On the Ia Immunogenicity of Mouse Corneal Allografts Infiltrated with Langerhans Cells

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Langerhans cells were induced to infiltrate the corneal epithelium of mice by pretreating the surface of the cornea with dinitrofluorobenzene (DNFB). Treated corneas were then used in heterotopic grafting experiments to determine their Ia immunogenicity. It was found that Langerhans cell containing cornea grafts sensitized their recipients to the Ia alloantigens of the donors. Moreover, corneas infiltrated with Langerhans cells were rejected at heterotopic sites by recipients presensitized to the Ia alloantigens of the graft donor. These results demonstrate that corneas, which normally lack both Langerhans cells and Ia-antigen expressing cells, can have their Ia immunogenicity restored if Langerhans cells are induced to infiltrate the epithelium. The implications of these results for improving the success of clinical, orthotopic, corneal allografts are discussed. Invest Ophthalmol Vis Sci 25:513–518, 1984

Although the success rate for human corneal allografts has been remarkably good compared with that of other solid tissue grafts, such as skin and kidney, a significant proportion of corneal allografts in humans are rejected. While a variety of cell surface molecules have been implicated in the induction of allograft immunity, it recently has become clear that in human kidney grafts, molecules encoded by the HLA-D/DR region play a dominant role. These strong transplantation antigens are the equivalent of class II (H-2 Ia) surface alloantigens described in mice. We previously have shown that H-2 Ia-disparate corneas are not rejected when grafted heterotopically in mice. Since we and others have reported that mouse corneas are devoid of epithelial Langerhans cells, it seemed reasonable to propose that the absence of Langerhans cells and the lack of Ia immunogenicity of mouse corneas were reflections of the same phenomenon. Ia-bearing Langerhans cells have been implicated in the process by which antigens are presented in skin. Accordingly, we have put forward the hypothesis that the absence of Langerhans cells from the cornea may account, in part, for the reduced immunogenicity of corneal allografts. We further reasoned that if Langerhans cells could be induced to infiltrate the corneal epithelium, then the Ia-immunogenicity of the cornea should be restored to that of conventional body wall skin. In this report, we describe our studies designed to test the validity of this hypothesis.

Materials and Methods

Mice

Adult male mice of inbred strains BALB/c, A.TH, and A.TL were used in these studies. BALB/c corneas were examined before and after perturbation with an irritant to document whether Langerhans cells had been induced to infiltrate the epithelium. The latter two strains were chosen because they are disparate across the I region of the mouse major histocompatibility complex, H-2, but are syngeneic at the remainder of the H-2 complex as well as other genetic loci. Studies using these animals were performed in a manner to conform to the ARVO resolution of the use of animals in research.

Irritation Reactions in Cornea

Corneas were exposed to 10 μl of 0.5% DNFB in carrier (4:1 acetone/olive oil) in the following manner. Mice were anesthetized with ether. A gauze pad, prefitted to the corneal surface, was placed on each cornea immediately after the surface had been wiped clear of the tear film. Ten microliters of the DNFB solution was placed on the pad, which was held in place for approximately 30 sec and then removed.

Identification of Langerhans Cells

Two histochemical staining reactions were used to identify Langerhans cells. (1) the ATPase reaction (as described previously) and (2) an immunofluorescence (IF) assay employing a monoclonal antibody directed

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Table 1. Density of Langerhans cells in corneas treated with DNFB

<table>
<thead>
<tr>
<th>Site</th>
<th>Treatment</th>
<th>Density of Ia-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonlimbic cornea</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Nonlimbic cornea</td>
<td>DNFB</td>
<td>237 ± 66</td>
</tr>
<tr>
<td>Limbic cornea</td>
<td>DNFB</td>
<td>536 ± 44</td>
</tr>
<tr>
<td>Body wall skin</td>
<td>None</td>
<td>810 ± 140</td>
</tr>
</tbody>
</table>

注: BALB/c mouse cornea was treated with 10 μl of 0.5% DNFB 7 days prior. Ia⁺ positive cells in the epithelium was assessed by immunofluorescence using a monoclonal anti-I-A<sup>+</sup> antibody.

