Corneal Epithelial Rejection in the Rat

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PURPOSE. To investigate clinical and histologic changes in the epithelium during corneal graft rejection in the rat.

METHODS. LEW (RT1l) or PVG (RT1c) strain corneas were transplanted to PVG strain recipients and examined by slit lamp for clinical signs of rejection. Recipients were killed, and corneal epithelial sheets were removed and examined by adenosine diphosphatase (ADPase) staining for Langerhans cells (LC) and by immunohistology for leukocytes and adhesion molecules (T cells, macrophages, granulocytes, major histocompatibility complex [MHC] class II, CD2 and CD54 intercellular adhesion molecule [ICAM]-1) at a range of time points before, during, and after rejection, depending on the cell type sought. Normal and contralateral eyes were examined for ADPase+ and MHC class II+ cells.

RESULTS. Clinical rejection, as defined by stromal opacity, occurred between days 10 and 15 after transplantation. In 94% of allografts, a curved clinical epithelial rejection line was observed in which ADPase+/MHC class II+, CD4+, or CD8+ T cells were identified. There were significantly more infiltrating cells of all types in epithelia of allografts than in those of isografts. The most numerous cells were CD4+ and CD8+ T cells, suggesting preferential migration of these cells into the epithelium from underlying layers. Expression of MHC class II and ICAM-1 was induced on epithelial cells.

CONCLUSIONS. Epithelial rejection in rats is clinically similar to that in humans and occurs simultaneously with stromal infiltration. It may be mediated by T cells rather than macrophages. In isolation, its recognition in humans may be a useful indication that the patient is at high risk of endothelial rejection. (Invest Ophthalmol Vis Sci. 2002;43:729–736)

Corneal epithelial rejection receives little attention, because clinical monitoring and intervention after corneal transplantation are directed primarily at protecting the most critical cell layer, the endothelium. Rejection of each of the three corneal cell layers can occur independently of the others or simultaneously in any combination, but the factors governing these different manifestations of immunity are unknown. In particular, the importance of the epithelium in relation to rejection of the endothelium is still unclear and controversial. Alldredge and Krachmer identified epithelial rejection in 10% of 156 grafts examined for at least 1 year, which represents 34% of those showing signs of rejection, compared with 72% showing endothelial rejection. One possible explanation for the observed lower incidence of epithelial rejection is that donor corneal epithelium is rapidly replaced after transplantation by host tissue and is therefore absent by the time a donor-specific immune response has developed. However, human epithelial rejection can occur at least a year after transplantation and in a careful experimental study in rabbits, Khodadoust and Silverstein observed that epithelium is “neither sloughed off rapidly in the immediate post-operative period, nor is it even slowly replaced by recipient epithelium in the technically successful corneal transplant.” Epithelial rejection, characterized by a rejection line beginning at the graft margin, was observed 6 months after transplantation and in vascularized corneal allografts, it occurred “in almost every instance.” Khodadoust and Silverstein further showed that grafts of the epithelial layer alone could rapidly sensitize the host, with rejection occurring as early as 2 weeks after transplantation, although because of its mode of preparation, the transplanted tissue would almost certainly have contained many more donor type Langerhans cells (LCs) than the epithelium of a normal donor. In pigmented rats, we also occasionally noticed an epithelial rejection line. The purpose of this study was therefore to establish accurately the incidence of clinical epithelial rejection in rats and to correlate it with clinical signs of stromal rejection and with the profile of cells infiltrating the epithelial layer.

