Herpes Simplex Virus–Specific T Cells Infiltrate the Cornea of Patients with Herpetic Stromal Keratitis: No Evidence for Autoreactive T Cells

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PURPOSE. Herpetic stromal keratitis (HSK) is a T-cell–mediated inflammatory disease initiated by a herpes simplex virus (HSV) infection of the cornea. Recently, studies in the HSK mouse model have shown that the immunopathogenic T cells are directed against the HSV protein UL6 cross-reacting with an unknown corneal autoantigen. Whether this type of autoimmunity plays a role in human HSK was analyzed.

METHODS. T-cell lines (TCLs) were generated from corneal buttons of 12 patients with different clinical stages of HSV-induced necrotizing stromal keratitis (n = 9) or immune stromal keratitis (n = 3). The initiating virus was identified by polymerase chain reaction and immunohistology performed on the corneal buttons. Peripheral blood mononuclear cells (PBMCs) were isolated, and B cell lines (BLCLs) were generated by transformation with Epstein-Barr virus. Proliferative responses of these intracorneal TCLs were determined by culturing T cells with autologous BLCLs infected with HSV-1, HSV-2, wild-type vaccinia virus (VV-WT), or VV expressing HSV-1 UL6 (rVV-UL6). Alternatively, T cells were incubated with PBMCs pulsed with human cornea protein extract.

RESULTS. Irrespective of clinical diagnosis or treatment, T cells were recovered from the corneal buttons of all the 12 HSK patients. The intracorneal TCLs of 9 of the 12 HSK patients showed HSV-specific T-cell reactivity. In none of the TCLs, T-cell reactivity against HSV-1 UL6 or human corneal antigens was detected.

CONCLUSIONS. These data suggest that the potentially immunopathogenic intracorneal T-cell response in HSK patients is directed to the initiating virus and not to a human corneal autoantigen or HSV-1 UL6. (Invest Ophthalmol Vis Sci. 2000;41:2607–2612)

Herpes simplex virus (HSV) infections of the cornea can elicit the development of herpetic stromal keratitis (HSK). HSK is a sight-threatening disease in which tissue destruction, edema, and corneal scarring are the result of an inflammatory response in the corneal stroma. Current knowledge on the immunopathogenesis of HSK is primarily based on studies performed in the experimental mouse model for HSK that closely mimics necrotizing keratitis in humans. In the HSK mouse model, CD4+ T helper 1 (Th1) cells have been demonstrated to play a pivotal role in this local immunopathogenic response.1,2 The other cell types involved are Langerhans cells (LCs),3 macrophages, and, notably, polymorphonuclear neutrophils (PMNs).3,5 The nature of the antigens recognized by these immunopathogenic cornea-infiltrating T cells is a matter of debate. A long-standing assumption has been that the intracorneal T-cell response in HSK is directed to HSV-encoded antigens.6 Recently, however, studies in the HSK mouse model have provided evidence that HSK is an HSV-induced autoimmune disease.7,8 HSK could be induced by CD4+ T cells directed to an epitope derived from the HSV-1 capsid protein UL6, that cross-reacts with an antigen uniquely expressed in the murine cornea.8

Recently, we have demonstrated the presence of HSV-specific CD4+ Th0-like cells in corneas of two patients with necrotizing ulcerative HSK. In this study no reactivity to human corneal antigens could be detected.9 Nevertheless, autoreactive T cells may still be involved in clinically distinct HSK entities. In the present study we determined the antigen specificity of cornea-infiltrating T cells, obtained from 12 patients with different clinical forms of HSK. T-cell reactivity was tested toward the HSV serotypes 1 and 2, recombinant HSV-1 UL6, and a soluble human corneal protein extract.