at the I-A<sup>+</sup> antigen (#1360, Becton-Dickinson Monoclonal Center Inc.; Mountain View, CA). Both assays were conducted on whole mounts of pure corneal epithelium, separated from the underlying stroma after incubation in 20 mM EDTA. In mice, the ATPase and IF assays detect comparable numbers of dendritic Langerhans cells in skin (Bergstresser, unpublished observations). In certain experiments, it was necessary to determine whether Ia-positive cells could be identified in the corneal stroma. Whole mounts of corneal stroma, separated from the overlying epithelium by incubation in 20 mM EDTA, were stained with the anti-I-A<sup>+</sup> antibody in fluorescence as described above. The stained specimen then was examined microscopically under UV light.

**Cornea Grafting to Heterotopic Sites**

Normal or DNFB-perturbed corneas were grafted heterotopically to wounds freshly prepared on the thoracic wall of recipient mice. This method has been described in detail previously. Plaster-of-Paris bandages were removed periodically for gross and slit-lamp inspection after which the wounds were redressed. Rejection indices were calculated as described previously to measure the survival of corneal allografts and the amount of inflammation.

In some experiments, grafting with conventional full thickness, orthotopic skin was performed as described previously. Bandages were removed at 7 days and daily observations made thereafter. Median survival times (MST) were calculated from these data. Statistical significance was determined by calculating the 95% confidence limits as originally described by Litchfield.

**Results**

The experimental approach designed to test the hypothesis that corneas infiltrated with Langerhans cells would effectively express Ia alloantigens was as follows. First, a method was employed that would reproducibly bring significant numbers of Langerhans cells into the epithelium of mouse corneas. Second, these corneas were tested for their capacity to sensitize naive mice to their putative Ia alloantigens. Third, similar Langerhans-cell containing corneas were tested for their capacity to be rejected by animals rendered previously immune to the relevant Ia alloantigens.

**Induction of Langerhans Cell Migration into Corneal Epithelium**

A variety of approaches have been reported to cause Langerhans cells to infiltrate into corneal epithelium beyond the limbus. After trying several methods in preliminary experiments, we selected the method originally described by Rowden in which dinitrofluorobenzene, used as a primary irritant, was applied directly to the external surface of the cornea. Rowden's method of application was modified such that precisely 10 μl of 0.5% DNFB was placed on a gauze pad preflitted to the surface of the mouse cornea. After 30 sec, the gauze pad was removed. Within the next several days, a moderately severe conjunctivitis appeared in eyes treated with DNFB. Using the ATPase histochemical stain, as well as an immunofluorescence assay for surface Ia molecules, as indicators of the presence of Langerhans cells, DNFB treated corneas were studied daily to determine the extent of Langerhans-cell infiltration. Ia and ATPase positive dendritic epithelial cells first appeared in the central region of the cornea beyond the limbus at 48 hr. By 6–7 days, significant numbers of these cells were present throughout the central regions of the cornea. A representative study is presented in Table 1. The number of Langerhans cells in the nonlimbic cornea usually approximated 50% of the number found at the limbus, a density almost 25% of that normally found in body wall skin. Frequently, ulceration of the central region of the corneas was observed 6–7 days after DNFB treatment. Histologic examination of parafin imbedded and hematoxylin and eosin stained specimens confirmed the central ulceration, even though the stroma was invariably intact and contained a modest infiltrate of polymorphonuclear leukocytes. The denuded area was covered with debris and polymorphonuclear cells. Few mononuclear cells were seen in the cellular infiltrate. We also examined whole mounts of deep epithelialized stroma at this time by immunofluorescence for the presence of Ia-positive cells. No Ia-positive cells were observed.

Thus, application of DNFB to mouse corneas in this fashion causes an inflammatory reaction that results in the infiltration of the corneal epithelium with Langerhans cells, but fails to induce other types of Ia-bearing cells into the stroma or other regions of the cornea. Since the only Ia-bearing cells to be found in these corneas were epithelial Langerhans cells, we
moved on to the next stage of the study to test the Ia immunogenicity of these corneas as allografts.

