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Virus-Induced Immunoinflammatory Lesions in the Absence of Viral Antigen Recognition

Shivapракаш Gangappa,* John Sam Babu,† Johnson Thomas,* Massoud Daheshia,* and Barry T. Rouse2*

Herpes simplex keratitis (HSK) is a CD4+ T cell-controlled immunopathologic lesion in the eye that results from infection with herpes simplex virus (HSV). Target Ags involved in HSK remain undefined. In this study, we determined if HSK could be induced in animals genetically incapable of generating HSV Ag-specific CD4+ T cells. Mice bearing transgenic TCR specific to OVA peptide 323–339 (DO11.10) were crossed to SCID mice whose offspring (Tg·SCID) possessed CD4+ T cells, >98% of which expressed the OVA peptide-specific TCR. HSV infection of Tg·SCID mice was lethal, and mice failed to generate detectable T cell responses even after repeated immunization with a mutant avirulent virus (AN-1). Immunization with AN-1 virus followed by ocular challenge with HSV resulted in ocular inflammation before encephalitis, in contrast to the protection conferred in the control BALB/c and DO11.10 mice. These results indicate that clinical HSK may not require viral Ag recognition by CD4+ T cells and that T cells of irrelevant specificity can be recruited, activated, and driven into effector function in the HSV-infected cornea. This is suggested to represent a bystander activation effect resulting from the presence of proinflammatory mediators resulting from HSV replication. The Journal of Immunology, 1998, 161: 4289–4300.

Material and Methods

Mice

Four- to five-week-old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN), OVA-TCR-transgenic mice (DO11.10 mice, kindly provided by Dr. Dennis Loh, Washington University School of Medicine, St. Louis, MO and Dr. Casey Weaver, University of Alabama, Birmingham, AL) were used.
were used. Transgenic mice were crossed to SCID mice for two generations, and, with brother-sister mating, it was possible to obtain Tg-SCID mice (as screened by PCR-tail DNA and serum IgM-ELISA). Tg-SCID mice thus obtained had only CD4+ T cells and few or no CD8+ T cells, as tested by FACS analysis, and were without any B cells, as screened by PCR.

Research in Vision and Ophthalmology (ARVO) resolution on the use and care of laboratory animals. The facilities used were accredited by the American Association for Accreditation of Laboratory Animal Care. All experimental procedures were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use and care of laboratory animals.

**Virus**

HSV-1 RE strain was propagated on vero cells and stored as infectious cell preparations at −70°C. Replication-defective virus AN-1 was kindly provided by Dr. Sandra K. Weller, University of Connecticut Health Center, Farmington, CT.

**Corneal infections, immunization, and passive transfer**

In each experiment, control eyes were sacrificed and treated with 4 μl of PBS or tissue culture extracts. The corneal surfaces of deeply anesthetized mice (methoxy flurane; Pittman-Moore, Mondelein, IL) were scarified with a sterile 27-gauge needle, and 5 × 10^5 TCID-50 HSV-1 RE strain was applied in a 4.0-μl volume and gently massaged onto the eye lids. For immunization with AN-1 virus or UV-inactivated HSV-1, prophylactic treatment of sulfatrim pediatric suspensions (Barre-National, Baltimore, MD) at the rate of 5 ml per 200 ml of drinking water. Antibiotic treatment was started 1 day before the beginning of the experiments. All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animal Resources, Commission Life Sciences, National Research Council. The facilities used were accredited by the American Association for Accreditation of Laboratory Animal Care. All oculocutaneous ulcers, and the results shown here represent one of the three experiments performed.

**Clinical evaluation**

Mice were scored according to their clinical severity by a person who was blinded to the experimental design using a slit lamp biomicroscope (Keeler Instruments, Bromall, PA) as follows: score 0, normal cornea; score 1, neovascularization at periphery, iris visible; score 2, partial opacity, iris not visible; score 3, neovascularization at center, opacity; score 4, bleb formation; score 5, necrosis. The data were plotted as the mean daily clinical score for all animals in a particular treatment group.

**Ig ELISA**

Serum collected was analyzed for HSV-specific total IgG, using standard ELISA as described previously (14). Basically, ELISA plates were coated with 100 μl of HSV Ag in carbonate buffer (pH 9.8), and, after overnight incubation at 40°C, the plates were washed three times in PBS containing 0.05% Tween 20, pH 7.2 (PBST), and then blocked using PBS (pH 7.2) with 3% dehydrated milk for 2 h at 37°C. A total of 200 μl of serially diluted serum samples (prediluted in PBST) were added in duplicate, and washed wells coated with goat anti-mouse IgG (0.25 μg/ml) were treated with serially diluted standard mouse IgG. Plates were incubated for 2 h at 37°C. After three washes, 100 μl of goat anti-mouse IgG horseradish peroxidase was added. After three washes, 2,2-azino-bis-3-ethyl-thiazolium salt (Sigma, St. Louis, MO, No. A1888) was added. The concentration of the Abs in the serum samples was determined from the standard curve.