METHODS

Clinical Materials and Reagents

Corneal buttons and peripheral blood mononuclear cells (PBMCs) were obtained from 12 patients, with HSV-induced necrotizing stromal keratitis (patients 1 through 9) or immune stromal keratitis (patients 10 through 12), after therapeutic
penetrating keratoplasty. HSK classification, and quiescent disease for at least 6 months or active disease, was defined on the basis of clinical criteria.10 The characteristics, diagnosis, and preoperative treatment of the patients studied are listed in Table 1. Patient 12 was transplanted because of a corneal graft rejection due to a necrotizing ulcerative HSK, and patients 1 and 2 have been described previously.9 Isolation of PBMCs and the generation of B cell lines (BLCLs), by transformation with Epstein-Barr virus, were performed as described previously.9 Virus stocks of the MacIntyre strain of HSV-1 (American Type Culture Collection [ATCC] VR-539) and the MS strain of HSV-2 (ATCC VR-540) were generated and titrated in Vero cells. Recombinant vaccinia virus (VV) rVV-UL6 expressing the HSV-1 UL6 gene (strain 17) has been described and kindly provided by Arvind H. Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK).11 Virus stocks of the rVV-UL6 and the wild-type strain WR (ATCC VR-1354) were generated and titered on RK13 cells (ATCC CCL-37). Protein was extracted from whole human cornes (n = 12) and donated for transplantation but found unacceptable because of senile changes of the endothelium, by sonification of a Tris-buffered cornea tissue lysate as described previously for the generation of a soluble murine cornea protein extract.7,12 The present study was performed according to the Declaration of Helsinki, and informed consent was obtained from all patients.

Immunohistochemistry

Corneal buttons, obtained within 1 hour after surgery, were divided in two equal parts for immunohistologic analysis and T-cell recovery. One quarter of the cornea was snap-frozen in optimal cutting tissue, and one quarter was fixed with formalin and embedded in paraffin. Routine histopathology (hematoxylin/eosin and periodic acid–Schiff staining) and immunoperoxidase staining were performed on cryostat and paraffin sections as described previously.13 Mouse anti-human monoclonal antibodies (MAbs) were used as primary antibodies. The following MAbs were used as recommended by the supplier: anti-CD3 (Dako, Glostrup, Denmark), anti-CD4 and -CD8 (Becton–Dickinson, San Diego, CA), and anti–HSV-1 (Dako). Peroxidase-labeled polyclonal rabbit anti-mouse IgG antibody (Dako) was used as secondary antibody and visualized using diaminobenzidine or 3-amino-9-ethyl carbazole (Sigma, St. Louis, MO).13

DNA Extraction and Polymerase Chain Reaction Analyses

The surplus half of the corneal specimens was minced and treated with collagenase essentially as described previously.9 DNA was isolated from one-fourth part of the corneal cell suspension lysed in a guanidine isothiocyanate buffer using Celite solution (Jansen Chemika, Beers, Belgium) according to the method of Boom et al.14 The polymerase chain reaction (PCR) primers and conditions for detection of HSV type 1 and 2 and varicella zoster virus specific DNA after Southern blot analysis have been described previously.15

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex*</th>
<th>Age†</th>
<th>Diagnosis</th>
<th>Disease Status</th>
<th>HSV-1 PCR on Cornea‡</th>
<th>Time Since Keratitis First Diagnosed§</th>
<th>Time Since Last Recurrence‡</th>
<th>Topical Preoperative Treatment</th>
<th>Steroids</th>
<th>Acyclovir</th>
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<td>+</td>
<td>352</td>
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<td>Quiescent</td>
<td>–</td>
<td>143</td>
<td>37</td>
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</table>

* M, male; F, female.
† Age in years.
‡ + or –, positive or negative HSV-1–specific PCR product detected. ND, not done. ?, unknown treatment.
§ Time in months. Times since last recurrence means time lapse since presentation of active disease in months, whereas active disease is defined as visible stromal infiltration with edema. In the patients with a time lapse of 1–1.5 months, patients 1, 8, and 11, the cornea was still showing evident cellular infiltration. Impending perforation made penetrating keratoplasty necessary.

Corneal-Derived T-Cell Lines

Cornea-derived T-cell lines (TCLs) were generated from the remaining corneal cell suspension as described previously.9 After one round of mitogenic stimulation, using phytohemagglutinin-L (PHA-L; Boehringer–Mannheim, Mannheim, Germany) and allogeneic feeder cells, the intracorneal TCLs were frozen in aliquots at −135°C. Control experiments with corneas histologically devoid of infiltrating T cells did not result in the generation of TCLs, indicating that the method applied facilitates the recovery and outgrowth of T cells compartmentalized to the cornea (data not shown). The TCLs were characterized for cell surface expression of CD3, CD4, and CD8 by triple color flowcytometry using fluorescein isothiocyanate (FITC)–, RPE–, and R-Phycocerythrin (RPE)-Cy5–conjugated MAbs, respectively (Dako).