**Immunogenicity of Cornea Grafts Containing Epithelial Langerhans Cells**

Inbred mouse strains A.TL and A.TH were selected for these studies because the immunogenetic differences between these strains is limited to the I region of the H-2 complex. A.TL carries the Ia\(^k\) alloantigens, while A.TH expresses Ia\(^d\). Skin grafts exchanged between these strains are rejected with a tempo very similar to that of grafts between strains differing only at a class I H-2 locus. We have demonstrated previously that normal corneas from these mice, when exchanged as heterotopic grafts, heal in place on the thoracic wall and fail to sensitize their recipients to the putative Ia alloantigens. In the experiments now to be described, the corneal surface of eyes of A.TL mice was treated with DNFB as described above. Seven days later, the corneas were removed surgically and the limbal attachments excised, a maneuver designed to avoid contaminating the grafts with the Langerhans cells that normally populate the epithelium of the limbus. Three corneal grafts were then placed, endothelial side down, on graft beds freshly prepared on the thoracic walls of normal A.TH recipient mice. Each graft was held in place with vaseline-impregnated gauze and covered with plaster-of-Paris bandages. Several control panels of mice were also prepared: (1) one panel of A.TH mice was grafted with normal corneas from A.TL donors; (2) another panel received full thickness skin grafts from normal A.TL donors; (3) a third panel served as the negative control and received no grafts. The protective dressings were removed from all mice after 10 days. Twenty days later (30 days after initial grafts were applied), each panel of mice was challenged with a graft of normal body wall skin from A.TL donors to determine whether the pattern of rejection would be normal or accelerated, and, therefore, whether the recipients had been sensitized to Ia\(^k\) alloantigens. Protective bandages were removed from this second group of grafts 7 days after application, and grafts were observed daily for evidence of rejection. The results are presented in Table 2. As we had shown previously, A.TH mice grafted with normal (untreated) A.TL corneas were not sensitized to the Ia\(^k\) alloantigens; they rejected their test A.TL grafts in typical first set fashion. By contrast, recipients of DNFB-treated A.TL corneas rejected their test A.TL skin grafts quite rapidly—MST = 6.6 days. This value is statistically similar to the MST of skin grafts rejected by mice whose first graft had been skin from A.TL donors. Thus, when Langerhans cells are induced to migrate into the corneal epithelium, they confer upon that tissue the capacity to immunize appropriate recipients to the Ia molecules which Langerhans cells express. Despite the fact that a moderate inflammatory reaction existed within the stroma of DNFB-treated corneas, no Ia-bearing cells were visualized upon fluorescent microscopy using an anti Ia reagent (vide supra). Since normal corneas completely lack this capacity to sensitize to Ia alloantigens, we conclude that Langerhans cells alone account for the allo-Ia immunogenicity of corneal tissue. If that tissue lacks these important cells, then its Ia antigenic load is virtually nonexistent.

**Susceptibility to Rejection of Langerhans Cell-containing Corneas**

The previous studies document the ability of Langerhans cells within DNFB-treated corneas to sensitize mice to Ia alloantigens. Since skin grafts exchanged between Ia-disparate mice are rejected, this means that some target cells within the graft bear Ia antigens. In skin, numerous cell types are thought to express Ia antigens, but in cornea, only Langerhans cells express these surface glycoproteins. It seemed important, therefore, to determine whether corneas that were treated with DNFB in order to induce the migration of Langerhans cells into the epithelium also could serve as the targets of an established Ia immunity. We recently have described methodology for evaluating heterotopic cornea grafts in mice by gross and slit-lamp microscopy. By this approach, it is possible to determine when rejection has taken place and to quantify the degree of inflammation attending the rejection process. Accordingly, panels of A.TH mice were sensitized to Ia\(^k\) alloantigens by a primary skin graft from A.TL donors. Four weeks later, these mice were used as recipients for corneal grafts that were obtained from A.TL mice whose corneas had been painted with DNFB 7 days before. The cornea grafts were placed heterotopically on the thoracic wall. Bandages were removed at 7, 10, and 14 days postgrafting at which time the grafts were evaluated and scored. Dressings...
Corneas of A.TL mice were treated with 10 μl 0.5% DNFB. Six days later, they were grafted heterotopically to allogeneic (A.TH) and syngeneic (A.TL) recipients. The grafts were examined by gross and slit-lamp microscopy, and a rejection index was calculated as described in Materials and Methods.