MATERIALS AND METHODS

Rats

Inbred female Lewis (LEW; RT1l) and PVG (RT1c) rats were purchased from Banting and Kingman (Hull, UK). LEW or PVG corneas were transplanted to PVG recipients aged 8 weeks. Procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal Transplantation and Clinical Evaluation

Transplantations were performed as previously described. Briefly, the donor eye was removed, and the central cornea was cut with a 3.5-mm trephine. Separation of the button was completed with curved scissors. A 3.0-mm button was removed from the recipient by a similar procedure and replaced by the donor button, which was sutured in place with 12 interrupted 11-0 nylon sutures. Atropine and chloramphenicol ointments were applied to the eye after the operation. The sutures' ends were cut as short as possible, and grafts were examined daily until day 15 and then twice weekly thereafter. An epithelial rejection line was sought, and corneas were scored centrally for opacity (scale of 1–4), edema (1–4), and vascularization (1–5). Graft rejection was defined as the day on which donor corneal opacity became moderate or severe (a score of at least 3), which was greater than that seen in isografts at any time.

Removal of Epithelial Sheets

Rats were killed by intraperitoneal injection of pentobarbitone sodium. Transplanted eyes were enucleated and incubated at 37°C in 5.0 mL prewarmed 2% EDTA for 3 to 4 hours. Under a binocular dissecting microscope, corneal–conjunctival epithelium was removed as a whole sheet with forceps. Footpad epidermis was similarly removed for use as positive control tissue.

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Adenosine Diphosphatase Staining

Epithelial sheets were stained for ADPase activity by the technique of MacKenzie and Squier with the modification of Chaker et al. Briefly, specimens were first washed in 0.9% saline, fixed in cacodylate (40%) and formaldehyde (10%) for 1 hour at 4°C, washed in three changes of 0.2 M rinsing buffer (Trismal; Sigma Chemical Co., Poole, UK), and left overnight in rinsing buffer at 4°C. Subsequently, they were incubated in freshly prepared prewarmed adenosine diphosphate (ADP; Sigma)-lead nitrate solution at 36°C for 70 minutes and washed twice in rinsing buffer. The color was developed in 10% ammonium sulfide solution on glass slides for 20 minutes. The sheets were washed twice in distilled water and mounted flat, basal layer upward, in Apathy’s medium (BDH Ltd., Poole, UK) and sealed with clear nail polish. Positive control tissue consisted of sheets of footpad epithelium.

Immunoperoxidase Staining

Epithelial sheets were washed twice in PBS and fixed in acetone for 5 minutes. Endogenous peroxidase activity was blocked using 0.1% hydrogen peroxide in 50% PBS and methanol, followed by 1.5% normal horse serum. Sheets were then incubated with a specific monoclonal antibody (major histocompatibility complex [MHC] class II [OX6]), CD4 [W3/25], CD8 [OX8], CD2 [OX34], macrophage [ED2], granulocyte [HIS48], CD54/ICAM-1 [1A29]) or a negative control IgG1 antibody (human complement factor 1 [OX21]) overnight at 4°C. All antibodies were IgG1 except HIS48, which was IgM and OX34, which was IgG2a. Positive control lymph node sections or footpad epithelium (for MHC class II only) were included in every staining run for each antibody. Control experiments were also included in which the primary antibody was omitted. Primary antibody was followed by biotinylated horse anti-mouse IgG (preadsorbed with rat serum; Vector Laboratories, Peterborough, UK) and ABC complex (Vector Laboratories, Peterborough, UK) and left in PBS for 1 hour at room temperature. Sections were rinsed in PBS and twice in distilled water and mounted.