T-Cell Proliferation Assays

Autologous BLCLs were infected with HSV-1 and HSV-2 at a multiplicity of infection (MOI) of approximately 5 at 37°C for
20 hours. The virus- and mock-infected cells were washed and UV-irradiated $(2.5 \times 10^{-2}$ mW/mm²). Alternatively, BLCLs were infected with the rVVs at an MOI of approximately 5 for 20 hours and fixed with 1% paraformaldehyde as described previously. The level of infection of the BLCLs with the respective viruses was determined by flowcytometry. The expression of HSV-1 UL6 in rVV-UL6-infected BLCLs was demonstrated using a rabbit anti-UL6 serum (2C2) and, subsequently, FITC-conjugated swine anti-rabbit serum (Dako). As for all viruses, approximately 70% to 90% of the BLCLs were shown to be infected (Fig. 1). T cells ($3 \times 10^4$/well), removed from culture at days 10 to 12 after one mitogenic stimulation, were cultured in triplicate together with virus- or mock-infected BLCLs ($2 \times 10^4$/well) in 96-well round-bottomed plates in 150 μl complete medium at 37°C in a CO₂ incubator. Complete medium consisted of RPMI 1640 (GIBCO-BRL, Breda, The Netherlands) supplemented with 10% heat-inactivated pooled human serum and antibiotics. Because of the limited ability of BLCLs to process and present exogenous antigens to T cells, T-cell reactivity to human soluble cornea protein extract (HuSoCo; at final protein concentrations of 50 and 100 μg/ml) was performed using 10⁵ autologous PBMCs (UV-irradiated) as antigen-presenting cells (APCs). The cells were cultured for 72 hours and pulsed with 0.5 μCi [³H]-thymidine over the last 18 hours of culture. The cells were harvested and the incorporated radioactivity was determined in a β-scintillation counter. Proliferation was considered positive when stimulation indices (counts per minute [cpm] incorporated in response to antigen/cpm incorporated in response to control) were more than 4. T-cell reactivity to all antigens tested for was assayed simultaneously, and PHA-L (1 μg/ml) was included as positive control for T-cell proliferation. The assays were performed at least two times, and the SD was always less than 30% of the mean counts per minute.

**Intracorneal HSV-Specific T Cells in HSK Patients**

**RESULTS**

**Immunohistochemistry on Corneas of HSK Patients**

Diagnostic analyses were performed on corneal buttons, obtained after therapeutic penetrating keratoplasty, from 12 patients with necrotizing stromal keratitis (patients 1 through 9) or immune stromal keratitis (patients 10 through 12). All patients had a history of recurrent episodes of HSV-1-induced stromal keratitis (Table 1). The corneal histopathology observed in the HSK patients (Fig. 2A) included granulomatous reactions at the level of Descemet’s membrane (Figs. 2B and 2C), suppurrative keratitis with edema (Figs. 2D and 2E), reactive hyperplasia of the epithelium, and breakdown of Bowman’s layer (Figs. 2F and 2G). A mononuclear cell infiltrate was observed in the corneas of all patients and consisted predominantly of CD4⁺ T cells (Fig. 2G).

PCR analyses revealed the presence of HSV-1 DNA in 8 of 11 corneas analyzed, implicating HSV-1 as initiating the disease. HSV-1 DNA-positive corneas were mainly obtained from patients with fulminant necrotizing stromal keratitis (Table 1). Evidence for an ongoing intracorneal HSV-1 infection was only found in patient 5, demonstrated by the presence of HSV-1-infected kerocytes (Fig. 2E).