were re-applied after inspections at 7 and 10 days. Controls comprised A.TL mice that received heterotopic grafts of syngeneic A.TL corneas that had been treated with DNFB. Corneal grafts were observed for 14 days. However, at this time, the intensity of this response was much greater in and around the allografted corneas. Subsequently, the intensity of inflammation mounted in the allografts but began to subside in the syngeneic grafts. Most importantly, the vast majority of allocorneas were no longer apparent in the graft beds on day 14; the five that remained, appeared as opaque remnants. By contrast, the majority of syngeneic corneas were no longer apparent in the graft beds on day 14; the five that remained, appeared as opaque remnants. By contrast, the majority of syngeneic corneas persisting on their graft beds were clear and displayed little evidence of inflammation. Since normal corneas from donors Ia-disparate with their recipients are not rejected, we conclude that the induction of Langerhans cells into corneas is sufficient to render these grafts susceptible to rejection by an established anti-Ia immunity. Recall that the only cells within DNFB-treated corneas that can be determined to express Ia antigens are Langerhans cells, and that these cells, in fact, comprise only a small minority of cells within these grafts. We interpret this to mean that the vulnerability of the entire cornea to allo-Ia immune rejection can be laid at the doorstep of epithelial Langerhans cells. Presumably, destruction of non-Langerhans-cell components of the cornea is achieved by nonspecific means.

Discussion

The ability of an allograft of solid tissue to elicit an immune rejection response is predicated upon two factors: (1) the number and strength of alloantigenic molecules expressed on the cells comprising the graft and (2) the presence within the graft of cells capable of functioning as specialized antigen-presenting cells. Class I molecules encoded by genes within the major histocompatibility complex (HLA-A, B, C in humans; H-2 K, D in mouse) and minor H antigens are expressed on virtually all nucleated cells. By contrast, class II molecules of the MHC (HLA-D/DR, H-2 Ia) are strong transplantation antigens with expression limited only to certain cells of the body. In normal solid tissues, such as skin, expression of class II determinants is limited to dendritic Langerhans cells, macrophages, and lymphoreticular cells delivered to the tissue from the peripheral blood. In mice, where H-2 recombinant congenic strains of mice are available for study, skin grafts exchanged between mice disparate only for H-2 Ia antigens are rejected briskly. Thus, when immunogenetic disparity between graft and recipient is limited to gene loci encoding class II molecules, the alloimmune response is elicited by a subpopulation of class II antigen-bearing cells; and rejection is achieved despite the fact that the target cell population is normally limited to a minor population of class II, antigen-bearing cells.

We have proposed previously that epidermal Langerhans cells alone account for the Ia immunogenicity of skin. This hypothesis was based, in part, on the observations that normal mouse corneas are devoid, in the central, nonlimbic portions, of Langerhans cells and that heterotopic corneal allografts that are disparate from their recipients only at H-2 Ia loci are neither rejected nor capable of inducing allo-Ia immunity.7 In this report, we describe the experiments to test this hypothesis directly. The epithelium of corneas destined to be used as allografts was perturbed with DNFB such that significant numbers of Langerhans cells were induced to infiltrate the epithelium. Grafts prepared from these Langerhans cells containing corneas then were shown to (1) induce allo-Ia immunity (as revealed by the fact that recipient mice rejected skin syngeneic with the immunizing cornea in a second set fashion) and (2) cause susceptibility to rejection when placed on mice previously immunized to the Ia alloantigens expressed on the Langerhans cells. Since under normal circumstances the corneal epithelium is devoid of Langerhans cells, except in the perlimbic region, then allografts prepared from
corneas would be expected to be deficient in expression of Ia alloantigens. The absence of Langerhans cells from the normal cornea has important implications beyond the ability of these cells to express Ia molecules as alloantigens. It has been proposed that Langerhans cells function as critical antigen presenting cells of skin. In grafts of skin, they are thought to be responsible for presenting to the recipient class I MHC and minor H loci antigens in such a manner that an effective alloimmune response is generated. A corollary of this hypothesis is that skin devoid of Langerhans cells would be ineffective at presenting major and minor alloantigens. This corollary has been partially tested—in that we have demonstrated previously that corneal allografts differing from their recipients only at class I MHC loci are rejected—in the absence of Langerhans cells in the grafts. This suggests that Langerhans cells are not required for anti class I rejection. However, this conclusion may be premature. Histologic sections of heterotopic corneal grafts within 4 days of grafting reveal that “clear” cells appear within the corneal epithelium, suggesting that host Langerhans cells can rapidly infiltrate the graft (data not shown). This raises the possibility that antigen processing capability within the grafts may be assumed by recipient Langerhans cells, even though the grafts were devoid of Langerhans cells at the time of surgery.