Experimental Protocol

In pilot clinical experiments, rejection was under way within 15 days. Sutures were therefore left in place throughout, because of the potential to cause further inflammation by their removal when rejection was imminent. In a further series of experiments, epithelial sheets were removed and stained on day 10 (just before rejection) and day 15 (during rejection) and at additional time points, depending on cell type, as follows: ADPase+ cells on days 2, 6, 30, and 100, in that these should be involved in early T-cell activation and may persist for longer than effector cells; CD4+ and CD8+ T cells on days 6, 8, and 30, to determine whether CD4+ cells infiltrates earlier or persists longer than CD8+ cells; and MHC class II and macrophages on day 30, to determine whether dendritic cells (DCs) express class II and whether antigen-presenting cells (APCs) persist as rejection resolves. Granulocytes, CD2, and ICAM-1 were sought only on days 10 and 15. For each cell type, sheets from contralateral eyes and eyes from untreated animals were stained on day 10 (just before rejection) and day 15 (during rejection) and at additional time points, depending on cell type. Blood vessels persisted in the vicinity of sutures in isografts, whereas in allografts they had progressed half way to the center of the donor cornea by day 15. In 94% of grafts, epithelial rejection was characterized by a curved, irregular white line (Fig. 3) that stained with 2% fluorescein. Typically, this was an arc of at least 180°, but the line occasionally formed a complete circle, appearing on day 11 after transplantation near peripheral blood vessels. The line progressed centripetally over the course of 3 to 5 days, disappearing when close to the center. This feature was never observed in isografts. During progression of the line, stromal opacity (Fig. 2) and edema increased. The epithelial defect was restricted to the line, with no evidence of epithelial denudation before or behind it. Stro-
Epithelial Rejection in the Rat

There was mild cell infiltration of the allograft epithelium by day 10 and heavy infiltration by day 15. Isografts showed only very mild infiltration. Epithelial sheets from rejected allografts treated with control antibody showed some diffuse background staining of epithelium, but not of infiltrating cells. A positive control section of lymph node showed characteristic staining for each antibody with minimal background staining. Infiltrating cells of all types were distributed throughout the sheet on day 15. Numbers decreased in parallel with a reduction in corneal opacity (Fig. 2), but LCs were still present in the central donor cornea on day 15. Histologically, a rejection line appeared as a curve of ADPase+ cells, CD4+ or CD8+ T cells, which expressed ICAM-1—seen on day 15, more densely packed in the line than elsewhere. The line was not seen in sheets stained for macrophages and granulocytes.

Quantification of Cells

ADPase+ and MHC Class II+ Cells. ADPase+ cells were typically of dendritic morphology (Fig. 4A). In epithelia from untreated rats and those contralateral to transplants, these were found only in a dense collar at the limbus, and the numbers remained unchanged after transplantation (data not shown). However, by day 2 such cells were already present in donor tissue of transplanted corneas (Fig. 5). They continued to increase in number in allografts until day 15 (Figs. 4A, 5), which correlated strongly with the onset of clinical rejection, whereas those in isografts remained constant (Figs. 4B, 5). The overall difference in number between allografts and isografts was significant (P < 0.0001 in both host and donor tissue). The change in cell numbers over time was also significantly different in allografts compared with isografts (P < 0.001 for both host and donor tissue), reflecting increased numbers of cells in allografts between days 10 and 30 (P < 0.0001 in the donor) but not in isografts (P = 0.3 in the donor). The mean numbers on day 15 in allograft host and donor tissue were 148 (95% CI 75-244; n = 6) and 46 (95% CI, 18-86; n = 6) cells/mm², respectively. Despite the increasing number of cells in the donor of allografts, there were consistently more ADPase+ cells in the host than in the donor, until day 30 (Fig. 5), by which time the number of cells in all parts of the epithelium was decreasing, as was the severity of clinical opacity.

To determine whether ADPase+ and MHC class II+ cells show a similar distribution of staining, and by inference, whether ADPase+ cells are capable of antigen presentation, further epithelial sheets were stained for MHC class II on days 10, 15, and 30. A blue substrate was used to distinguish clearly cells from brown melanocytes. A majority of the class II+ cells were dendritic. The pattern and number of infiltrating cells in both allografts and isografts were virtually identical with that after ADPase staining (data for day 15 shown in Fig. 6). Many DCs also stained for the costimulatory-adhesion molecule CD2 (Fig. 4E).

Figure 2. Clinical central corneal opacity (top) and vascularity (bottom) scores after transplantation (n = 6, except for day 100, when n = 4). Opacity scores: 1, very mild with iris detail visible; 2, mild with some iris detail visible; 3, pupil visible but no iris detail; and 4, white opaque. Scores of leading edge of vessels: 1, less than half way to the margin; 2, between half way to the margin and the margin; 3, within graft at sutures; 4, beyond sutures but in peripheral half of donor; and 5, in central half of donor.