**Cytokine assay**

For cytokine (IFN-γ) assay, splenocytes from mice were suspended in 10% RPMI 1640, and 10^5 cells in 1 ml were stimulated in vitro with 1.5 MOI (multiplicity of infection, before inactivation) of UV-inactivated HSV-1 (KOS strain). Similar number of cells were Con A stimulated (5 μg/10^5 cells/ml) in 12-well culture plates. Plates were incubated at 37°C for 72 h. The supernatant fluid was collected and stored at −20°C until use. These supernatants were screened for the presence of IFN-γ by ELISA as described previously (14).

**Histopathology**

Eleven days after infection, sections of eye were prepared for histopathology according to standard procedures. Briefly, at the termination of experiments, whole eyes were fixed in 10% buffered neutral formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Sections were observed for thickness of cornea, presence of inflammatory infiltrates, neovascularization, epithelial erosions, superficial or deep ulcers, and corneal perforation.

**Proliferation assay**

Splenocytes (responders) at day 11 p.i. were collected and restimulated in vitro with HSV-1 KOS (MOI of 1.5 before UV irradiation) or OVA peptide (Research Genetics, Huntsville, AL). The HSV-1-specific lymphoproliferative response was measured as described previously (15). Briefly, responders: stimulator ratios tested ranged from 10:1 to 0.625:1. Responders plus stimulator mixtures were incubated at 37°C for 4 days. [3H]Thymidine (1.0 μCi/well) was added, harvested 18 h later, and read using a beta scintillation counter (Trace 96, Inotech, Lansing, MI). The results were expressed as mean plus SD.

**RNA isolation and RT-PCR**

On day 11 p.i., splenocytes and cornea were collected and transferred to Tri-reagent (Molecular Biology, Cincinnati, OH). The corneas were teased with a sterile 21-gauge needle and titrated with a sterile 1-ml syringe plunger to expose the cells to lysis action of the Tri-reagent. The total cellular RNA was isolated from the Tri-reagent cellular lysate by adding chloroform, and centrifugation followed by ethanol/isopropanol precipitation of the aqueous RNA solution according to the manufacturer’s instructions. The RNA thus obtained was reverse transcribed using oligo(dT) primers and super script (Life Technologies, Bethesda, MD) according to standard protocol (16). The cDNA thus obtained was used as a template in subsequent qualitative and quantitative PCRs. Primers for different Vα1, Vα2, Vα5, Vα7, Vα13.1 expression in Tg-SCID and BALB/c samples. For transgenic TCR-positive T cells, KJ-1-26.1 (anti-OVA TCR Ab), a kind gift from Dr. Phillipa Marrack, National Jewish Hospital, Denver, CO) Ab was used and detected by adding goat anti-mouse IgG2a-FITC Ab. To

**Flow cytometric analysis**

Isolated cervical lymph node cell populations were analyzed for cell surface markers by flow cytometry. Viable cells were blocked with heat-inactivated FBS and washed three times with FACS buffer (1× PBS with 1% BSA and 0.05% NaN₃). For Tg-SCID mice characterization, cells from cervical lymph nodes were stained with anti-CD4-FITC and anti-CD8-PE. For transgenic TCR-positive T cells, KJ-1-26.1 (anti-OVA TCR Ab), a kind gift from Dr. Phillipa Marrack, National Jewish Hospital, Denver, CO) Ab was used and detected by adding goat anti-mouse IgG2a-FITC Ab. To
check for activation markers on CD4⁺ T cells, lymph node cells were blocked with FBS and double stained with FITC or PE-labeled anti-CD4 and anti-CD45 RB-PE or anti-L-selectin-PE. Dual Vα TCR analysis was done using TCR Vα2-PE and KJ1-26.1 Ab. Events were collected and analyzed using a Becton Dickinson (Mountain View, CA) FACScan analyzer.

**Immunohistochemical staining**

At the termination of experiments, eyes were nucleated and snap frozen in OCT compound (Miles, Elkhart, IN). Eight-micron-thick sections were cut, air dried, and fixed in cold acetone for 5 min. The sections were then blocked with heat-inactivated goat serum and stained with biotinylated anti-CD4, anti-Thy1.2 (PharMingen, San Diego, CA) or biotinylated KJ1-26.1 Ab (biotinylated anti-OVA TCR, a kind gift from Dr. Jerold Woodward, University of Kentucky, Lexington, KY). Sections were then treated with horseradish peroxidase-conjugated streptavidin (1:1000) and 3,3'-diaminobenzidine (Vector, Burlingame, CA) and counterstained with hematoxylin. For OVA-TCR (KJ1-26.1) staining, sections were also treated with a tyramide signal amplification kit (TSA Indirect, Dupont NEN, Boston, MA) before treatment with diaminobenzidine.