The results of our experiments raise questions concerning human corneal grafting:

1. Why do the majority of histoincompatible corneas grafted orthotopically survive and function? One traditional answer to this query is that the orthotopic site is an immunologically privileged one. As a consequence, the immune system remains unaware of the existence of the alien corneal graft, or, more likely, the immune response to the incompatible graft is largely down-regulated by the induction of a deviant immune response such as has been postulated to follow the introduction of alloantigenic tissues into the anterior chamber of the eye. Based on the findings reported here, we would suggest the possibility that normally the donor corneas used in grafting are essentially devoid of Langerhans cells as well as other antigen presenting cells. In successful orthotopic allografts, we would presume that recipient Langerhans cells are not induced to enter into the graft, a situation that greatly diminishes the immunogenicity of the graft itself. It is even possible that the privileged site conspires with the Langerhans cell deficient cornea to produce an ineffectual, suppressed alloimmune response.

2. Why do a significant proportion of corneal allografts fail in humans? A certain proportion of these grafts undoubtedly fail for nonimmunologic reasons, and discussion of these causes is beyond the scope of this report. For failures that are immunologically based, we would offer the possibility that, unbeknownst to the surgeon, the donor cornea contains Langerhans cells within the epithelium. It is possible that when placed at the orthotopic site, these cells may prove to be effective at immunizing to their unique HLA-DR antigens; in addition, they may serve to process and present other alloantigens on the graft via linked recognition in a manner that prejudices the host response in favor of rejection. The role of HLA-D/DR antigens in human renal grafts now has been shown to be immunodominant. When patient and donor are disparate at this component of HLA, renal graft failure is much more likely than when donor and recipient are HLA-D identical. There is no direct evidence that this effect is mediated by HLA-DR bearing dendritic cells within the grafted kidneys; but circumstantial evidence from laboratory animal experiments strongly supports this contention. A similar situation may apply to corneal allografts.

The value of experiments conducted in animal model systems lies in the hope that the results may suggest improvements in the practice of clinical medicine. It would be rash to extrapolate directly to humans from our experiments conducted in mice, since the method and site of grafting are completely unrepresentative of clinical corneal grafting. Nevertheless, the following matters seem worthy of consideration. Given the dominant role of HLA-D/DR disparity in human renal grafting failure and the pivotal role of Ia-bearing Langerhans cells in the immunogenicity of mouse corneas, prospective matching of cornea donor and recipient for HLA-D antigens may offer a significant advantage over mismatched cornea grafts. There might also be merit in a prospective study of the content of Langerhans cells in corneas to be used for grafting. The statement that normal corneas essentially are devoid of Langerhans cells is based on relatively few examinations of human corneas and rests mainly on studies of corneas in laboratory animals. Such an analysis would verify (or refute) the claim that normal human corneas are devoid of Langerhans cells. One might even imagine discarding, prior to grafting, corneas known to contain significant numbers of Langerhans cells. Finally, if the incidence of donor corneas significantly contaminated with Langerhans cells turns out to be prohibitively great, then development of strategies to rid the cornea of these cells would be in order. Possibilities such as in vitro treatment with ultraviolet light B or denuding the donor cornea of epithelium are examples of this type of approach.

Key words: cornea, Langerhans cell, allograft, Ia immunogenicity, rejection
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References