T6-Positive Langerhans Cells in Diseased Corneas

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Langerhans cells (LC) in normal human corneas (with the exception of newborns) lack thymocyte antigen T6, a highly specific marker for noncorneal LC. Because corneal LC could not be induced to express T6 antigen when cultured with various cytokines including interleukin-1 (shown to modulate T6 expression on gingival LC), some authors assume that corneal LC may represent a distinct LC subpopulation that is innately inactive. In this study, 62 corneas from patients with various corneal diseases were investigated for the presence of T6 and histocompatibility antigen HLA-DR on LC in the central and pericentral epithelium. Both T6- and HLA-DR-positive LC at a high density similar to that observed in normal epidermis could be detected in the epithelium of five corneas with epidermalization after alkali burns. Furthermore T6- and HLA-DR-positive LC at smaller densities also were detected in corneas from patients with chronic herpetic stromal keratitis, zoster keratitis, chronic allograft rejection, and bacterial corneal ulcers. Although the functional significance of T6 expression on corneal LC remains to be determined, the induction of T6 antigen on corneal LC may represent an important event for the antigen-presenting function of these cells in various corneal diseases including corneal allograft rejection. Invest Ophthalmol Vis Sci 32:2492-2497, 1991

Materials and Methods

Tissues

Sixty-two human corneas were obtained at the time of penetrating keratoplasty in various inflammatory and degenerative corneal diseases (Table 1). Immediately after trephination, the corneal buttons were bisected with a razor blade, snap frozen, and stored in liquid nitrogen until processed further.

Antibodies

Two monoclonal antibodies, anti-HLA-DR (Dako-HLA-DR/alpha) and anti-T6 (Dako-T6; Dakopatts, Copenhagen, Denmark), were used for the detection of LC. The specificity and characteristics of these antibodies were reported previously.6,7 In brief, Dako-HLA-DR/alpha reacts with the alpha chain of monoclonal histocompatibility antigen class II (HLA-DR), which can be detected on various cells including monocytes, B-cells, activated T-cells, and LC. Dako-T6 reacts with 70% of the cortical thymocytes and has been recognized as a highly specific marker for intraepidermal LC.3,4

For the characterization of mononuclear cell infiltrates, we used a panel of monoclonal antibodies (Dakopatts). Dako-T11 was used to detect mature peripheral T-cells; Dako-T4, to mark helper/inducer T-cells; Dako-T8, to detect cytotoxic/suppressor T-cells; Dako-EBM11, to detect monocytes/macrophages; and Dako-CD22, to mark B-cells.
Table 1. Expression of HLA-DR and T6 antigens on Langerhans cells (LC) in diseased corneas

<table>
<thead>
<tr>
<th>Number of corneas n = 62 (15)*</th>
<th>Diagnosis</th>
<th>T6-negative/HLA-DR-positive LC</th>
<th>T6-positive/HLA-DR-positive LC</th>
</tr>
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<tbody>
<tr>
<td>10 (-)</td>
<td>Keratoconus</td>
<td>-/+§</td>
<td>-</td>
</tr>
<tr>
<td>6 (-)</td>
<td>Fuch’s dystrophy</td>
<td>-/+§</td>
<td>-</td>
</tr>
<tr>
<td>8 (-)</td>
<td>Bullous keratopathy</td>
<td>-/+§</td>
<td>-</td>
</tr>
<tr>
<td>6 (-)</td>
<td>Corneal scars</td>
<td>-/+</td>
<td>-</td>
</tr>
<tr>
<td>8 (2)</td>
<td>Bacterial keratitis</td>
<td>++/++</td>
<td>-/+</td>
</tr>
<tr>
<td>7 (3)</td>
<td>Herpetic stromal keratitis</td>
<td>++/++</td>
<td>-/+</td>
</tr>
<tr>
<td>4 (2)</td>
<td>Zoster keratitis</td>
<td>++/++</td>
<td>-/+</td>
</tr>
<tr>
<td>2 (-)</td>
<td>Rheumatoid corneal ulcers</td>
<td>+/+</td>
<td>-</td>
</tr>
<tr>
<td>6 (3)</td>
<td>Chronic allograft rejection</td>
<td>++/++</td>
<td>-/+</td>
</tr>
<tr>
<td>n 5 (5)</td>
<td>Alkali burns</td>
<td>+++H</td>
<td>+++H</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate corneas with T6-positive/HLA-DR-positive LC.
† Results indicate the number of positive cells per linear 5 mm of central corneal epithelium: -, negative; +, 1-5; ++, 6-15; ++++, >16.
§ LC occasionally found.