**Antigen Specificity of Cornea-Derived TCLs from HSK Patients**

We have recently developed a protocol that enables the recovery and expansion of in vivo activated corneal infiltrating T cells from corneal buttons of HSK patients. This method facilitated the generation of intracorneal TCLs from 12 HSK patients studied. All TCLs consisted predominantly of CD3⁺ T cells and the ratio of CD4⁺ and CD8⁺ T cells varied interindividually. Interestingly, the TCLs of 2 patients with quiescent necrotizing stromal keratitis consisted almost exclusively of CD4⁺ T cells (patients 7 and 9; Table 1). The reactivity of the cornea-derived TCLs toward the triggering virus was analyzed in T-cell proliferation assays using mock-, HSV-1-, and HSV-2-infected autologous BLCLs as APCs. The intracorneal TCLs of 9 of 12 patients showed HSV-specific T-cell reactivity (Table 2). Illustrative for the high sequence homology between the HSV serotypes, the majority of these TCLs recognized both HSV-1- and HSV-2-infected BLCLs. In the case of patient 8, however, the HSV-specific intracorneal T-cell response was restricted to HSV-2.

To test the hypothesis that an HSV-induced autoreactive intracorneal T-cell response is involved in the immunopathogenesis of HSK in humans, the reactivity of the TCLs to recombinant HSV-1 UL6 and a HuSoCo protein extract was determined. In repeated experiments, none of the TCL showed significant responses to HSV-1 UL6 or human corneal antigens (Table 2).

**DISCUSSION**

HSV infection of the cornea can result in the development of stromal keratitis, a leading infectious cause of blindness worldwide. The adult cornea is an ocular tissue without constitutive lymphoid components. Therefore, any intracorneal T cell found in HSK patients must have migrated into the cornea upon infection and subsequent inflammation. Experimental
HSK animal models have been developed to investigate the immunopathogenesis of HSK. Based on these studies, HSK is considered to represent an immunopathologic reaction in the corneal stroma coordinated by CD4\(^+\) Th1 cells.\(^1,2\)

Although the processes orchestrated by cornea-infiltrating CD4\(^+\) T cells have been studied extensively, the target antigens recognized remain unclear. Given the involvement of HSV in the etiology of HSK, HSV antigens are the most likely candidates. To address this notion we analyzed the antigen specificity of cornea-infiltrating T cells in 12 patients with HSV-induced stromal keratitis. After one round of mitogenic stimulation, cornea-derived TCLs were successfully generated from corneas.
of the 12 patients studied. Intracorneal HSV-specific T-cell reactivity, mainly HSV-type common, was observed in 9 of 12 corneas tested. These data indicate that T cells specific for the triggering virus infiltrate corneas of HSK patients. In patient 8, however, the HSV-specific response was solely directed to HSV-2. Possibly, the determinants recognized by these T cells are HSV-type common and are not efficiently processed and presented in HSV-1– compared with HSV-2–infected BLCs. Surprisingly, HSV-reactive T cells could also be detected in TCLs of patients in a quiescent phase, treated with steroids, and even from HSV DNA–negative corneas. These data suggest that HSV-specific T cells can reside for longer periods of time and, even under steroid treatment, in corneas of patients with HSV-induced stromal keratitis.