Figure 3. Clinical epithelial rejection line (arrow) in an allograft 15 days after transplantation.
Macrophages and Granulocytes. Many ED2⁺ macrophages had dendritic morphology. Although ED2 marks a different population of cells, their distribution resembled that of ADPase⁺ and class II⁺ cells, in that up to day 15 they were consistently more numerous in the recipient than in the donor (Fig. 7). There was again a significant overall difference in numbers between allografts and isografts in both host (P < 0.0001) and donor (P = 0.02) tissues, because the number of cells in allografts increased up to day 15 (Fig. 7; P = 0.03 in donor tissue), whereas those in isografts remained relatively constant (P = 0.8 in donor tissue). The change in number of cells with time was significant only in the host tissue (P = 0.005).

Granulocytes in the epithelium as a whole were fewer in number than other cell types. There was a significant difference overall in numbers between allografts and isografts in the donor tissue only (P = 0.01), because of an influx of cells in allografts by day 15 (Fig. 6; P = 0.01), representing 22 cells/mm² (95% CI 4.0–52, n = 5). The recipient consistently contained fewer cells (data not shown).

T Cells. By day 6 after transplantation, the earliest time point investigated, CD4⁺ and CD8α⁺ cells were scattered throughout in the donor epithelium of allografts (Fig. 7) and isografts. The numbers increased in allografts until day 15, when both subsets were more concentrated in a curved line (Figs. 4C, 4D) interpreted as the epithelial rejection line observed during slit lamp microscopy. Overall, there were significantly more CD4⁺ and CD8⁺ T cells in the donor allografts than in isografts (P < 0.0001 in both cases, corresponding to a mean count of 142 (95% CI 77–226, n = 6) CD4⁺ and 167 (95% CI 58–332, n = 5) CD8⁺ cells/mm² in the allograft donor cornea on day 15 (Fig. 6). T cells of both subsets were more numerous than macrophages, DCs, or granulocytes. The pattern of infiltration was consistent with overall movement in one direction from recipient to donor; in that in allografts they were more numerous in recipients on day 6, but were three- to sixfold more numerous in the donor cornea on day 15 (Fig. 7). The number of cells changed significantly with time in allografts compared with isografts (P < 0.0001 and P = 0.001 for CD4⁺ and CD8⁺ cells, respectively), because numbers in the donor allografts increased to a peak on day 15 (P < 0.0001 for CD4⁺ and P = 0.004 CD8⁺ cells), whereas those in isografts remained at a constant low level (P > 0.05 and P = 0.2 for CD4⁺ and CD8⁺ cells, respectively). Many small round cells, presumed to be T cells, expressed CD2 (Fig. 4E).

MHC Class II and ICAM-1 Expression on Epithelial Cells

Neither MHC class II nor ICAM-1 was detected on the epithelium of normal corneas and MHC class II was not detected on isografts. However, in the donor allografts, patchy expression of MHC class II was observed, delineating the epithelial cell membrane (Fig. 4F). ICAM-1 was expressed de novo in both allografts and isografts, but by analysis of gray levels, expression was significantly greater on allografts (P = 0.02). ICAM-1 was more evenly distributed and homogeneous on epithelial cells than class II expression. ICAM-1 was also expressed on infiltrating cells, including in the rejection line.

DISCUSSION

Epithelial rejection in this rat model resembled the rejection line seen in humans and rabbits, being manifest as a white,
A curved line that traversed the donor cornea within a few days. Unlike humans, it occurred simultaneously with stromal infiltration. As in rabbits, no area of epithelial denudation was observed, illustrating the remarkable ability of epithelial cells to proliferate and maintain a continuous covering of the stroma. Infiltrating cells were not enumerated in the line, because it was irregular and variable in size and was not always identified histologically, so that numeric comparisons could not be accurately made. We interpret the absence of macrophages and granulocytes in the line to be because they were too few in number to be perceived as a line in the stained sheet, rather than that they were entirely absent; therefore no particular cell type was disproportionately represented in the line.

