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Bystander Activation Involving T Lymphocytes in Herpetic Stromal Keratitis

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Herpes simplex virus infection of mouse corneas can lead to the development of an immunopathological lesion, termed herpetic stromal keratitis (HSK). Such lesions also occur in TCR-transgenic mice backcrossed to SCID (TgSCID) that are unable to mount detectable HSV-specific immune responses. The present study demonstrates that lesion expression in such mice depends on continuous viral replication, whereas in immunocompetent mice, lesions occurred even if virus replication was terminated at 4 days after infection. The continuous replication in TgSCID mice was considered necessary to produce an activating stimulus to CD4+ T cells that invade the cornea. Lesions in TgSCID were resistant to control by cyclosporin A, but were inhibited by treatment with rapamycin. This result was interpreted to indicate that T cell activation involved a non-TCR-mediated cytokine-driven bystander mechanism. Bystander activation was also shown to play a role in HSK lesions in immunocompetent mice. Accordingly, in immunocompetent DO11.10 mice, lesions were dominated by KJ1.26 OVA-specific CD4+ T cells that were unreactive with HSV. In addition, KJ1.26 HSV nonimmune cells parked in ocularly infected BALB/c mice were demonstrable in HSK lesions. These results provide insight for the choice of new strategies to manage HSK, an important cause of human blindness. The Journal of Immunology, 2001, 167: 2902–2910.

Inflammatory lesions caused by viruses involve many events. In some instances, tissue damage is the direct consequence of virus replication, whereas in others, lesions are the sequel to immune responses against viral components (1, 2). In such instances, either CD4+ or CD8+ T cells usually act as the principal mediators of the immunopathology. A less frequent, and still poorly understood, outcome of virus infection is the induction of an autoimmune inflammatory reaction (1). Explanations for the mechanism of viral-induced breakdown in self-tolerance include unveiling of “hidden self,” molecular mimicry between viral and host components, determinant spreading, and bystander damage associated with recruited cells and aberrant cytokine production (1–3). One viral-induced immunopathology that could involve an autoimmune inflammatory reaction is herpetic stromal keratitis (HSK) (4, 5). This blinding chronic inflammatory reaction results from HSV infection of the eye. The pathogenesis of HSK, as studied in the mouse, involves numerous components, but CD4+ cells appear to be the principal orchestrators of the immunopathology (4).

Currently, the agonists that drive CD4+ T cell activation in the eye remain ill defined. Peptides derived from viral proteins represent the logical candidate Ags, but this mechanism has been difficult to prove, and the identity of such peptides remains unknown (5). Another idea is that autoantigens, unmasked by HSV infection of the cornea, serve to drive the inflammation (6, 7). The best evidence for this hypothesis was provided by Avery et al. (6), who indicated that a corneal peptide, whose sequence was represented in the IgG2a Iγ isotype, appeared as the inflammatory agonist. Curiously, mice that expressed the IgG2a Iγ isotype, and hence were tolerant to the peptide, were resistant to HSK development (6). More recently, HSV ocular infection was suggested to set off an inflammatory response by expressing a molecular mimic of the Ig peptide (7). These observations await confirmation and have been questioned (8, 9).

An alternative idea to explain HSK lesion development is that the response involves bystander activation of T cells. Support for this mechanism came from studies with TCR-transgenic (Tg) mice backcrossed to SCID or RAG−/− (10–12). Such animals possessed a limited repertoire of CD4+ T cells and were unable to generate detectable immune responses to HSV Ags. However, unlike SCID or RAG−/− mice, TCR Tg SCID (TgSCID) or TCR Tg RAG−/− (TgRAG−/−) mice could develop HSK lesions upon ocular infection with HSV (10–12). The mechanism by which HSK occurred in such mice was assumed to involve non-TCR-mediated activation of CD4+ T cells in the cornea. The present study provides more evidence about the mechanism of HSK expression in TgSCID mice. Our results demonstrate that lesion expression requires continuous viral replication most likely necessary to drive production of proinflammatory cytokines. We indicate that activated T cells are involved in HSK expression and that, on the basis of studies with immunosuppressive drugs, CD4+ T cell activation in TgSCID lesions appears as a non-TCR-mediated event. Finally, bystander recruitment and activation are shown to play a significant component in HSK lesions of immunocompetent mice. The results are discussed in terms of the choice of strategies to manage HSK in the clinic.