Immunoperoxidase technique: Serial frozen sections (6-µm thick) were cut in a Reichert Jung cryostat (Leica–Reichert Co.) at −20°C and mounted on poly-L-lysine-coated slides. The sections were air dried overnight, fixed in acetone at 4°C for 20 min, and stained by the peroxidase-antiperoxidase method. Before staining, the sections were immersed in a solution of 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 min to block endogenous peroxidase activity. They then were washed in PBS and incubated in 20% normal rabbit serum diluted in PBS for 20 min to reduce background staining. Excess serum was shaken off, and the sections then were incubated with the selected primary mouse monoclonal antibody for 60 min at room temperature. The optimal dilution of antibody in PBS containing 0.1% bovine serum albumin was determined by titration. The sections then were incubated for 30 min with rabbit anti-mouse immunoglobulin G (Dakopatts) and finally with peroxidase-mouse-antiperoxidase complex (Dakopatts) for 30 min at room temperature. All steps were separated by three 5-min washes in PBS at room temperature. Antibody binding was visualized by incubating the sections in a solution of 3-amino-9-ethylcarbazole, dimethylformamide, and hydrogen peroxide in acetate buffer (pH 5.2; Dakopatts). The reaction was terminated by washing the slides in PBS. The specimens were counterstained with Mayer’s hematoxylin, mounted in liquid glycerol gelatin, and examined with an Olympus microscope (Olympus Co., Austria, Vienna). Sections of normal skin, processed identically, served as positive controls. Negative controls were prepared by substituting nonimmune mouse serum for the primary antibody. Any T6- or HLA-DR-positive intraepithelial cells with definite dendritic morphology were classified as LCs. The LC were counted in a total of 5 mm of the central corneal epithelium from the sections of each cornea using an ocular micrometer on an Olympus microscope. At least ten sections of each specimen were examined.

Double Immunofluorescence Technique

In addition to immunoperoxidase staining, corneas with T6-positive LC in the epithelium or stroma were examined by two-color immunofluorescence to investigate the coexpression of HLA-DR and T6 antigen on corneal LC. We cut 6-µm thick frozen sections, mounted them on poly-L-lysine-coated slides, air dried them, and fixed them in acetone in a manner identical to that used for the immunoperoxidase staining procedure. After rehydration with PBS, the sections were overlaid for 1 hr with a 1:1 mixture of Dako-HLA-DR/alpha monoclonal antibody (immunoglobulin Gl subclass) and Dako-T6 monoclonal antibody (immunoglobulin G2a subclass). The final dilution of both primary antibodies was 1:40 in PBS containing 0.1% bovine serum albumin. After a buffer wash (three 5-min washes in PBS), the sections were incubated for 30 min with the second layer of antibodies consisting of a 1:1 mixture of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G1 subclass and Dako-T6 monoclonal antibody (immunoglobulin G2a subclass). The final dilution of both primary antibodies was 1:40 in PBS containing 0.1% bovine serum albumin. After a buffer wash (three 5-min washes in PBS), the sections were incubated for 30 min with the second layer of antibodies consisting of a 1:1 mixture of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G1 subclass-specific antibody and rhodamine (TRITC)-conjugated goat anti-mouse immunoglobulin G2a subclass-specific antibody (Southern Biotechnology, Birmingham, AL). The final dilution of both anti-mouse antibodies was 1:20. The sections then were rinsed again with PBS (three 5-min rinses) and covered slipped with fluoromount G (Southern Biotechnology). They were examined under an Olympus fluorescence microscope with epillumination using different appropriate sets of filters for the
two labels (fluorescein and rhodamine) on the same section.

Double immunofluorescence staining of normal skin from the eyelid served as the positive control and revealed coexpression of HLA-DR and T6 antigen on LC in the epidermis as described previously (Figs. 1A–B). To exclude cross-reactivity of anti-mouse immunoglobulin G1 and G2a subclass-specific antibodies, the sections were either incubated with Dako-HLA-DR/alpha monoclonal antibody (G1 subclass) followed by TRITC-conjugated goat anti-mouse immunoglobulin G2a subclass-specific antibody, or they were incubated with Dako-T6 monoclonal antibody (G2a subclass) followed by FITC-conjugated goat anti-mouse immunoglobulin G1 subclass-specific antibody. Using this technique, no specific staining and, thus, no cross-reactivity between FITC- and TRITC-conjugated anti-mouse immunoglobulin G subclass-specific antibodies could be detected (Figs. 1C–D).