**Delayed-type hypersensitivity (DTH) assays**

On the day of testing, each mouse was injected with 20 μl of 10^5 UV-inactivated HSV-1 KOS (10^6 pfu before inactivation) in the right ear and the same volume of vero cell extract in the left ear. The right and left ear thicknesses of each mouse were measured with a screw gauge Odimeter (Oditest, H. C. Kroeplin, Schluechtern, Germany) and recorded individually. The thickness was measured at 1 h before ear injection, then at 24, 48, and 72 h after injections, and the values were represented as n × mm². The mean ear thickness of each ear from each group of animals was calculated, and the mean increase between before and 48 h after injections was compared among BALB/c mice, DO11.10 mice, and Tg-SCID mice.

**Virus recovery**

At various time points p.i., eye swabs were taken using sterile swabs soaked in McCoy medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY). The swabs were then placed in tubes containing 800 μl of the above mentioned transport medium and stored at −80°C. For detection of HSV in swabs, the samples were thawed and vortexed, and 100 μl of each sample from individually marked mice was used for quantification of virus recovery by standard pfu assay on vero cell cultures as described elsewhere (18).

**Statistical analysis**

Wherever specified, data obtained were analyzed for statistical significance by Student’s t test.
Results

Stromal keratitis in BALB/c and DO11.10 mice

As reported previously, infection of the scratched cornea of BALB/c mice with HSV-1 RE is followed after 8 to 10 days by the development of a clinically evident inflammatory response in the cornea (19). Typically, responses are at their peak around 3 wk p.i., and they may persist for several weeks. A similar response profile is recorded in Figure 1. In addition, Figure 1 shows the response pattern that occurred in DO11.10 mice. It is apparent that the clinical pattern was much the same. Sample BALB/c and DO11.10 mice were killed at 14 days p.i., and their eyes were examined histologically and additionally analyzed by immunohistochemistry for the presence of CD4 Te T cells (Fig. 2). Essentially no differences were observed between the two animal strains.

Initially, we had expected that the DO11.10 mice might fail to develop HSK since their limited TCR repertoire might compromise their development of an immune response to the virus. Accordingly, if HSK was the consequence of a CD4 Te T cell antiviral response, lesions would be absent in TCR-transgenic mice. However, as recorded in Table I, the DO11.10 mice responded immunologically to HSV and developed both T cell and Ab responses. Moreover, the DO11.10 mice recovered normally from infection and did not succumb to viral encephalitis. The explanation for such patent anti-HSV responses and protective immunity was presumably because of the extensive TCR a-chain leakage evident in the DO11.10 mouse (17). Thus, as shown in Figure 3, the DO11.10 mice expressed most of the TCR a-chains expressed by BALB/c at least as detected by RT-PCR for a- and b-chain mRNA. Quantitative comparisons of a- and b-chain expression in transgenic and BALB/c animals were not made.

HSK in Tg-SCID mice

In an attempt to reduce or perhaps eliminate TCR a-chain leakage in the transgenic mice, DO11.10 mice were backcrossed to SCIDs, and the offspring were selected that were TCR Tg1 and SCID1 (Tg-SCID). Such mice were shown by FACS analysis to possess T cells, all of which were CD4 Te and expressed the KJ1-26.1 Id marker (Fig. 4). The mice possessed no B cells and failed to generate IgM Ab to environmental Ags (data not shown). These Tg-SCID mice were highly susceptible to HSV infection and rapidly succumbed to encephalitis following ocular infection with HSV-1 RE at 4 to 5 wk of age. Animals infected via the cornea at 7 to 8 wk of age survived for as long as 13 days before dying from lethal encephalitis. Notably, however, most animals of this age groups

Table I. Immune responses in HSV-RE-infected BALB/c mice and DO11.10 mice

<table>
<thead>
<tr>
<th></th>
<th>Ag-Specific Proliferation (cpm)</th>
<th>OVA stimulators</th>
<th>HSV-Specific IgG (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mice</td>
<td>Responders</td>
<td>Responders</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ uninfected</td>
<td>+ HSV stimulators</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Responders</td>
<td>Responders</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>200 ± 99</td>
<td>155 ± 60</td>
</tr>
<tr>
<td></td>
<td>DO11.10</td>
<td>116 ± 20</td>
<td>43 ± 20</td>
</tr>
</tbody>
</table>

Ag-specific proliferation in HSV-1 RE-infected BALB/c and DO11.10 mice. Five mice/group were individually assayed for proliferation in response to UV-HSV and OVA peptide (323-339) by incorporation of 3H. The values represent the mean ± SD.

