Histology and Immunohistology of Igh-1-Restricted Herpes Simplex Keratitis in BALB/c Congenic Mice

E. Mitchel Opremcak,* Beverly A. Rice,† Peter A. Wells,† and C. Stephen Foster†

In order to characterize the local ocular immunologic milieu of Igh-1-restricted herpes simplex keratitis (HSK), we investigated histologic and immunohistologic correlates of disease over a 21-day time course. Clinically observable keratitis began 10 days postinoculation in susceptible C.AL-20 (Igh-1d) and moderately susceptible BALB/c (Igh-1a) mice, whereas HSV-1-resistant C.B-17 (Igh-1b) mice rarely developed disease. Igh-1-restricted histologic differences were observed by day 11 postinoculation; C.AL-20 and BALB/c mice showed augmented recruitment of neutrophils and mononuclear cells in conjunctival, limbal, and corneal tissues compared to C.B-17 mice. On immunohistologic study, Lyt-1 to Lyt-2 cell ratios by day 11 postinoculation were 7:1, 2:1, and 1:8 in corneas from C.AL-20, BALB/c, and C.B-17 mice, respectively. Macrophages and neutrophils were absent in corneas from C.B-17 mice at this time, but could be found in large numbers in the corneas of susceptible mouse strains through day 21. These data demonstrate a strong relationship between Igh-1 phenotype and inflammatory cell recruitment in response to corneal infection with HSV-1, and support a role for T cell subpopulations in mediating Igh-1-restricted HSK.


The severity of herpes simplex keratitis (HSK) is determined largely by characteristics of the viral isolate and the host’s immune response. The importance of host genetics in determining resistance patterns to herpes simplex virus (HSV)-1-mediated immunopathema has been established previously by employing inbred mice in animal models of HSV infection.1–5 C57BL/6J mice are resistant to corneal and intraperitoneal HSV challenge, whereas BALB/c and A strain mice routinely develop severe disease. Work in our laboratory has established that gene products linked to the Igh-1 locus on chromosome 12 in the mouse play an important role in the determination of these resistance patterns and in the development of severe, necrotizing stromal HSK.6–8 Igh-1 disparate, C.AL-20 (Igh-1d), BALB/c (Igh-1a), and C.B-17 (Igh-1b) congenic mice develop significantly different HSK frequencies, 82%, 40%, and 12% respectively (P < 0.00001). The mechanism responsible for Igh-1 influence is not completely understood.

Igh-1-encoded gene products are known to control both the constant and variable regions of immunoglobulin heavy chains, and as such could influence HSK via humoral mechanisms.9,10 Alternatively, the IgT-c locus, mapped adjacent to the Igh-1 region on chromosome 12, controls T cell differentiation, immunomodulation, and function.9,10 Influence from these gene products could restrict disease development via T-cell-mediated processes. In order to define further the mechanism(s) of Igh-1 influence, we employed a murine model of HSK using Igh-1 disparate BALB/c congenic mice, and studied histologic and immunohistologic correlates of their distinct disease patterns.

Materials and Methods

Virus

The HSV-1 KOS strain was obtained from Dr. David Knipe (Harvard Medical School, Boston, MA) and passed twice in Vero cells (CCL 81 American Type Cell Collection, Rockville, MD). Infected Vero cell monolayers were harvested when a 4+ cytopathic effect was observed. The infected cells were freeze-thawed three times and centrifuged at 1500 g.7 Supernatants were aliquoted and stored frozen at −70°C. Aliquots were selected at random and assayed with a standard plaque assay technique on

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Vero cells, as described previously. Titer were verified in triplicate.

**Animals**

BALB/cByJ (Igh-1<sup>b</sup>) mice were obtained from Jackson Laboratories (Bar Harbor, ME). C.AL-20 (Igh-1<sup>a</sup>) mice were obtained from Dr. Alfred Nisonoff (Brandeis University, Waltham, MA) and bred in microisolators mounted in a ventilated animal rack in our animal facility. C.B-17 (Igh-1<sup>b</sup>) mice were provided by Dr. Charles Sidman (Jackson Laboratories, Bar Harbor, ME). Animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research and with National Institutes of Health guidelines.

**Inoculation**

Animals were anesthetized with ether, and under binocular microscopy, the right eye of each mouse was scratched with a 23-gauge needle (eight horizontal and eight vertical scratches). Five microliters of diluted HSV-1 (KOS strain) were deposited onto the scarified cornea to effect a viral challenge dose of 2.5 × 10<sup>6</sup> plaque-forming units (PFU) per eye. Four representative mice from each congenic strain at postinoculation (pi) days 4, 8, and 21, and 10 animals at pi day 11 were killed by anesthesia overdose. The inoculated eyes were removed and processed as described below.