**Results**

Sixty-two corneas from patients with various corneal diseases were examined for HLA-DR- or T6-positive LC in the central and pericentral epithelium (Table 1). As expected, LC could only be detected sporadically in corneas from patients with keratoconus, Fuchs' dystrophy, bullous keratopathy, and corneal scars without vascularization. However, all corneas with acute or chronic inflammatory diseases (including corneas with a history of perforating injury) contained varying numbers of HLA-DR-positive cells with definite dendritic morphology in their central epithelium. These cells were assumed to be LC (Fig. 2).

The T6- and HLA-DR-positive LC could be detected in 15 of the 62 corneas examined in this study (Table 1, Figs. 3, 4). The most numerous of such cells, with a density similar to that in normal epidermis (300–500 cells/mm²), were found in the epithelium of five corneas with epidermalization after severe alkali burns (Fig. 3). Both T6- and HLA-DR-positive LC in the central and pericentral corneal epithelium, at smaller densities than in alkali-burned corneas, were found in three of seven patients with chronic herpetic stromal keratitis, in two of four patients with zoster keratitis, in three of six patients with chronic allograft rejection, and in two of eight patients with bacterial corneal ulcers (Table 1, Fig. 5).

Corneas with LC in the central or pericentral epithelium commonly had other, particularly mononuclear, infiltrating cells in their stroma and to a lesser extent also in their epithelium. In alkali-burned corneas, mononuclear cell infiltrates, consisting mainly of varying densities of helper/inducer T-cells, cytotoxic/suppressor T-cells, and macrophages were present particularly in the anterior stroma. There was evidence of marked neovascularization in virtually all of these alkali-burned corneas. The cellular infiltrates in the rejected corneal allografts and in corneas with herpetic stromal keratitis also were composed predominantly of macrophages and helper/inducer and cytotoxic/suppressor T-cells, most of which were T-helper/inducer cells. However, there was considerable individual variation in the density and composition of the cellular infiltrates, probably according to the time course and severity of the disease. No correlation could be found between the presence or density of a particular T-cell population and the presence or absence of T6-positive LC. It was apparent, however, that corneas with T6-positive LC generally contained dense mononuclear cell infiltrates particularly in the anterior stroma and often also in the epithelium; corneas with T6-negative LC usually had only sparse cellular infiltrates.

**Discussion**

Corneal LC normally do not express T6 antigen, a characteristic marker for noncorneal LC and indeterminate cells (IC) of the skin. The T6 antigen consists of a 49-kilodalton cell-surface glycoprotein associated with β2-microglobulin, and it is considered by some authors to represent a nonclassic fraction of the class I antigens. Moreover, it has been shown that T6 antigen and HLA-DR antigen can be cointernalized in LC and IC through common receptor-mediated endocytosis organelles. It is assumed by some investigators that this phenomenon might be involved in antigen-processing events.

In a recent study, corneal LC could not be induced to express T6 antigen when cultured with various cytokines including interleukin-1 (IL-1) and thymopentin (TP-5). These cytokines have been shown to modulate T6 expression on gingival LC and thymocytes, respectively. Thus, some authors hypothesized that corneal LC might represent a distinct LC population that is innately inactive.

Using double immunofluorescence staining techniques and immunoelectron microscopy, T6+/HLA-DR-positive, T6-positive/HLA-DR-negative, and T6-negative/HLA-DR-positive LC can be distinguished in the normal epidermis. Both T6+/HLA-DR-positive and T6-positive/HLA-DR-negative LC populations were detected in human gingival epithelium and human cervical epithelium. However, the most noncorneal LC are T6+/HLA-DR-positive cells, and some investigators assume that this subset may represent an activated state of LC or a functionally distinct subtype. The reason why LC (derived from the bone marrow) express thymocyte...
Fig. 1. (A) Normal human skin from the eyelid shows HLA-DR-positive LC in the epidermis labeled with fluorescein. (B) Same section viewed through a rhodamine filter shows that the fluorescein-labeled HLA-DR-positive cells are also rhodamine labeled (T6-positive). Cryostat section that is 6-μm thick shows the indirect immunofluorescence double-staining technique. Original magnification × 400. (C) Normal human skin from the eyelid incubated with anti-HLA-DR monoclonal antibody (Dako-HLA-DR/alpha, IgG, subclass) followed by rhodamine-conjugated goat anti-mouse IgG₂ subclass-specific antibody. No specific staining is seen in this 6-μm-thick cryostat section. Original magnification × 500. (D) Normal human skin from the eyelid. No specific staining is seen in the epidermis after the use of Dako-T6 (IgG₃ subclass) as primary antibody followed by fluorescein-conjugated goat anti-mouse IgG₁ subclass-specific antibody. Nonspecific autofluorescence of collagen is seen in the dermis of the 6-μm-thick cryostat section. Original magnification × 400.