In allografts the predominant infiltrating cells throughout the donor epithelium were T cells. These findings are consistent with the electron microscopic study of Kanai and Polack in rabbits, which also showed large numbers of lymphocytes in the allograft epithelium. They found macrophages in areas where the epithelial basement membrane was damaged, which is consistent with a macrophage function to phagocytose cell debris rather than as a primary cause of damage. Previous immunohistologic studies of rejection in humans, sheep, and rats have concentrated on the stroma, which is easily viewed in sectioned corneas. We found reference to the epithelium only in Larkin et al., who observed...
mononuclear cells and cells expressing ICAM-1 within the tissue. It is difficult to compare the cell profile we identified in the epithelium with those in the stroma identified by previous investigators, because, even in studies of a single species, a different combination of antibodies has been used in each study. However, insofar as comparison is possible, it appears that the epithelium contains more T cells than macrophages and granulocytes than does the stroma. Our more recent parallel, unpublished observations (Figueiredo et al., 1998) on stromal cell infiltrates in sectioned tissue in this strain combination, using the same monoclonal antibodies as in this study, confirm this.

Altogether, the data support the notion of differential migration of T cells into the epithelial layer. This would occur initially at the limbus, but as vessels ingress, it may also occur through the corneal stroma. Access to the epithelium may be facilitated at the graft-host junction, where there is more likely to be discontinuity in the epithelial basement membrane. This migration into the epithelium may be mediated by ICAM-1 (CD54) expressed on epithelial cells, through its ligand lymphocyte function-associated antigen (LFA)-1 (CD18/CD11a) on lymphocytes, in a manner similar to the ICAM-1-mediated extravasation of lymphocytes across vascular endothelium. This predominance of T cells in the donor epithelium, including in the rejection line, suggests that epithelial rejection may be mediated primarily by T cells, rather than by macrophages. We did not stain for CD25 (interleukin-2 receptor), but have observed such cells similarly concentrated in the epithelium of sectioned corneas (Figueiredo et al., unpublished data, 1998), showing that many of the T cells were activated.

The T-cell response could theoretically be directed either against MHC class I- or class II-expressing cells, because we noted in the current study and have observed in sectioned corneas (Figueiredo et al., unpublished data, 1998) that class II is induced on epithelial cells. However, in the case of a fully mismatched graft, it is difficult to reconcile specific cytotoxicity (by either CD4+ or CD8+ cells) as a major mechanism of rejection with the fact that T cells activated by the indirect route (i.e., through recipient APC and biased toward recognition of non-MHC antigens) play a major, if not a decisive, role in rodent corneal graft rejection.4,17-20 Such recipient-restricted cells would not be of the correct specificity to recognize either MHC class I or II expressed on graft epithelium. T cells with antigen specificity would have to be primed by direct presentation (i.e., MHC class II presented on donor DCs). Such DCs, although present in the rat cornea, are few.21 It is possible that within the indirectly activated T-cell population there is sufficient cross-reactivity for donor MHC to account for donor recognition. Alternatively, invading T cells kill through some as yet undefined, non-antigen-specific mechanism, a possibility also suggested by Sano et al.19 to explain their finding that clinical rejection of fully allogeneic mouse corneal grafts correlates with indirect activation of CD8+ T cells. However, if this is the case, it is difficult to explain how such precise specificity for the donor epithelium is achieved. The significance of the observed MHC class II expression on epithelium therefore remains uncertain. An alternative possible role for such expression in an established T-cell response and accumulation in the donor to perform their effector functions, eventually dying in situ. By contrast, although their numbers were increasing in the donor up to day 15, all categories of APC were more numerous in the recipient than in the donor until rejection was resolving (i.e., day 30). Although there may be other explanations for this, such a profile would be expected if these cells were trafficking in both directions: into the cornea and out again. Both DCs27 and T cells expressed the costimulatory molecule CD2. It has been shown that cross-linking of CD2 stimulates sessile T cells to migrate.28 Its strong expression is thus consistent with high activation and mobility of both cell types. As well as APC trafficking to the lymph node to prime T cells, others that infiltrate the graft may function to promote the ongoing effector response by actively processing and presenting graft antigens to T cells in situ.29