SI, stimulation index.

Serum IgG were measure by standard capture ELISA assay with five mice per group. The values represent the mean ± SD.

FIGURE 3. TCR Va and Vb analysis in splenocytes of DO11.10 and BALB/c mice. Total RNA was isolated from the splenocytes and reverse transcribed as described in Materials and Methods. Equal amounts of cDNA were used for analysis of different Va (A) and Vb (B) using specific primers corresponding to variable regions along with a unique primer corresponding to either constant region of Coa or Cb.
expressed mild ocular lesions (clinical score 3 at day 10) that were approximately equal to those observed in BALB/c and DO11.10 mice at the same time frame (Fig. 5). Analysis of lesions by histopathology as well as for the presence of CD4 T cells by immunohistochemistry revealed a situation comparable to that observed in BALB/c mice (Fig. 6). In addition, the T cells in the cornea were clonotype positive (KJ1-26.1). Accordingly, we judge that the Tg-SCID mice had lesions of HSK, although all animals died of HSV encephalitis within 4 to 5 days of their lesions being manifest. Control SCIDs lacking T cells failed to express lesions when examined around day 10 p.i. (Fig. 6) and also died of encephalitis by 14 days. Analysis of virus recovery from the infected eyes of Tg-SCID (Table II) revealed that infectious virus was demonstrable up to day 11 whereas, in the BALB/c mice, infectious virus was not demonstrable beyond 6 days p.i. In another set of experiments to determine whether the disease was indeed a result of HSV replication, Tg-SCID mice were infected with HSV and, after 24 h, given anti-HSV antiserum. This procedure not only protected mice from death by encephalitis, but also, as shown previously by Pepose and colleagues (20), prevented the development of primary HSK. These data are shown in Figure 7, as are the results in Tg-SCID mice that received normal mouse serum. The latter animals developed HSK and died of encephalitis.

**FIGURE 4.** Flow cytometric analysis of draining lymph node cells for CD4+ T cells, CD8+ T cells, and CD4+ KJ1-26.1+ T cells from BALB/c mice (left column), DO11.10 mice (middle column), and Tg-SCID mice (right column). Numbers indicate relative percentages of cells within a quadrant.

**FIGURE 5.** Clinical score from HSV-1 RE-infected BALB/c, DO11.10, and Tg-SCID mice on day 6 (left panel), day 8 (middle panel), and day 11 (right panel). Each dot represents the value for an individual mouse, and the horizontal line represents the mean clinical score for the group. Each group of mice consisted of six animals, and the results shown here represent one of the three experiments performed.
Immune responsiveness of Tg-SCID mice

The fact that Tg-SCID mice could express HSK, even though they failed to survive HSV infection, could mean that they too had sufficient Vα-chain leakage and developed anti-HSV CD4+ T cells that in turn orchestrated the ocular inflammatory response. Alternatively, it could be that the HSK responses in the Tg-SCID mice were the consequence of a nonvirus-specific, perhaps autoreactive, response, set off in the cornea as a consequence of HSV infection.

Following HSV ocular infection, evidence was sought for lymphoid expansion in Tg-SCID mice. Mice with HSK had enlarged and more cellular cervical lymph nodes (Table III) and, upon analysis by FACS for activation markers, showed a higher percentage of CD4+ T cells expressing the activated phenotype, i.e., CD45RB low and L-selectin low, than cells from uninfected mice (Fig. 8).

Table II. Duration of the presence of infectious virus in the eye swabs following ocular HSV-1 RE infection

<table>
<thead>
<tr>
<th>Mice</th>
<th>Days Postinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>BALB/c</td>
<td>$0.38 \times 10^5$</td>
</tr>
<tr>
<td>Tg-SCID</td>
<td>$3 \times 10^3$</td>
</tr>
</tbody>
</table>

HSV-1 RE ($5 \times 10^5$ pfu) was inoculated on the scarified cornea, and at the indicated times the presence of infectious virus in eye swabs was determined by the agarose overlay method. The number of pfu/ml was estimated. At each time point, eye swabs were collected from four mice (n = 4), and viral titers were determined.

² pfu/ml, plaque-forming unit per ml.
³ UD, undetectable, i.e., below the sensitivity of the assay (< 10 pfu).
spleens were tested for HSV-specific lymphoproliferation and cytokine secretion. As controls, infected BALB/c and DO11.10 splenocytes were tested at the same time p.i. The results shown in Table IV indicate that the Tg-SCID mice failed to respond to HSV, but they did respond to OVA stimulation. In addition, mice were infected in the food pad with UV inactivated HSV-1 KOS and tested 2 wk later for HSV-1-specific DTH response. Such DTH responses were lacking in Tg-SCID mice but were present in similarly immunized BALB/c control mice (Table V).