Materials and Methods

Mice

BALB/c mice (4–6 wk) were purchased from Harlan Sprague Dawley (Indianapolis, IN). DO11.10 (OVA-TCR Tg mice), DO11.10 × SCID (TgSCID), and CB.17 SCID mice were bred in our special pathogen-free facility. The DO11.10 OVA-TCR Tg mice were kindly provided by C.
Weaver (University of Alabama, Birmingham, AL). Generation and immunological profile of the TgSCID mice have been previously described (10). Do11.10 mice were backcrossed to CB.17 SCID mice for four generations. Offspring from the first breeding ($F_1$) were screened for the transgene by PCR from tail-genomic DNA, and peripheral blood serum was used to screen for IgM by ELISA. Screened offspring from each generation were backcrossed to CB.17 SCID mice. After four generations of backcrossing, brothers and sisters (screened for the transgene and IgM levels) were mated to develop the TgSCID mice on a homozygous background. Only were mated to develop the TgSCID mice on a homozygous background. Crossing, brothers and sisters (screened for the transgene and IgM levels) were backcrossed to CB.17 SCID mice. After four generations of backcrossing, brothers and sisters (screened for the transgene and IgM levels) were mated to develop the TgSCID mice on a homozygous background. All food, water, bedding, and instruments were autoclaved, and all manipulations were done in a laminar flow hood. To prevent bacterial superinfections, all mice were treated prophylactically with sulfathiazole pediatric suspension (Barre-National, Baltimore, MD) at the rate of 5 ml/200 ml drinking water. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. The facilities used were accredited by the American Association for Accreditation of Laboratory Animal Care.

**Virus and reagents**

HSV-1 RE and HSV-1 KOS strains were propagated and titrated on monolayers of Vero cells (ATCC CCL81) using standard protocols (13). All virus stocks were aliquoted and stored at −80°C. The thymidine kinase-negative (TK−) virus from the HSV-1 RE-KOS chimera was a generous gift of D. M. Coen (Harvard Medical School, Cambridge, MA) (14). Cyclosporin A (Sigma, St. Louis, MO) was diluted to 30 μg/ml in castor oil and administered to 100 μl i.p. to achieve doses of 100 mg/kg (15–17). Rapamycin (Sigma) was diluted in ethanol to 1 mg/ml and administered i.p. at a dose of 0.2 mg/kg (16). Acyclovir (ESI Lederle, Philadelphia, PA) was administered in drinking water at 1 mg/ml concentration (18). Cpg6 sequence 1826 (kindly provided by A. Krieg, University of Iowa, Iowa City, IA) was administered at 30 μg/ml in a 100-μl volume in PBS i.p.

**Corneal HSV infections and clinical observations**

Corneal infections of all mice groups were conducted under deep anesthesia with 10 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY) and stored at −80°C. For detection and quantification of HSV in the corneas, the samples were thawed and vortexed. Duplicate 200-μl aliquots of each sample of thawed swab medium were plated on Vero cells grown to confluence in 24-well plates at 37°C in 5% CO₂ for 1 h and 30 min. Medium was aspirated, and 1 ml of 2× DMEM containing 1% low-melting point agarose was added to each well. Cultures were observed daily for the development of typical cytopathic effect. The titers were calculated as PFU/ml as per standard protocol (13).

**Ag-specific lymphoproliferation assay**

Individual spleens and cervical and submandibular draining lymph nodes (DLN) were used as responders for lymphoproliferation assays. This method has been described in detail elsewhere (19). Briefly, these responders were restimulated in vitro with irradiated syngeneic splenocytes pulsed with UV HSV-1 KOS (multiplicity of infection (MOI), 1.5) or irradiated naïve splenocytes and incubated for 5 days at 37°C. Eighteen hours before harvesting, [3H]thymidine (1 μCi/well) was added to all culture wells, and the plates were read using a beta scintillation counter (Tracor 9C; Inotech, Lansing, MI). The results were expressed as mean cpm ± SD for six replicates per sample.

**Quantification of cytokines by ELISA**

Single-cell suspensions of splenic, cervical, and mandibular DLN cells (2 × 10⁶ cells/ml) were in vitro restimulated with syngeneic stimulators pulsed with 1.5 MOI UV-HSV-1 RE or 10 μg/ml OVA₃₂₃-₃₃₅ peptide and incubated for 48–72 h at 37°C. Con A-stimulated (5 μg/10⁶ cells/ml) and unstimulated cells were used as positive and negative controls, respectively. The supernatants were analyzed for IFN-γ cytokine production by ELISA. Microtiter plates were coated with 2 μg/ml rat anti-mouse IFN-γ Ab (BD Pharmingen, San Diego, CA) at 4°C overnight. The plates were then washed three times with PBS containing 0.5% Tween 20 and blocked with 5% nonfat dry milk for 1 h at 37°C. After washing, serially diluted samples and standards (rIFN-γ) were added to the plates and incubated overnight at 4°C. After washing with PBS, 1 μg/ml biotinylated anti-IFN-γ Ab (BD Pharmingen) was added to wells and incubated at 37°C for 2 h. Peroxidase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) was added and incubated at 37°C for 1 h. The color was developed by adding the substrate solution (11 mg 2,2’-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid in 25 ml of 0.1 M citric acid, 25 ml of 0.1 M sodium phosphate, and 10 μl of hydrogen peroxide). Quantification was performed using a Spectramax ELISA reader (softmax version 1.2).

