injected with suspensions of 10 × 10^6 or 20 × 10^6 allogeneic Class II+ cells also mounted second-set skin allograft reactions (Table 1).

### Discussion

The cellular constituents of the normal cornea primarily express Class I histocompatibility antigens.1-7,10,11,27,28 Class II histocompatibility antigens have restricted expression and occur primarily on the plasma membranes of B lymphocytes, macrophages, various dendritic cells, and Langerhans cells of the skin and conjunctiva.29,30 Under ordinary conditions, the corneal epithelial, stromal, and endothelial cells do not express Class II antigens. Also, there are very few migratory Class II+ cells in the normal cornea.

The normal human cornea and the corneas of some animal species may contain a few Class II+ Langerhans cells in the periphery.1-13 The number of such cells, however, is vanishingly small; in an extensive analysis, not reported here, it was found that young adult rats of the strains used in this study have, on the average, 500 or fewer Class II+ cells in their corneas (Gebhardt, unpublished).

Rubsamen et al 31 and Williams et al 7,9 speculate that cells expressing Class II antigens in the cornea could be expected to be potent immunogens and that the larger the number of these cells present in the cornea, the more likely it is that an immune graft reaction will occur. The results of this investigation indicate that while Class II+ cells may elicit local immune reactions in the cornea they do not generate systemic immunity in the recipient.

It is known that certain anatomic locations in the body are immunologically privileged and that allografts in these sites are less likely to be subjected to immunologic attack.32-37 The cornea is one such site.38 In contrast, there are sites in the body, such as the peritoneal cavity, in which immune recognition of alloantigens is facilitated.25,33 In agreement with this concept, the current study showed that large numbers of allogeneic Class II+ cells injected directly

<table>
<thead>
<tr>
<th>Graft recipient</th>
<th>Number of cells (×10^6)</th>
<th>Mean survival time (days)</th>
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<tr>
<td>Normal F344</td>
<td>12</td>
<td>13.3 ± 1.6</td>
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<tr>
<td>F344 recipient/intrastromal injection of W/F Class II+ cells</td>
<td>8 0.01</td>
<td>12.1 ± 1.9</td>
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<td></td>
<td>8 0.1</td>
<td>13.4 ± 0.8</td>
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<td></td>
<td>8 1.0</td>
<td>11.9 ± 1.5</td>
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<tr>
<td></td>
<td>8 5.0</td>
<td>13.9 ± 2.0</td>
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<td></td>
<td>8 10.0</td>
<td>12.6 ± 1.7</td>
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<tr>
<td></td>
<td>8 20.0</td>
<td>12.7 ± 1.6</td>
</tr>
<tr>
<td>F344 recipient/intraperitoneal graft of F344 cornea containing W/F Class II+ cells</td>
<td>8 0.01</td>
<td>12.0 ± 1.9</td>
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<td></td>
<td>8 0.1</td>
<td>13.7 ± 2.1</td>
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<td></td>
<td>8 1.0</td>
<td>14.5 ± 1.4</td>
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<td></td>
<td>8 5.0</td>
<td>11.8 ± 1.3</td>
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<td></td>
<td>8 10.0</td>
<td>10.1 ± 1.1‡</td>
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<td>8 10.0</td>
<td>8.7 ± 0.7‡</td>
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<td>8 20.0</td>
<td>8.4 ± 0.9‡</td>
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* F344 strain rats were grafted with W/F skin. The grafts were observed daily after the dressing was removed. Graft rejection was recorded as the day the graft was judged inviable.
† N, the number of skin allograft recipients in each group.
‡ Second set graft reactions were characterized by an earlier onset of graft discoloration, hemostasis, and scab formation. The differences in mean survival time for these grafts, compared to the other groups, were statistically significant, P < 0.005.
into the peritoneum or implanted in a syngeneic carrier cornea produce a measurable systemic cellular and humoral immune response. In situ intrastromal injection of allogeneic Class II+ cells resulted in no measurable systemic response, regardless of the number of cells used. The failure of allogeneic Class II+ cells in situ to stimulate an immune response is not the result of the death of the cells injected into the cornea. Previous studies established that such cells must be viable in order to mediate immunologic reactions.24 Furthermore, it has been shown that inviable cells are very poorly antigenic.39

Mononuclear cells, including lymphocytes, migrate into the cornea under various circumstances, and they emigrate with equal facility.7,9,10,24 Thus, the difference between the immunogenicity of corneas bearing allogeneic Class II+ cells placed intraperitoneally or in situ would not appear to be due to the failure of the cells to emigrate from the cornea in situ and gain exposure to host immunocompetent cells.

Resident cornea cells may be induced to express Class II antigens as a consequence of an alteration in the homeostasis of the tissue. In particular, lymphokines such as gamma interferon may induce Class II antigen expression in cornea cells.23 The syngeneic F344 corneas used as carriers for the allogeneic Class II+ cells in these experiments contained very few resident Class II+ cells. Immunohistochemical staining of corneas retrieved from the peritoneal cavities of syngeneic recipients revealed that the expression of Class II antigens is restricted to the injected passenger cells and that the carrier corneal cells remain Class II antigen negative (Gebhardt, unpublished).

In the model used in this study spleen cells "present" their endogenous Class II antigens directly. This model mimics the classic T-cell mediated recognition of alloantigens in which foreign major histocompatibility complex molecules act as foreign antigens and elicit an allograft reaction.40

Direct evidence for the theory that Class II+ cells are a potent source of antigen in the generation of corneal allograft immune reactions is lacking at the present time. The results of this study suggest that Class II+ cells are not an important source of the antigens that generate an allograft reaction. It may be that coexpression of Class I and II antigens is necessary to initiate such reactions. It is also possible that the corneal allograft immune response that is initiated and carried out in the local ocular environment does not engender systemic cellular and humoral antibody responses; previous work supports this idea.25

Corneal allograft rejection continues to be a major clinical problem in patients whose graft beds are highly vascularized before transplantation.27 In such grafts, host blood vessels provide a route by which immunologically competent cells can enter the graft, recognize graft alloantigens, and mount an immune attack. These graft reactions may take place regardless of the Class II antigen content of the allograft. The results of the present investigation indicate that factors in addition to Class II antigen-expressing cells may be operative in the initiation of corneal allograft immune rejection.

Key words: allograft, Class II antigens, cornea, immunity, inbred rats

References