The gradual clearing of the cornea after rejection can be attributed to the capacity of rat endothelial cells to proliferate.30 As opacity subsided, we noted that ADPase+ cells diminished in number in the donor cornea less rapidly than in the host and less rapidly than T cells, with the result that in ADPase+ cells in the donor cornea exceeded those in the recipient on day 30 (Fig. 3). Because statistical comparisons could not be made at individual time points, it is impossible to determine whether this difference was significant, but it may reflect a reduction in APC trafficking to the lymph node in parallel with clearance of antigen. ADPase+ cells remained in the donor epithelium 100 days after transplantation. Continued secretion of chemoattractant factors due to the inflammatory stimulus of suturets may have been a contributory factor.

The relatively large number of epithelial cells in a graft and the accessibility of epithelium to indirect presentation through host APCs are strong a priori reasons to suppose that the epithelium plays an important role in host sensitization. Antigen in human epithelium may also include a few LCs in a fresh central corneal button.31,32 In humans, attempts have been made to address the question of epithelial immunogenicity and to improve graft survival by removal of the epithelium before transplantation, but results have been conflicting. Tuberville et al.33 found that this reduced the number of graft reactions, whereas later studies by Sundmacher34 and Stulting et al.35 did not confirm this finding. Removal of epithelium from corneas transplanted heterotopically into subcutaneous pockets in the...
mouse prolonged survival,39 whereas replacement of epithelium of an orthotopic corneal allograft with epithelium syngeneic with the recipient significantly prolonged survival, provided that the epithelium was deprived of LCs.37 An interesting finding of Hori and Streilein57 was that allogeneic grafts deprived of epithelium undergo enhanced rejection compared with full-thickness grafts. This was attributed to enhanced inflammation and more intense stromal vascularization in denuded grafts and may explain the poor performance of grafts in humans when deprived of epithelium.34,35 These study results are not inconsistent with an immunogenic role for allogeneic epithelium, but also reveal a conflicting protective effect that is related to an ability to inhibit neovascularization.

A further complicating consideration is the strong non-MHC component of target antigens in corneal graft rejection, which may be cell type specific. However, there is no evidence from the rat that the three layers are separately targeted, because they are rejected more or less simultaneously, with infiltration of the endothelium also reaching maximal levels around day 15 in this strain combination38 (although the infiltration does not begin until day 11 or 12, and isografts are not infiltrated). Data from rabbits, without immunosuppression, suggest that all three layers are likely to be targeted, either simultaneously or in close succession, if, as in these experiments, corneas are vascularized.39 In humans, the effect of immunosuppressants, in combination with the unique layered structure of the cornea wherein the endothelium is more accessible to effector cells from the iris, rather than antigenic differences, may explain why differential rejection so frequently occurs.

The observation of epithelial rejection as long as 13 months after transplantation in humans1 is strong evidence that epithelium is not rapidly replaced by host tissue unless rejected. Because rejected epithelium is rapidly replaced by recipient cells, loss of rejected tissue poses no direct threat to vision. Consequently, the incidence of epithelial rejection is almost certainly underestimated, because symptoms are mild and may be missed between outpatient appointments.1 However, recognition of epithelial rejection can show that the recipient is sensitized and that activated cells are reaching the graft. The fact that, if it does not coincide with endothelial rejection, epithelial rejection always precedes it1 means that, seen alone, it may be a valuable signal of an increased risk of endothelial rejection and a need for closer monitoring of the patient and prolonged prophylactic treatment with topical steroids. Because of this, detection of epithelial rejection should perhaps be given greater clinical priority, especially in patients known to be in a high risk category.

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