**Intracorneal cytokine staining**

DLN cells from HSV-1 RE-infected Do11.10 or BALB/c mice were cultured in a 96-well flat-bottom plate and pulsed with OVA₃₂₃-₃₃₅ (10 μg/ml), HSV-UV (MOI 5.0), or unstimulated naïve syngeneic splenocyte for 15 h at 37°C in 5% CO₂. Cell surface staining for FITC CD₄²⁺ (BD Pharmingen) was performed, followed by intracellular staining for PE-IFN-γ (BD Pharmingen) using a Cytofix/Cytoperm kit (BD Pharmingen) in accordance with the manufacturer’s instructions. Golgistop (BD Pharmingen) was added for the last 4 h of the incubation period. All samples were collected on a FACScan (BD Biosciences, Mountain View, CA), and data were analyzed using CellQuest 3.1 software (BD Biosciences).

**Flow cytometry**

**Cell preparation.** Single-cell suspensions were prepared from eyes, DLN, popliteal lymph nodes, and spleens of mice at different days postinfection. Eyes were digested with collagenase D, as described elsewhere (20), with some modifications. Briefly, enucleated whole eyes were incubated with collagenase for 60 min at 37°C in a humidified atmosphere of 5% CO₂. After incubation, eyes were disrupted by grinding with a syringe plunger.

**Table I. Viral titration in mice treated with acyclovir at day 1 or day 4 following HSV infection on scarified corneas**

<table>
<thead>
<tr>
<th>Mice</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgSCID (control)</td>
<td>5.2 ± 0.4</td>
<td>4.9 ± 0.6</td>
<td>4.4 ± 0.5</td>
<td>4.2 ± 0.6</td>
<td>4.3 ± 0.8</td>
<td>4.4 ± 0.9</td>
<td>5.0 ± 0.6</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>TgSCID (day 1 p.i.)</td>
<td>4.9 ± 0.6</td>
<td>1.6 ± 1.0</td>
<td>1.0 ± 0.8</td>
<td>UD³</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>TgSCID (day 4 p.i.)</td>
<td>5.4 ± 0.6</td>
<td>4.8 ± 0.4</td>
<td>4.4 ± 0.9</td>
<td>2.8 ± 0.8</td>
<td>1.6 ± 0.8</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>BALB/c (control)</td>
<td>4.6 ± 0.9</td>
<td>3.2 ± 0.6</td>
<td>2.4 ± 1.6</td>
<td>2.0 ± 0.8</td>
<td>1.8 ± 0.6</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>BALB/c (day 1 p.i.)</td>
<td>4.7 ± 0.4</td>
<td>2.8 ± 0.2</td>
<td>1.2 ± 0.6</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>BALB/c (day 4 p.i.)</td>
<td>4.8 ± 0.6</td>
<td>4.6 ± 0.8</td>
<td>3.2 ± 0.9</td>
<td>2.0 ± 1.4</td>
<td>1.5 ± 0.9</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>DO11.10 (control)</td>
<td>4.3 ± 0.9</td>
<td>4.0 ± 0.7</td>
<td>3.5 ± 1.2</td>
<td>2.1 ± 0.7</td>
<td>2.0 ± 0.9</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>DO11.10 (day 1 p.i.)</td>
<td>4.0 ± 0.8</td>
<td>3.0 ± 0.5</td>
<td>2.2 ± 1.2</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>DO11.10 (day 4 p.i.)</td>
<td>4.2 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>2.9 ± 0.9</td>
<td>2.5 ± 1.6</td>
<td>1.9 ± 0.4</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
</tbody>
</table>

* HSV-1 RE was inoculated on the scarified cornea, and at indicated times the presence of infectious virus on the eye swabs was determined using the agarose overlay method.
* UD, Undetectable, i.e. below the sensitivity of the assay (<10 PFU/ml); p.i., postinfection.
FIGURE 1. HSK lesion expression in BALB/c, TgSCID, and DO11.10 mice treated with acyclovir. Groups of BALB/c mice (A, n = 6), TgSCID mice (B, n = 6), or DO11.10 (C, n = 4) were treated with acyclovir (1 mg/ml in drinking water) daily from either day 1 or day 4 postinfection with HSV-1 RE (5 × 10^5 PFU) on scarified corneas. Mice were scored clinically for HSK on a score of 0–5. Data are plotted for TgSCID mice for days 7, 9, and 12 postinfection as they succumbed to encephalitis by days 10–12 postinfection. For BALB/c and DO11.10 mice, data are plotted for days 7, 9, 12, 15, 18, and 21 postinfection. Results are expressed as mean clinical score ± SD. *, p < 0.5 by Student’s t test between untreated and day 1 acyclovir-treated BALB/c and DO11.10 groups, and also between untreated TgSCID and day 1 and day 4 acyclovir-treated TgSCID mice.