Fig. 2. Central cornea from a 53-yr-old patient 25 months after allotraft rejection stained with monoclonal antibody, anti-HLA-DR (Dako-HLA-DR/alpha), by the PAP method. One HLA-DR-positive dendritic cell, which is probably an LC, is seen in the epithelium (arrow). Other HLA-DR-positive cells in the epithelium have no definite dendritic morphologic features and thus cannot be classified as presumable LC. The cryostat section is 6-μm thick. Original magnification × 200.

Fig. 3. Central cornea with epidermalization from a 40-yr-old patient 12 months after alkali burn stained with monoclonal antibody, anti-T6 (Dako-T6), by the PAP method. Many T6-positive LC at a density similar to that seen in normal epidermis are seen in the epithelium. Mononuclear cell infiltration is dense in the superficial stroma (arrows). Arrowhead marks Bowman’s membrane. The cryostat section is 6-μm thick. Original magnification × 400.
Fig. 5. Central cornea from a 42-yr-old patient 16 months after alkali burn, shows HLA-DR-positive LC in the epithelium by fluorescein immunofluorescence. (B) Same section viewed through a rhodamine filter shows that the fluorescein-labeled HLA-DR-positive cells are also rhodamine-labeled (T6-positive). The 6-µm thick cryostat section shows indirect immunofluorescence double-staining technique. Original magnification x 500.

Fig. 5. Central cornea from a 42-yr-old patient with chronic herpetic stromal keratitis stained with monoclonal antibody, anti-T6 (Dako-T6), by the PAP method. T6-positive LC are scattered in the epithelium of the 6-µm-thick cryostat section. Original magnification x 400.

T6 antigen is not understood fully. Some authors suggest that keratinocyte production of epidermal cell-derived thymocyte-activating factor (ETAF), an IL-1-like cytokine with similar biologic activities as IL-1, may be the cause of the squamous influence thought to be associated with the expression of T6 antigen on epidermal LC. Indeed, Walsh and co-workers showed that IL-1 and ETAF can induce T6 expression on LC in human gingival organ cultures. By analogy with ETAF (which is produced by epidermal keratinocytes), a similar hormone-like substance has also been detected in the cornea. This cytokine, named corneal epithelial cell-derived thymocyte-activating factor, is produced by corneal epithelial cells and is similar in its molecular weight and biologic activities to ETAF and IL-1. However, the reason why corneal LC normally do not express T6 antigen is unknown. Some investigators believe that the same unknown factors that hinder LC from existing in the central portion of normal adult corneas also may be responsible for the lack of T6 expression on corneal LC.

We showed that corneal LC can express T6 antigen in certain corneal diseases. In general, T6-positive LC could be detected in diseased corneas with dense mononuclear cell infiltrates, particularly in the anterior stroma and the epithelium, e.g., in chronic herpetic stromal keratitis, zoster keratitis, or allograft rejections. The most numerous T6-positive LC, however, were found in alkali-burned corneas with epidermalization. Why do LC express T6 antigen in such corneas with dense mononuclear cell infiltrates or epidermalization? A simple explanation might be that the inflammatory cells (particularly macrophages and T-cells) found in these corneas may produce various cytokines that might be responsible for the T6 expression. However, we did not reach any definitive conclusions as to the nature of either the cell types or the cytokines directly or indirectly involved in the T6 expression on corneal LC. Furthermore, we could not determine whether epidermalization also influences T6 expression on corneal LC because dense inflammatory cell infiltrates also were found in the stroma and epithelium of these corneas.

However, we also must consider another explanation for the presence of T6-positive LC in these corneas with considerable neovascularization. Although various stimuli have been shown to induce both corneal neovascularization and migration of LC from the periphery into the central regions of the cornea, there also is the possibility that the T6-positive corneal LC we detected are derived directly from the blood stream and may represent a population of LC that normally would not reside in the cornea.

Although the functional significance of T6 expres-
sion on corneal LC (particularly in regard to their antigen-presenting capacity and the immunostimulatory potential of both T6-negative and T6-positive LC in the cornea) still remains to be determined, the induction of T6 antigen on corneal LC may represent an important event for the antigen-presenting function of these cells in the initiation of T-cell-mediated immune responses in certain corneal diseases, including corneal allograft rejection.

Further studies will be needed to clarify which factors are responsible for the T6 expression on corneal LC, and in particular, whether various infiltrating cells producing a variety of cytokines are responsible for the T6 expression or whether other factors, eg, corneas with epidermalization, also may induce T6 expression on LC in some corneal diseases.

Key words: Langerhans cell, thymocyte (T6) antigen, HLA-DR antigen, corneal diseases

References