In contrast to corneal patients with necrotizing stromal keratitis\(^7\) (Fig. 2E), murine HSK corneas are devoid of HSV antigens.\(^1,2\) Nonetheless, HSV-specific T cells have been demonstrated in whole-eye cell suspensions of mice with fulminate HSK.\(^3\) Similarly, in 8 of the 9 HSK patients, from which intracorneal HSV-specific T cells were recovered, the corneas were devoid of HSV antigens. On infiltration of the cornea, these HSV-specific T cells may have been activated by viral peptides devoid of HSV antigens. On infiltration of the cornea, these corneal HSV-specific T cells were recovered, the corneas were HSK.\(^3\) Similarly, in 8 of the 9 HSK patients, from which intracorneal HSV-specific T cells were recovered, the corneas were HSK. In none of the TCLs generated from corneas of any of the HSK patients studied here, reactivity to HSV-1 UL6 or a HuSoCo protein extract could be demonstrated. Stimulation of the intracorneal TCLs with PHA-L resulted in high proliferative responses, indicating that this is not due to a low viability of the TCLs tested (Table 2). The lack of reactivity toward HSV-1 UL6, harboring the cross-reactive epitope, is not surprising given the constraints of major histocompatibility complex allele-specific peptide binding.\(^19\) In the case of the HuSoCo protein extract, the negative results could be due to a true lack of autoreactive T cells or an inappropriate corneal antigen preparation used. The putative corneal autoantigen could be located in the buffer-insoluble part of the human cornea extract, it may be genetically polymorphic or the intracorneal autoreactive T-cell responses are mediated by CD8\(^+\) T cells. Given the nature of the HuSoCo protein extract and the type of assay used (i.e., exogenous antigen preparation in a T-cell proliferation assay), the potential role of CD8\(^+\) corneal autoantigen–specific T cells in HSK could not be addressed. In the HSK mouse model, the keratogenic T-cell clone recognized an unknown Tris–buffer soluble cornea-specific antigen\(^7\) and was able to induce the disease in HSK-resistant mice, arguing against genetic polymorphism of the autoantigen. The HuSoCo protein extract used here, obtained from 12 human cornea buttons and similarly generated as described in the murine HSK study,\(^7,12\) was a heterogeneous protein preparation in which the major soluble cornea protein BCP54\(^12\) was predominantly present (sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis; data not shown). Positive peripheral blood T-cell responses, using similar concentrations of an equivalent HuSoCo protein extract or purified BCP54, have been obtained in patients with inflammatory corneal diseases.\(^20,21\) These data suggest that the HuSoCo protein extract used in the present study may be considered immunogenic. Although not formally excluded, the lack of intracorneal T-cell reactivity to HSV-1 UL6 and human corneal antigens does not support the hypothesis that human HSK is an HSV-induced autoimmune disease. The cloning and identification of the putative HSK-related murine cornea autoantigen, and its human homologue will be needed to further address the validity of the molecular mimicry hypothesis at the single antigen level.

In conclusion, the present study demonstrates T cells specific for the triggering virus in the corneas of the majority of the 12 HSK patients studied. On antigenic stimulation, the cornea-derived HSV-specific T cells from HSK patients secrete

### Table 2. Phenotype and Antigen-Specific Proliferative Responses of Cornea-Derived TCLs from HSK Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CD3(^+)</th>
<th>CD4(^+)</th>
<th>CD8(^+)</th>
<th>[(\text{H})]-Thymidine Incorporation, cpm*</th>
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</thead>
<tbody>
<tr>
<td>BLCL/ Mock</td>
<td>BLCL/ HSV-1</td>
<td>BLCL/ HSV-2</td>
<td>BLCL/ rVV-UL6</td>
<td>PBMC/ Medium</td>
</tr>
<tr>
<td>1</td>
<td>92</td>
<td>68</td>
<td>23</td>
<td>1,260 ± 122</td>
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<td>98</td>
<td>84</td>
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<td>19 ± 9</td>
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<tr>
<td>12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>699 ± 172</td>
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* T cells were incubated with autologous BLCs infected with HSV-1 or -2, VV-WT, or VV expressing HSV-1 protein UL6. Alternatively, T cells were cultured with autologous PBMCs in the presence of HuSoCo, medium or PHA-L (1 μg/ml) as negative and positive controls. Results using 50 μg/ml HuSoCo are presented. Data are expressed as mean cpm ± SD of triplicate cultures. ND, not done.
both interferon gamma (IFN-\(\gamma\)) and interleukin 2 (IL-2)\(^9\) (data not shown). In the mouse HSK model, both cytokines have been shown to be pathologic in the cornea of HSV-1-infected mice.\(^1,5,22\) IFN-\(\gamma\) has been shown to facilitate migration of PMNs from the blood into the cornea, and on activation by IL-2, and perhaps IFN-\(\gamma\) secretes proteolytic enzymes that contribute to destruction of the cornea.\(^1,2,23\) We hypothesize that HSV-specific T cells have an important role in the local immunopathogenesis of HSK in humans. On entry into the cornea they are activated by HSV-infected corneal cells or by viral peptides retained by corneal cells like LC corneal cells, and, subsequently, initiate a cytokine-mediated immunopathogenic response in the cornea.

Acknowledgments

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References