As shown before, whereas immunocompetent mice eliminate virus from the eye following infection, SCID and TgSCID mice do not, and usually die of herpetic encephalitis at about day 11–12 postinfection (10, 11). As shown in Table I and Fig. 1, if TgSCID mice were treated with the antiviral drug acyclovir, continually starting on day 1 or day 4 post-HSV infection, lesions failed to manifest and animals treated at day 1 survived infection for at least 3 wk (late time studied). In experiments with ocularly infected BALB/c or DO11.10 mice (both competent to respond immunologically to HSV), antiviral drug acyclovir treatment begun on day 1 markedly diminished or abrogated HSK lesion development, but treatment begun on day 4 was without effect (Fig. 1). These data indicate the necessity for continuous viral replication in the eyes of TgSCID mice to produce lesions, but only a brief, but necessary, requirement for replication to induce HSV in immunocompetent animals. The experiment shown in Fig. 2 demonstrates a failure of replication-competent TK− virus to induce HSK in TgSCID mice. The virus mutant fails to replicate in ganglionic tissue (14), perhaps implying that virus persists in the eye by spreading to the stroma following replication in the ganglion.

Previous studies showed that TgSCID mice fail to recognize HSV Ags, and that >97% of their CD4+ T cells recognize OVA peptide (10, 11). Accordingly, we hypothesize that T cell activation in TgSCID mice occurs other than by TCR recognition. The data in Fig. 3 add support to this concept. In these experiments, both BALB/c and TgSCID mice were ocularly infected with HSV and then treated daily with either cyclosporin A or rapamycin. As is evident in Fig. 3B, HSK lesions were markedly diminished in BALB/c mice treated with CsA. In contrast, the drug had no apparent effect on lesions in TgSCID mice (Fig. 3A). Rapamycin and through passing cell scrapers. Cells were washed and suspended in RPMI 1640 with 10% FBS. Cells were counted by trypan blue exclusion with high viability.

Staining for flow cytometry. A total of 2 × 10^6 cells was first blocked with unconjugated anti-CD32/16 for 10 min. Samples were incubated with PE anti-CD4, or PE anti-CD44, or PE anti-CD62L, or PE anti-CD25 (BD Pharmingen); PE anti-CCR5 or RPE anti-CD45RB (Calbiochem, La Jolla, CA); and KJ1-26.1 (anti-OVA TCR Ab), followed by biotinylated rat anti-mouse IgG2a and streptavidin PerCP. All samples were collected on a FACScan (BD Biosciences), and data were analyzed using CellQuest 3.1 software (BD Biosciences).

Histopathology and immunohistochemical staining

Eyes were frozen in optimum cutting temperature (OCT) compound (Miles, Elkart, IN) at different days postinfection. Six-micrometer-thick sections were cut, air dried, and fixed in cold acetone for 5 min. Sections were then blocked with heat-inactivated goat serum and stained with CFSE by incubation with 1 × 10^6 cells/ml. Excess CFSE was quenched by adding 10% FBS. Cells were washed three times before i.v. adoptive transfer via tail vein. Cells were stained and analyzed, as described previously.

Statistical analysis

Wherever specified, data obtained were analyzed for statistical significance by Student’s t test and Wilcoxon rank sum test.

Results

HSK lesions in TgSCID and immunocompetent mice involve different immune mechanisms

As shown before, whereas immunocompetent mice eliminate virus from the eye following infection, SCID and TgSCID mice do not, and usually die of herpetic encephalitis at about day 11–12 postinfection (10, 11). As shown in Table I and Fig. 1, if TgSCID mice were treated with the antiviral drug acyclovir, continually starting on day 1 or day 4 post-HSV infection, lesions failed to manifest and animals treated at day 1 survived infection for at least 3 wk (late time studied). In experiments with ocularly infected BALB/c or DO11.10 mice (both competent to respond immunologically to HSV), antiviral drug acyclovir treatment begun on day 1 markedly diminished or abrogated HSK lesion development, but treatment begun on day 4 was without effect (Fig. 1). These data indicate the necessity for continuous viral replication in the eyes of TgSCID mice to produce lesions, but only a brief, but necessary, requirement for replication to induce HSV in immunocompetent animals. The experiment shown in Fig. 2 demonstrates a failure of replication-competent TK− virus to induce HSK in TgSCID mice. The virus mutant fails to replicate in ganglionic tissue (14), perhaps implying that virus persists in the eye by spreading to the stroma following replication in the ganglion.

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treatment, however, dramatically reduced lesions in TgSCID mice. This effect was not the result of antiviral activity, since virus persisted in ocular washings and animals succumbed to herpetic encephalitis at the normal time period (data not shown). In rapamycin-treated BALB/c mice, lesions were also reduced in severity (Fig. 3, C and D). These results are consistent with the notion that HSK in TgSCID and BALB/c mice involve different primary mechanisms of CD4⁺/H11001 T cell activation.