**Histology**

For standard histologic examination, the challenged eyes were fixed in Karnovsky’s fixative (1% paraformaldehyde and 1.25% glutaraldehyde, in 0.2 M sodium cacodylate buffer, pH 7.2) for 48 hr at 4°C prior to 30-gauge needle opening and an additional 48-hr fixation. After fixation, the tissue was rinsed in buffer, dehydrated through ascending concentrations of ethanol, infiltrated with glycol methacrylate solutions overnight, and then embedded in LKB Historende buffer. The sections were incubated with a 1:500 dilution of biotinylated affinity-purified goat anti-mouse IgG and scored by relative intensity of peroxidase staining on a scale of 1 to 4+.

The eyes were analyzed for the presence of neutrophils, mononuclear cells consisting of lymphocytes, plasma cells, and macrophages in the central cornea, limbus, and conjunctiva. The number of inflammatory cells was counted by means of brightfield microscopy in three representative high-power (500×) fields in two separate serial sections from the central cornea, limbus, and conjunctiva for each BALB/c congenic mouse strain. Group means and standard errors were calculated in the usual way.

**Immunopathology**

An immunoperoxidase technique was used to further characterize the cell subpopulations involved in the disease process after corneal inoculation. Whole eyes from two animals at pi days 4, 8, and 21, and eight animals from pi day 11 were snap-frozen and embedded in Tissue-Tek OCT compound (Ames, Division of Miles Laboratory, Elkhart, IN) immediately after enucleation. With a Minotome (International Equipment Co.) cryostat, the eyes were sectioned at a thickness of 4 μm. Whole-eye cross sections were fixed to gelatin-coated 12-well microscope slides and stored at −70°C until analysis. The slides were then air-dried and fixed for 10 min in acetone prior to staining. The sections were incubated for 45 min with the primary antibody at various dilutions. After incubating with the primary antibodies, sections were washed three times with 0.01 M phosphate-buffered saline (PBS) and blocked for endogenous peroxidase with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS for 30 min. Optimal dilutions of the primary antibodies were obtained by staining sections of mouse spleen and lymph node with the immunoperoxidase procedure. Primary antibodies consisted of the following reagents: anti-Thy 1.2 (T lymphocytes), 1:200; anti-Lyt-1 (T helper/TH lymphocytes), 1:100; anti-Lyt-2 (T suppressor/cytotoxic lymphocytes), 1:10; and anti-L3T4 (T inducer lymphocytes), 1:10 (Becton Dickinson, Mountain View, CA); antimurine/ human Mac-1 antigen (macrophages), 1:25 and antimurine Ia (antigen-presenting cells), 1:100 (Hybritech, San Diego, CA); and horse antimouse IgG (H&L) (Vector Laboratories, Burlingame, CA). Sections were incubated with a 1:500 dilution of biotin-conjugated affinity-purified mouse anti-rat IgG (H&L) (Jackson Immunoresearch) for 45 min. After three rinses in PBS, the tissue was incubated 45 min with 1:1000 dilution of peroxidase conjugated Streptavidin (Jackson Immunoresearch Laboratories, Avondale, PA). The tissue was rinsed in PBS and overlaid with peroxidase substrate containing 3-amino-9-ethyl-carbazole and H<sub>2</sub>O<sub>2</sub> in 0.1 M Na-acetate buffer. The sections were fixed in 4% formalin, counterstained with Gill’s no. 3 hematoxylin, rinsed, and coverslipped with Gelvatol (Monsanto, Springfield, MA). Experimental controls included tissue sections without the addition of the primary antibody.

In certain experiments, the eyes were evaluated for the presence of IgG via peroxidase-labeled, horse antimouse IgG and scored by relative intensity of peroxidase staining on a scale of 1 to 4+.

**Enumeration**

Positively stained cells in the cornea, limbus, and conjunctival tissue were counted with a Zeiss (Ober-
kochen, West Germany) photomicroscope III. Three separate high-power fields from two serial sections of three ocular tissues (cornea, limbus, and conjunctiva), from three different murine strains (C.AL-20, BALB/c, and C.B-17 mice), at four different time points (days 4, 8, 11, and 21), were analyzed. Individual inflammatory cell subpopulations from two animals of each murine strain at each time point were enumerated by means of H&E staining and, in a similar fashion, characterized with seven separate primary antibodies in two mice of each strain at days 4, 8, and 21 and in eight mice of each strain at day 11 (n = 4104 high-power experimental fields). All counts were compared with positive and negative control tissues stained and mounted on each slide.

The results were tabulated for each pi day and each mouse strain. Group means and standard errors of the means were calculated in the usual way. Cell counts for HSV-1-susceptible C.AL-20 and -resistant C.B-17 mice were compared by calculating student t-test statistics for significance differences. All cell counts and evaluations were performed by two different masked observers.