To further evaluate the HSV-specific immune competence of Tg-SCID mice, 5- to 6-wk-old mice were immunized with a replication-competent but package-defective HSV mutant (AN-1) virus, known to induce excellent immune responses in BALB/c mice, but neither lethal nor in fact capable of inducing HSK lesions (21). Animals were immunized two times at 14-day intervals, and, after a further 8 days, animals were sacrificed and their spleens were tested for HSV-specific T cell immunity. It is evident from Table VI that the Tg-SCID mice still failed to develop HSV-specific immunity detectable either in vivo by DTH or by in vitro Ag stimulation of their splenocytes. Further attesting to the failure of such AN-1-immunized mice to generate immune responses to HSV was the fact that the mice still succumbed to encephalitis upon ocular infection with HSV-1 RE. Before death, however, the immunized and ocularly infected mice all developed HSK lesions (Table VII).

**Repertoire expression and evaluation of dual TCR-containing T cells in Tg-SCID mice**

The above results indicate that Tg-SCID mice fail to recognize HSV Ags, yet such animals still expressed HSK lesions. Virus infection did result in an increase in T cell activation, but the issue arose as to how the repertoire-restricted Tg-SCID T cells could mediate HSK. One possibility was that Tg-SCID T cells showed Vα or Vβ leakage and that such dual Vα TCR-1 T cells recognized corneal Ags and mediated HSK. Evidence for Vα segment leakage was searched for by RT-PCR using splenic RNA collected from both uninfected and HSV-infected Tg-SCID mice. As is apparent in Figure 9 and Table VIII, evidence for Vα (but not Vβ) leakage was present in splenic samples in both groups of animals. Interestingly, examination of individual animals showed that all of them expressed Vα1, Vα2, Vα5, and Vα11 in addition to the Vα13.1 transgene. We obtained no evidence that HSV infection changed the nature of the Vα leakage, but the expression level appeared increased in samples from HSV-infected Tg-SCIDs (data not shown). By semiquantitative PCR, Vα2 showed the most leakage of the four extra Vα segments expressed. Evidence for cells in the draining lymph node of HSV-infected mice that expressed surface Vα2 in addition to Vα13.1 was sought for by FACS analysis. Such dual Vα-positive T cells were present in DO11.10 samples

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**Table III.** Cell count from cervical lymph nodes of BALB/c and Tg-SCID mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Naive (number of cells/ml)</th>
<th>HSV immune (number of cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>2.2 × 10⁶</td>
<td>7.3 × 10⁶</td>
</tr>
<tr>
<td>Tg-SCID</td>
<td>1.0 × 10⁶</td>
<td>2.3 × 10⁶</td>
</tr>
</tbody>
</table>

HSV-1 RE (5 × 10⁵) was inoculated on scarified cornea of mice, and, on day 10, draining lymph node cells were counted by trypan blue exclusion method. Data represent one of the four analyses performed.

---

**FIGURE 7.** Clinical score from HSV-1 RE-infected Tg-SCID mice and DO11.10 mice on day 6 (left panel), day 8 (middle panel), and day 11 (right panel). Tg-SCID mice were given either anti-HSV serum or naive serum i.v., 24 h p.i. Each dot represents the value for an individual mouse, and the horizontal line represents the mean clinical score for the group. Each group of mice consisted of five animals, and the results shown here represent one of the two independent experiments.

**FIGURE 8.** Phenotypic analysis of draining lymph node cells from naive and HSV-infected mice. Cervical lymph node cells from BALB/c (upper panel) and Tg-SCID (lower panel) mice were stained with anti-CD4 FITC in combination with either anti-CD45RB PE or anti-L-selectin PE Abs and analyzed by flow cytometry. Bold line, profile of the naive mice; shaded area, profile of the HSV-infected mice; dotted line, isotype Ab control. Data shown are representative of four experiments.
and OVA peptide (323-339) by incorporation of 3H. The values (expressed as counts per minute (cpm)) represent the mean ± SD. The data presented in this report, however, indicate that, whereas HSIs results from HSV infection and requires the presence of CD4+ T cells, the lesion occurs regularly in animals in which T cell response to HSV Ags could not be demonstrated. Our results are interpreted to support the notion that effector CD4+ T cells, perhaps of any specificity, entering the proinflammatory environment of the HSV-infected cornea may receive bystander activation and further contribute to the orchestration of an immunoinflammatory lesion. We consider an alternative idea that T cells of Tg-SCID mice cross-react with an HSV-induced autoantigen in the eye as less likely, given the marked repertoire handicap of Tg-SCID mice.

(but the 2.9% value is likely not significant) but were not detectable in either Tg-SCID or BALB/c samples (Fig. 10). Furthermore, perhaps arguing against a pathogenic role for Vero dual-positive cells in mediating HSIs, we were unable to find evidence for leaked Vα expression in corneal samples from Tg-SCID mice with early HSIs (Fig. 11).