FIGURE 3. HSK lesion expression in BALB/c and TgSCID mice treated with cyclosporin A or rapamycin drugs. Groups of TgSCID (n = 4) and BALB/c mice (n = 6) infected with HSV-1 RE (5 × 10⁵ PFU) on scarified corneas were treated with cyclosporin A (100 mg/kg, CsA) or rapamycin (0.2 mg/kg Rap) daily. A and B, TgSCID mice (A) and BALB/c mice (B) were scored clinically for HSK on a score of 0–5, as described in Materials and Methods. Data are plotted for TgSCID mice for days 7, 9, and 12 postinfection as they succumbed to encephalitis by days 10–12 postinfection. For BALB/c and D011.10 mouse, data are plotted for days 7, 9, 12, 15, 18, and 21 postinfection. Results are expressed as mean clinical score ± SD. Data represent one of three experiments with similar results. *, p < 0.5 by Student’s t test between control- and rapamycin-treated TgSCID, and also between control- and rapamycin- or CsA-treated BALB/c at days 15 and 21 postinfection. C and D, TgSCID mice were terminated at days 10–12 postinfection, and BALB/c mice at days 18–21 postinfection, and splenocytes were stimulated with syngeneic APCs pulsed with UV-inactivated HSV (MOI 1.5) or OVA₁₂₃–₃₃₉ (10 µg/ml) in a lymphoproliferation assay (C) or IFN-γ cytokine assay (D). Results are means of three independent experiments. *, p < 0.5 by Student’s t test between groups.

FIGURE 4. Ag-specific or nonspecific activation affects HSK lesion severity in TgSCID mice. Groups of TgSCID (n = 6) were infected with HSV-1 RE (5 × 10⁵ PFU) on scarified corneas. Mice were either treated daily with bioactive CpG 1826 (30.6 µg at days 0, 3, 5, and 7 postinfection) or immunized day 9 before with OVA in CFA (100 µg s.c. at the base of the neck). DLN cells from mice given treatment prior to virus infection were analyzed for activation phenotype by flow cytometry analysis. CpG-treated mice showed 56% of CD44 high cells, and OVA in CFA-treated mice showed 78% CD44 high KJ/H11001 CD4⁺ cells. A, TgSCID mice were scored clinically for HSK on a score of 0–5, as described in Materials and Methods. Data are represented as percentage of mice showing lesions ≥3 at day 11 postinfection. Result represents one of two experiments with similar results. *, p < 0.05 by Wilcoxon rank sum test between untreated and OVA- or CpG-treated groups. B, Percentage survival of mice at different days postinfection. C and D, TgSCID mice were terminated at days 10–12 postinfection, and splenocytes were stimulated with syngeneic APCs pulsed with UV-inactivated HSV (MOI, 1.5) or OVA₁₂₃–₃₃₉ (10 µg/ml) in a lymphoproliferation assay (C) or IFN-γ cytokine assay (D). Results are means of two independent experiments.
Activation requirements for HSK lesion expression

To measure the role of T cell activation, experiments were done to compare the severity of HSK lesions in groups of TgSCID mice that were unmanipulated, with groups immunized previously with OVA peptide in CFA or exposed daily to immunostimulatory CpG motifs. As is evident in Fig. 4A, animals exposed to either activation protocol developed more severe lesions than control-untreated animals. These differences were not striking, but they were significant (Wilcoxon rank sum test). Interestingly, TgSCID mice exposed to activation survived for 1–3 days longer than did the control-untreated animals, most likely the consequence of increased IFN-γ production (Fig. 4, B–D). In separate experiments, both immunized and CpG treatment were shown to result in an increased percentage of KJ7 splenic T cells that expressed the activation phenotype (see legend to Fig. 4).

A second series of experiments compared the efficacy of adoptive transfers of activated vs nonactivated OVA-specific CD4+ T cells at conferring lesions in ocularly infected SCID recipients (Fig. 5A). In this model, major differences were observed. Thus, whereas adoptive transfers of nonactivated cells resulted in mild HSK and many negative responses, the transfer of T cells from Ag-stimulated TgSCID resulted in severe HSK. Moreover, SCID recipients of nonactivated cells also developed more severe HSK lesions if such mice were additionally immunized with OVA peptide in CFA.

Interestingly, if SCID mice were given adoptive transfers of purified OVA-specific CD4+ T cells and recipients were rested for 15 days before infection, such animals developed significantly more severe HSK lesions than did SCID recipients of similar adoptive transfers but immediately infected with virus (Fig. 5B). During the 15 days hiatus, the adoptively transferred cells presumably underwent homeostatic expansion and activation, as described by others (21, 22). In support of this, a decrease in CFSE intensity and an up-regulation of the CD44 marker were seen in CD4+ KJ7 cells in mice terminated at day 20 postadoptive transfer (Fig. 5, C and D).

**FIGURE 5.** Activated OVA-specific T cells are capable of transferring increasing HSK severity in SCID recipients. SCID mice were adoptively transferred with 4–5 × 10^6 KJ7 CD4+ cells, affinity column purified from naive TgSCID mice or cells having undergone in vitro stimulation with OVA\textsubscript{323–339}. A, Mice were infected with HSV-1 RE (5 × 10^5 PFU) on scarified corneas 24 h prior to adoptive transfer. Mice were scored clinically for HSK on a score of 0–5, as described in Materials and Methods. Data are represented as percentage of mice showing lesions ≥3 at day 11 postinfection. Result represents one of several experiments with similar results. *, p < 0.05 by Wilcoxon rank sum test between naive OVA and both activated groups.