Errors intrinsic to the experimental design include the inability to directly correlate individual Histoestin-embedded eyes and H&E staining patterns with frozen tissue immunohistochemical results. Because whole eyes at each time point were processed for either one or the other histologic technique, direct numerical comparisons were difficult. In this animal model, disease frequency is rarely 100% for any of the BALB/c congenic strains used, and therefore it was impossible to determine whether susceptible animals sacrificed at pi days 4, 8, and 11 were destined to develop HSK. Furthermore, clinical disease could be detected around pi day 10 but not infrequently could be found several days earlier or later. These two variables are acknowledged as potential errors in this type of a histologic and immunohistologic kinetic study.

Results

Postinoculation Day 4

Histology: Uninfected normal corneas from C.AL-20, BALB/c, and C.B-17 mice had identical baseline histology. Four days after corneal scarification and inoculation with \( 2.5 \times 10^4 \) PFU KOS, no significant differences were noted between the BALB/c congenic mouse strains on H&E stained, plastic sections. Polymorphonuclear neutrophils were not found in any ocular tissue in any congenic strain. Central corneas showed only an occasional mononuclear cell. Limbal cell populations paralleled conjunctival tissues in all murine strains and consisted primarily of mononuclear cells without neutrophils: \( 21+/ -6 \), \( 36+/ -9 \), and \( 9+/ -6 \) for C.AL-20, BALB/c, and C.B-17, respectively.

Immunohistology: Immunoperoxidase staining at pi day 4 did not show significant differences between the mouse strains in either number or phenotype of the mononuclear cell populations. The conjunctival mononuclear cell infiltrates in all congenics consisted of equivalent numbers of Thy-1.2-positive T cells without Lyt-1, Lyt-2, or L3T4 surface markers: \( 64+/ -26 \), \( 43+/ -15 \), and \( 73+/ -50 \) for C.AL-20, BALB/c, and C.B-17 mice, respectively. Central cornea and limbal regions demonstrated only an occasional Thy-1.2-positive cell in all congenic mouse strains. MAC-positive mononuclear cells were not observed in the conjunctiva, cornea, or limbus. IA-positive cells were noted in conjunctival tissues in all strains: \( 30+/ -12 \), \( 11+/ -7 \), and \( 26+/ -20 \) for C.AL-20, BALB/c, and C.B-17 mice, respectively. Staining for IgG was mild (1–2+) in all strains and tissues examined.

Postinoculation Day 8

Histology: Although not statistically significant, Igh-1-influenced cellular recruitment patterns and trends in mononuclear cell subpopulations first became recognizable by pi day 8. HSV-1-susceptible C.AL-20 and BALB/c mice began to show increasing numbers of neutrophils and mononuclear cells migrating into the limbus from the conjunctiva, as compared to HSK-resistant C.B-17 mice on H&E (Fig. 1).
Immunohistology: Characterization of the conjunctival mononuclear cells by cell surface phenotype found all BALB/c mouse strains to have T cells present by pi day 8, but in slightly lower numbers than on pi day 4: 35+/−9, 50+/−10, and 37+/−8 for C.AL-20, BALB/c, and C.B-17 mice, respectively. T cells and MAC-positive cells now were present in the limbal area but not in the central cornea (Fig. 1). Cell surface markers for Lyt-1 and Lyt-2 were not detected in corneas or limbi but could be found on mononuclear cells in the conjunctivae of BALB/c mice at a 3:1 ratio. In all sections, the numbers of Lyt-1 cells paralleled the numbers of L3T4-positive cells.

IA-positive cells were found in all murine strains in the conjunctiva and limbi on pi day 8, but as with T cells, tended to be fewer in number than on pi day 4: C.AL-20 mice from 30 cells per high-power field on pi day 4, to 8 on pi day 8; BALB/c mice from 11 to 7 cells per high-power field; and C.B-17 mice from 26 to 11 cells per high-power field. For the first time after corneal infection, IA-positive cells now were present in low numbers in the central cornea.

Staining for mouse IgG showed an increase in intensity in C.AL-20 (3+) and BALB/c mice (4+) as compared to C.B-17 mice (2+).

Postinoculation Day 11

Histology: By pi day 11, keratitis was evident in susceptible C.AL-20 and BALB/c mice. Histologic differences in inflammatory cell infiltration and phenotype were observed among the congenic mouse strains (Fig. 2). C.AL-20 and BALB/c mice showed increasing numbers of mononuclear cells without large numbers of neutrophils in the cornea and limbus (Fig. 3). C.B-17 mice were found to have fewer mononuclear cells and no neutrophils in the cornea. All murine strains maintained an equivalent mononuclear infiltration in the limbus and conjunctiva.