**Discussion**

This report analyzes the pathogenesis of the ocular inflammatory lesion, herpetic stromal keratitis, a sequel to corneal infection with HSV and an important vision-imparing lesion in mankind. HSIs is a CD4+ T cell-dependent immunoinflammatory process presumed to involve recognition of HSV-derived Ags (13). The data presented in this report, however, indicate that, whereas HSIs results from HSV infection and requires the presence of CD4+ T cells, the lesion occurs regularly in animals in which T cell response to HSV Ags could not be demonstrated. Our results are interpreted to support the notion that effector CD4+ T cells, perhaps of any specificity, entering the proinflammatory environment of the HSV-infected cornea may receive bystander activation and further contribute to the orchestration of an immunoinflammatory lesion. We consider an alternative idea that T cells of Tg-SCID mice cross-react with an HSV-induced autoantigen in the eye as less likely, given the marked repertoire handicap of Tg-SCID mice.

**Table IV. Immune responses in HSV-RE-infected BALB/c mice and DO11.10 mice, and Tg-SCID mice**

<table>
<thead>
<tr>
<th>Ag-Specific Proliferation (cpm)*</th>
<th>HSV stimulators</th>
<th>OVA stimulators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responders + uninfected stimulators</td>
<td>Responders + HSV stimulators</td>
</tr>
<tr>
<td>BALB/c</td>
<td>104 ± 47</td>
<td>62 ± 19</td>
</tr>
<tr>
<td>DO11.10</td>
<td>88 ± 54</td>
<td>33 ± 08</td>
</tr>
<tr>
<td>Tg-SCID</td>
<td>63 ± 18</td>
<td>39 ± 11</td>
</tr>
</tbody>
</table>

* Ag-specific proliferation in HSV-1 RE-infected BALB/c, DO11.10, and Tg-SCID mice. Five mice/group were individually assayed for proliferation in response to UV-HSV and OVA peptide (323-339) by incorporation of 3H. The values (expressed as counts per minute (cpm)) represent the mean ± SD.

**Table V. DTH responses in HSV-infected BALB/c and Tg-SCID mice**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Immunized with</th>
<th>Left ear (vero extract)</th>
<th>Right ear (UV HSV-1 KOS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg-SCID</td>
<td>HSV-1 KOS</td>
<td>2.3 ± 0.5*</td>
<td>2.8 ± 2.1*</td>
</tr>
<tr>
<td>Tg-SCID</td>
<td>PBS</td>
<td>2.8 ± 1.3</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>BALB/c</td>
<td>HSV-1 KOS</td>
<td>1.8 ± 1.0</td>
<td>13.5 ± 1.3*</td>
</tr>
<tr>
<td>BALB/c</td>
<td>PBS</td>
<td>2.2 ± 1.0</td>
<td>2.0 ± 1.1</td>
</tr>
</tbody>
</table>

* BALB/c or Tg-SCID mice were immunized with 1 × 10^6 UV-inactivated HSV-1 KOS virus in the hind foot pads. Mice received PBS (pH 7.2) served as the negative control. Two weeks after immunization, mice were challenged with 20 μl of 1 × 10^6 UV HSV-1 KOS in the right ear pinna, and the same volume of vero extract was injected into the left ear pinna. Results shown above represent the mean increase in ear thickness after challenge. Values (expressed as n × mm -1) represent the mean ± SD.

**Table VI. HSV-induced Ag-specific proliferative responses of splenocytes and DTH responses of mice immunized with HSV mutant (AN-1)**

<table>
<thead>
<tr>
<th>Ag-Specific Proliferation (cpm)*</th>
<th>HSV stimulators</th>
<th>OVA stimulators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responders + uninfected stimulators</td>
<td>Responders + HSV stimulators</td>
</tr>
<tr>
<td>BALB/c</td>
<td>29,463 ± 5,615*</td>
<td>2,908 ± 1,125</td>
</tr>
<tr>
<td>DO11.10</td>
<td>19,029 ± 6,805*</td>
<td>941 ± 534</td>
</tr>
<tr>
<td>Tg-SCID</td>
<td>394 ± 89*</td>
<td>387 ± 84</td>
</tr>
</tbody>
</table>

* Mice were immunized with AN-1 virus (5 × 10^5 TCID50) through foot pad injection and were boosted with the same dose of virus on day 14. On day 35 p.i., splenocytes were individually assayed for Ag-specific proliferation as described in Materials and Methods. The values (expressed as counts per minute (cpm)) represent the mean ± SD.