B, Mice were infected 24 h before transfer with HSV-1 RE (5 × 10^5 PFU) on scarified corneas or at day 21 postadoptive transfer. Naive BALB/c or HSV-immune CD4+ T cells were used as controls. Mice were scored clinically for HSK on a score of 0–5, as described in Materials and Methods. Result represents one of two experiments with similar results. Control included SCID mice adoptively transferred with naive cells with no parking (SCID:Nv), in vitro activated CD4+ KJ7 cells (SCID:Act), HSV-immune CD4+ T cells from BALB/c mice (SCID:B/c), and SCIDs with no adoptive transfer (SCID:none). Homeostatically activated group of SCID mice denoted by SCID:Homeo. *, p < 0.05 by Wilcoxon rank sum test. C and D, SCID mice were adoptively transferred with 5 × 10^6 KJ7 CD4+ T cells from naive TgSCID mice. A group of mice was terminated at day 20 postadoptive transfer, and cells from DLN and spleen were analyzed for CFSE expression (C) and CD44 expression (D). Data are representative of three mice tested.
Do bystander recruitment and activation contribute to HSK lesions in immunocompetent mice?

Two types of experiments were performed to evaluate whether or not bystander recruitment and activation contribute to HSK lesions in immunocompetent mice. In the first approach, DO11.10 mice were ocularly infected with HSV, and the phenotype of CD4+ T cells was measured in HSK lesions, DLN, and spleen. In such mice, about 70% of the CD4+ cells was measured in HSK lesions, DLN, and spleen. In such mice, indicating that the majority of CD4+ T cells in DO11.10 ocular lesions are KJ+ bystanders reactive with OVA peptide. In fact, an analysis of such cells by flow cytometry revealed that the majority of intraocular KJ+ CD4+ T cells expressed activation markers (Fig. 8). Accordingly, HSK in DO11.10 mice contains a major component of bystander recruitment and activation.

The second approach used to assess the relevance of bystander activation as a contributing mechanism in HSK pathogenesis in immunocompetent animals was to measure the presence and activation status of nonimmune CFSE-labeled DO11.10 cells parked in BALB/c mice undergoing HSK lesions. Analysis of input cells revealed that 69% of cells were KJ+ and 96.4% expressed the CD44highCD62Lhigh naive cell phenotype (see footnote to Table III). Sample animals showing HSK lesions were killed at intervals subsequently to isolate cell infiltrates from the inflamed cornea as well as to determine the phenotype of cells in both DLN, distal lymph nodes, and spleens (Table III). In corneal samples, on day 16 postinfection, 14.7% of the total recovered cells were CD4+ (Fig. 9A and Table III). Of these CD4+ T cells, 10.2% were KJ+ and CFSE+. Moreover, 97.4% of the recruited KJ+ ocular cells expressed the CD44highCD62Lhigh naive cell phenotype (Fig. 9B), yet were nondividing, as indicated by the CFSE data (Fig. 9C). In distal LN and spleen samples, 0.9 and 1.6%, respectively, of the CD4+ cells were KJ+, and such cells did not show the activation phenotype observed with KJ+ cells derived from corneal or DLN samples (Table III).

Discussion

These investigations further define mechanisms that result in immunoinflammatory lesions in the corneal stroma following ocular
infection with HSV. Lesion development was analyzed in immunocompetent mice as well as in TCR Tg mice backcrossed to SCID. Almost all of the T cells in the latter animals were KJ⁺ and reactive with the OVA peptide (10, 11), and mice failed to generate detectable T cell responses to HSV Ags. HSK lesions in both immunocompetent and TgSCID mice appeared identical, yet the principal events responsible for the pathology were shown to differ. Lesion expression in the TgSCID model depended on continuous viral replication, an event most likely necessary to produce high levels of proinflammatory cytokines and chemokines. In such animals, OVA-specific CD4⁺ T cells, especially those with the effector/memory phenotype, invade the cornea and were activated to orchestrate the inflammatory reaction by a non-TCR-mediated mechanism. Thus, lesion severity in TgSCID mice was unaffected by cyclosporin A treatment, a procedure that was highly inhibitory to HSK in immunocompetent animals. Lesions in TgSCID mice were abrogated by rapamycin treatment, a drug whose action largely affects signals induced by cytokine receptor engagement (15–17). The results were interpreted to mean that HSK in TgSCID mice principally involves bystander recruitment and activation mechanisms, whereas lesions in immunocompetent mice involve CD4⁺ T cells recognizing specific Ags in the cornea. However, in immunocompetent mice, the bystander recruitment and activation mechanism was also shown to represent a significant component of HSK lesions.

The evolution of HSK lesions following HSV ocular infection involves numerous events (4, 5). Lesion expression appears strictly dependent on HSV replication in the eye. Thus, infection with replication-defective mutants or UV-inactivated virus, even though immunogenic, fails to cause HSK (24). In immunocompetent mice, the period of viral replication is brief, usually no more than 5 days (24, 25), but as shown in this work, it cannot be condensed to 1 day, since mice infected for only that time prior to antiviral drug treatment failed to develop lesions. In the TgSCID model, however, viral replication for longer periods is necessary to induce HSK. Accordingly, antiviral treatment at day 4 prevented the expression of HSK, although not death from herpetic encephalitis. In other studies, virus removal even as late as day 6 had an inhibitory effect on HSK expression (our unpublished observations). Normally, in untreated animals, virus can be demonstrated in the infected cornea until animals die of encephalitis at about day 11–12 postinfection (11). Of interest, whereas the location of viral Ags in immunocompetent mice was the corneal epithelium, after the first few days in TgSCID mice, viral Ag was demonstrable mainly in the stroma in which the inflammatory reaction occurs (11). More than likely, virus arrives at the stromal site by zosteriform spread from the ganglion, an idea supported by the observation that TK⁻ mutant virus, which cannot replicate in ganglionic neurons (14), failed to access the stroma. Furthermore, the mutant virus also failed to cause lesions in TgSCID mice.

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**FIGURE 8.** Activation markers on KJ⁺ CD4⁺ T cells derived from HSK lesions of DO11.10 mice. DO11.10 mice were infected with HSV-1 RE (5 × 10⁵ PFU) on scarified corneas. At day 15 postinfection, mice were sacrificed and cells were isolated from the ocular tissue and the DLN. Cells were stained with Abs to CD4 and OVA-TCR (KJ) and activation markers CD62L, CD44, CD45RB, CCR5, and CD25. Figure shows activation profiles for cells gated on KJ⁺ CD4⁺ and KJ⁺ CD4⁺ T cells in the ocular tissue and DLN. Data are representative of one of two similar experiments.

**Table II. Phenotypic analysis of CD4⁺ T cells recovered from DO11.10 mice**

<table>
<thead>
<tr>
<th>Site</th>
<th>CD4⁺</th>
<th>CD4⁺+</th>
<th>CD4⁺</th>
<th>CD4⁺+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ocular tissue</td>
<td>0</td>
<td>2.6</td>
<td>5.26</td>
<td>14.64</td>
</tr>
<tr>
<td>DLN</td>
<td>0</td>
<td>0.05</td>
<td>0.16</td>
<td>1.46</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>1.92</td>
<td>3</td>
<td>9.97</td>
</tr>
</tbody>
</table>

* Mice were infected on scarified corneas with 5 × 10⁵ PFU HSV-1 RE. At days 16–18 postinfection, mice were terminated and CD4⁺ T cells from ocular tissue, DLN, and spleen were analyzed for the presence of KJ⁺ or KJ⁻ cells by flow cytomtery analysis. Results are plotted as percentage of cells from total population ± SD from eight mice analyzed.

**Table III. Phenotypic analysis of DO11.10 cells derived from adoptively transferred BALB/c mice**

<table>
<thead>
<tr>
<th>Site</th>
<th>Days Postinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ocular tissue</td>
<td></td>
</tr>
<tr>
<td>CD4⁺</td>
<td>0  2.6  5.26  14.64</td>
</tr>
<tr>
<td>KJ⁺ CD4⁺</td>
<td>0  0.05  0.16  1.46</td>
</tr>
<tr>
<td>KJ⁺ CD4⁺/CD4⁺</td>
<td>0  1.92  3  9.97</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>CD4⁺</td>
<td>22.01 26.2 24.1 23.8 22.6</td>
</tr>
<tr>
<td>KJ⁺ CD4⁺</td>
<td>0.39 0.28 0.4 0.32 0.4</td>
</tr>
<tr>
<td>KJ⁺ CD4⁺/CD4⁺</td>
<td>1.8 1.06 1.6 1.34 1.76</td>
</tr>
<tr>
<td>DLN</td>
<td></td>
</tr>
<tr>
<td>CD4⁺</td>
<td>41.45 40.8 44.21 46.1 44.3</td>
</tr>
<tr>
<td>KJ⁺ CD4⁺</td>
<td>0.49 0.45 0.56 0.62 0.54</td>
</tr>
<tr>
<td>KJ⁺ CD4⁺/CD4⁺</td>
<td>1.25 1.1 1.26 1.34 1.21</td>
</tr>
</tbody>
</table>

* BALB/c mice adoptively transferred with DO11.10 cells were terminated at different days postinfection. Cells were isolated from the ocular tissue, DLN, spleen and the popliteal lymph nodes as described in Materials and Methods. Cells were stained with fluorescein FITC-labeled Abs for CD4⁺ and mAb against OVA TCR KJ. Shown are percentages of CD4⁺ KJ⁺ CD4⁺ cells isolated from each site. * Ratio of KJ⁺ CD4⁺ to total CD4⁺ T cells is represented. Data are representative of two experiments each involving three to four mice per group.
Although yet to be formally proven, it is likely that the stromal virus is responsible for inducing the proinflammatory cytokines and chemokines assumed to play a central role in the pathogenesis of TgSCID lesions. This idea was supported by the results of studies on the inhibitory effects of two types of immunosuppressive drugs. Thus, cyclosporin A inhibited HSK in immunocompetent mice, but had no effect on the severity of lesions in TgSCID mice. Lesions in the latter animals were inhibited by rapamycin, a drug that inhibits the response of T cells to cytokine stimulation (15, 16). Since the effect of cyclosporin A on T cells is directed at those triggered by engagement of the TCR, we interpret our results to imply that T cell activation in TgSCID lesions proceeds by a non-TCR activation mechanism. However, this notion requires further evaluation.