**Table VII.**

<table>
<thead>
<tr>
<th>Mice</th>
<th>HSV stimulators</th>
<th>OVA stimulators</th>
<th>DTH Response (mm -2)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responders + uninfected stimulators</td>
<td>Responders + HSV stimulators</td>
<td>Uninfected stimulators</td>
</tr>
<tr>
<td>BALB/c</td>
<td>29,463 ± 5,615*</td>
<td>2,908 ± 1,125</td>
<td>10</td>
</tr>
<tr>
<td>DO11.10</td>
<td>19,029 ± 6,805*</td>
<td>941 ± 534</td>
<td>20</td>
</tr>
<tr>
<td>Tg-SCID</td>
<td>394 ± 89*</td>
<td>387 ± 84</td>
<td>1</td>
</tr>
</tbody>
</table>

* Mice were immunized with AN-1 virus (5 × 10^5 TCID50) through foot pad injection and were boosted with the same dose of virus on day 14. On day 35 p.i., splenocytes were individually assayed for Ag-specific proliferation as described in Materials and Methods. Results shown above represent the mean increase in ear thickness after challenge. Values (expressed as n × mm -1) represent the mean ± SD.

By Student’s t test: c vs e, p < 0.00004; d vs e, p < 0.001; f vs h, p < 0.001; g vs h, p < 0.003.
responding neutrophil response (9, 13). The host Ag target hypothesis receives strongest support by studies of Avery et al. (12), who investigated the basis for the marked differences in susceptibility between two congenic mouse strains. Their observations were interpreted to indicate that HSV infection caused the availability of a corneal Ag that cross-reacted with the IgG2a isotype of Ig. Other more indirect lines of evidence supporting a role for autoantigens in HSK include the observance of antiself peptide-reactive T cells following HSV infection and the adoptive transfer of HSK to infected SCID mice with an autoreactive cell line (Ref. 25; S. J. Babu and B. T. Rouse, manuscript in preparation).

Our observation that viral Ag-unreactive Tg-SCID mice still develop HSK could be explained by their T cells recognizing non-viral Ags, perhaps even host-derived autoantigens expressed as a consequence of virus replication in the cornea. Corneal Langerhans cells presenting nonviral autoantigens that may be cross-reactive to the OVA-specific TCR may present such a possibility. Although we cannot formally reject the autoantigen hypothesis, we consider the explanation as unlikely. Thus, it is difficult to accept the idea that a T cell repertoire-handicapped mouse would still be able to recognize an autoantigen but not one or more of the abundant Ags derived from a virus that encodes more than 70 proteins (26). Alternatively, some viral or virus-induced self proteins could act as a superantigen stimulating CD4+ T cells via binding to Vβ region of TCR (27). We, in fact, observed a mild lymphocytosis in mice following HSV infection and showed that lymphocytes expressing activation markers were more abundant in infected mice. HSV itself is not known to express superantigen activity, so the explanation for the lymphocyte stimulation remains unknown. A possible source of stimulation is host heat shock proteins (HSP), some of which are up-regulated as a sequel to HSV infection (28). There is some evidence that host HSPs may act as Ags in certain autoimmune diseases and that some of them may act as polyclonal activators (29, 30). Conceivably, T cells expressing more than a single TCR could exhibit loose recognition characteristics that include virus-induced HSPs. Indeed some have advocated that T cells with multiple Vα expression could be involved

| Mice \n| \n| (n = 4) | HSV-Specific IgGb (ng/ml) | Mean Clinical Scorec | HSV-infected stimulators | Uninfected stimulators | OVA stimulators | Uninfected stimulators |
|---|---|---|---|---|---|---|
| BALB/c | 16,050 ± 1,350 | 0.8 | 32,877 ± 5,018d | 1,763 ± 694 | 19 | 1,856 ± 90 | 1,430 ± 596 | 1 |
| DO11.10 | 12,525 ± 1,878 | 0 | 13,182 ± 3,256e | 2,719 ± 1,452 | 5 | 27,907 ± 9,175 | 1,291 ± 293 | 22 |
| Tg-SCID | UD | 2.5 | 76 ± 2f | 110 ± 49 | 1 | 8,746 ± 5,842 | 122 ± 66 | 72 |

*On day 11 postchallenge, mice splenocytes were individually assayed for Ag-specific proliferation as described in Materials and Methods. The values (expressed as counts per minute (cpm)) represent the mean ± SD.

*On day 14 p.i., serum IgG were measured by standard capture ELISA assay as described in Materials and Methods. The values (expressed as ng/ml) represent the mean ± SD. UD, undetectable, i.e., below the sensitivity of the assay (100 pg/ml).

*Values represent mean clinical score of mice on day 11 postchallenge with HSV-1RE.

*By Student’s t test: d vs f, p < 0.0003; e vs f, p < 0.002.