The cells involved in TgSCID HSK would seem to be mainly OVA peptide-specific CD4+ T cells, activated by as yet unidentified chemokines and cytokines. Initially, it was suspected that some HSV protein might contain peptides cross-reactive with OVA. A search of the gene bank, however, found no such homology. In addition, other TCR Tg models backcrossed to RAG-/- were also shown to generate HSK (S. Deshpande, manuscript in preparation). It remains unclear why OVA-specific CD4+ T cells should enter the proinflammatory ocular environment. Experiments reported in this work demonstrate that in fact only cells of the effector phenotype appear to participate in the bystander activation process. Curiously, when comparing the efficacy of adoptive transfers of naive with stimulated KJ’ T cells to SCID mice, the stimulated cells supported more severe lesions. In fact, recipients of naive KJ’ cells developed trivial lesions, unless recipient animals were also immunized with OVA peptide in adjuvant. Interestingly, however, KJ’ cells could be induced to express the eye-seeking phenotype in at least two additional ways. One was to expose mice to immunostimulatory CpG motifs. This procedure generated significantly increased numbers of KJ’ cells with the effector phenotype, most likely a consequence of CpG motif-induced cytokine production by APCs.

Perhaps of even more interest was the observation that SCID mice given adoptive transfers of naive KJ’ cells, then rested for 2–3 wk before HSV ocular infection, readily developed HSK. It was recently recognized in such circumstances that naive T cells undergo homeostatic expansion and adopt the phenotype of memory cells (21, 22). They have been referred to as false memory cells (21, 22). Our observations that such cells appear able to mediate immunopathological reactions are, we believe, novel.

The observation that bystander activation can be the principal mechanism of immunopathology is made less interesting by the fact that the model used to show such an effect is unrepresentative of naturally occurring circumstances. However, bystander activation and recruitment were also shown to contribute a significant component of HSK lesions in normal immunocompetent animals. For example, the majority of T cells in HSK lesions of immunocompetent DO11.10 mice were in fact KJ’ cells unreactive with HSV Ags. In such animals, the 30% of CD4+ T cells that are KJ’ account principally for the reactivity to HSV Ags. However, such cells were barely more represented in ocular lesions than they were in unstimulated lymphoid tissue. We conclude from such observations that the bulk of the activated CD4+ T cells in the DO11.10 model are in fact bystanders.

Similarly, bystander recruitment and activation were implicated to occur in BALB/c mice that possessed adoptive transfers of KJ’ CFSE-labeled cells. When such animals were infected to induce HSK, at least 10% of cells were bystanders derived from the adoptive transfers. Presumably, many KJ’ CD4+ cells from the host itself were also bystanders. Both sets of recruited CD4+ T cells were expected to help orchestrate the inflammatory reaction, most likely by reacting with activating cytokines. However, in the immunocompetent animal model, the source of the activating cytokines is likely to differ, since virus does not continue to replicate in such animals as it does in TgSCID mice. In immunocompetent animals, the T cells themselves, reacting to the so far unidentified Ags, are the likely sources of cytokines.

The ultimate relevance of understanding the inflammatory mechanisms at play during HSK should impact on the choice of treatment. Accordingly, there seems little point in using antiviral drugs since virus is rarely present after the early phase, except in immunocompromised animals. Currently, in humans, corticosteroids are a popular treatment choice (26). However, a combination of other drugs such as cyclosporin and rapamycin would seem to deserve a trial. Indeed, recently, it was reported that cyclosporin A is useful for the control of human HSK (27).

**Acknowledgments**

We express our thanks for the help of Teresa Sobhani.

**References**


**FIGURE 9.** Role of bystander activation in HSK expression in immunocompetent BALB/c mice. Naive DO11.10 CD4+ T cells (10⁵) were adoptively transferred into naive BALB/c recipients. Cells were purified following positive selection on affinity column, and the cells transferred consisted of 69% KJ’ CD4+, of which 96.4% were CD44low and Lselhigh. One group of BALB/c mice was infected with HSV-1 RE (10⁶ PFU) on scarified corneas, and one was mock infected. Mice were sacrificed at different time points postinfection, and cells were isolated from ocular tissue and DLN. Data are represented for day 16 postinfection. A, KJ’ and CD4 expression in ocular tissue of infected and uninfected mice and DLN of infected mouse. B, Activation phenotype of cells gated for KJ’ CD4+. C, CFSE labeling in KJ’ CD4+ cells in adoptively transferred with mice infected and mock infected.

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