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**FIGURE 9.** Vα expression in BALB/c and Tg-SCID mice splenic T cells. Total RNA was isolated from splenocytes and reverse transcribed as described in Materials and Methods. The nomenclature of Vα and the Cα primer used for amplification are as mentioned in Reference 17. Upper panel, Lanes 1-13: Marker (100-bp DNA ladder), Vα1, Vα2, Vα3, Vα4, Vα8, Vα10, Vα11, Vα12, Vα13.1, and Vα34.S-281, negative control. Lower panel, Lanes 1-11: Marker (100-bp DNA ladder), Vα1, Vα2, Vα3, Vα4, Vα8, Vα10, Vα11, Vα12, Vα13.1, and Vα34.S-281. Vα6, Vα7, and Vα9 were also analyzed and found to be not expressed in BALB/c. Therefore, they were omitted in reanalysis. Not shown here are the data for Vβ analysis in BALB/c and Tg-SCID splenocytes and the data for extent of Vα expression in HSV-infected BALB/c and Tg-SCID mice splenocytes.

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**Table VIII.** Quantitative comparison of leaky Vα chain in Tg-SCID samples with BALB/c splenic T cells

| Vα | % Expression Levels |
|---|---|---|
| | Tg-SCID | BALB/c |
| 1 | 2.5 | 98 |
| 2 | 6.3 | 76 |
| 5 | 4.5 | 53 |
| 11 | 3.2 | 93 |

*Equal amounts of cDNA samples were amplified with Vα1, Vα2, Vα3, and Vα11 primers and compared with Vα13.1 expression in Tg-SCID and BALB/c splenocytes. The Vα13.1 expression in each sample is taken as 100%, and the results of other Vα expression were compared. (Vα8 expression in both samples was also analyzed. The expression levels of Vα8 in BALB/c samples in relation to Vα13.1 is 94%. Since Vα8 is unproductively rearranged and expressed in Tg-SCID samples (Fig. 8), it was omitted in quantitative comparison.)
in mediating certain autoimmune diseases (31). In our study, we demonstrated Va leakage in lymphoid tissue in Tg-SCID mice and noted that the extent, although not the spectrum, of leakage appeared elevated after HSV infection. However, arguing against the pathogenic role of dual Va cells in HSK was our failure to demonstrate Va leakage in corneal tissue taken from Tg-SCID mice with HSK. In addition, the lymphoid tissue from such mice did not contain significant number of cells with dual Va expression.

An alternative mechanism by which CD4+ T cells become activated to mediate immunoinflammatory lesions in Tg-SCID mice could involve a process akin to bystander activation. Accordingly, in the presence of a “cytokine storm,” cells with appropriate receptors may become activated and in turn contribute to the inflammatory response. This is thought to be a mechanistic event in the disease Dengue hemorrhagic fever (32). This bystander activation is usually considered to largely involve mononuclear phagocytes (33), but it has also clearly been shown to apply in some circumstances to CD4+ T cells (34, 35). Since we did not observe any detectable anti-HSV reactivity even after AN-1 immunization, it is tempting to speculate that CD4 T cells in the cornea are activated into effector function by mechanisms other than those involving TCR occupancy. One prominent consequence of HSV replication

**FIGURE 10.** Cell surface expression of dual Va T cells in Tg-SCID mice. Draining lymph nodes cells from BALB/c (A), DO11.10 (B), and Tg-SCID (C) mice were stained with FITC or PE-conjugated mAb for Va2TCR, CD4, and KJ1-26.1 (anti-OVA-TCR). Data shown are representative of three experiments.
in the mouse eye is the expression of many chemokines and cytokines, contributed by corneal epithelium and perhaps by recruited inflammatory cells (19, 21, 36). Therefore, we speculate that, in the HSV-infected eye, this proinflammatory microenvironment could act to trigger ingressing CD4 T cells. Indeed, it has been reported previously that resting naive human CD4+ T cells could be activated to proliferate by a cytokine combination consisting of IL-2, TNF-α, and IL-6 (34). Such a combination also causes effector function in resting memory CD4 T cells, as measured by lymphokine synthesis and providing help for Ig production by B cells (34). Alternatively, Ag-independent T cell activation could also occur through accessory molecules on the T cells. One such mechanism includes CD2-mediated interactions on the T cells with ligands such as LFA-3 and CD59. Indeed, it has been reported that LFA-3 and CD59 expression in corneal samples of HSK-positive mice. Total RNA was isolated from corneal lysate and reverse transcribed as described in Materials and Methods.

FIGURE 11. Leaky Vα expression in corneal samples of HSK-positive BALB/c and Tg-SCID mice. Total RNA was isolated from corneal lysate and reverse transcribed as described in Materials and Methods. Lanes 1-7: Marker (100-bp DNA ladder), Vα1, Vα2, Vα5, Vα11, Vα13.1, and Vα8.

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References