Immunohistology: On immunostaining, MAC-positive cells were noted in large numbers in the corneas, limbi, and conjunctivae of C.AL-20 mice as

Table 1. Lyt-1 to Lyt-2 Cell Ratios in Corneas from Congenic Mice 11 Days After Inoculation with HSV-1

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Experiment 1 (n = 2)</th>
<th>Experiment 2 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.AL-20</td>
<td>7:1</td>
<td>10:1</td>
</tr>
<tr>
<td>BALB/c</td>
<td>2:1</td>
<td>9:1</td>
</tr>
<tr>
<td>C.B-17</td>
<td>1:3</td>
<td>3:1</td>
</tr>
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</table>
compared to C.B-17 mice (P < 0.01) (Fig. 3). Thy 1.2, Lyt 1, and Lyt 2 cells now were detectable in the central cornea. Lyt-1 to Lyt-2 cell ratios in the central cornea (n = 8 animals) were higher for C.AL-20 and BALB/c mice than for C.B-17 mice (Table 1 and Fig. 4). Similar Igh-1-specific helper-to-suppressor ratios were found in limbal and conjunctival mononuclear cell populations, with C.AL-20 having 8:1 and 19:1 and BALB/c mice having 2:1 and 9:1 for limbus and conjunctiva, respectively. C.B-17 mice did not stain positive for T cell subsets in these tissues.

IA-positive cells were still at low levels in the central cornea of all strains but showed an increase in numbers in the limbal region: 22+/−13, 18+/−7, and 16+/−12 for C.AL-20, BALB/c, and C.B-17 mice, respectively. C.AL-20 and BALB/c mice demonstrated relatively more staining for IgG (4+) as compared to C.B-17 mice (2+).

Postinoculation Day 21

Histology: Keratitis was severe in C.AL-20 mice and in BALB/c mice by pi day 21. C.B-17 mice rarely developed significant HSK. Histologic correlates included a dramatic increase in the number of neutrophils and mononuclear cells in C.AL-20 corneas (P < 0.001), limbi (P < 0.01), and conjunctivae (P < 0.01), as compared to those in C.B-17 mice (Figs. 5, 6). BALB/c mice tended to have fewer polymorphonuclear cells in the corneas than did C.AL-20 mice.

Immunohistology: The mononuclear cell population consisted predominantly of macrophages in the corneas of C.AL-20 and BALB/c mice (Fig. 6). High numbers of Thy-1.2-positive cells also were found in the corneas of C.AL-20 mice and, to a lesser extent, in BALB/c mice. C.B-17 mice had negligible infiltration with MAC- or Thy-1.2-positive cells in their corneas, as compared to C.AL-20 mice (P < 0.001). T cell subsets in C.AL-20 mice continued to show a high T helper/DTH to T suppressor/cytotoxic ratio at 10:1, 6:1, and 5:1 in the cornea, limbus, and conjunctiva respectively. BALB/c mice also showed a high Lyt-1 to Lyt-2 ratio at 5:1, 15:1, and 3:1 in these same tissues. C.B-17 mice did not stain positively for these subpopulations in the cornea and only an occasional cell in the limbal area.

IA-positive cells paralleled the numbers of mononuclear cells in the corneas of all strains. C.AL-20 had the highest number of cells (93 cells per high-power field), compared to 2 cells per high-power field in C.B-17 (P < 0.01), BALB/c were intermediate, with 25 cells per high-power field. Conjunctival IA-positive cells returned to pi day 4 levels in all BALB/c congenics, whereas limbal IA-positive cells demonstrated a strain-specific pattern related to the corneal disease.

All congenic strains continued to show relatively more intense staining for IgG in the tissues on pi day 21 as compared to pi day 4, with susceptible strains showing relatively more intense IgG staining than did C.B-17.
Discussion

Strain-specific differences in severity and frequency of HSK in mice have been attributed to differences in host immunoreactivity and cellular permissivity to HSV. We have demonstrated recently that the Igh-1-linked gene products regulate the development of HSK by means of mechanisms unrelated to either host cell permissivity or ganglionic latency. The mechanisms responsible for the observed disease patterns therefore may be a function of disparate immunoregulation by gene products closely linked to the Igh-1 locus. In order to determine whether T lymphocyte subset and inflammatory cell recruitment play a role in the murine model of HSK, we used Igh-1 disparate BALB/c congenic mice and studied histologic and immunohistologic correlates of the disease patterns observed.

Igh-1-restricted differences in mononuclear cell subpopulation recruitment can be noted after p.i. day 8. Significantly greater numbers of MAC-positive cells and neutrophils were detected in the corneas of C.AL-20 mice, as compared to C.B-17 mice. T helper/DTH to T suppressor/cytotoxic cell ratios were consistently higher in susceptible C.AL-20 and BALB/c mice during this interval, a result that supports a role for T helper cell mediated recruitment of these cell subpopulations. Distinct T cell subpopulation compartmentalization and activity therefore may play a role in generating Igh-1-restricted inflammatory cell responses to HSV corneal challenge. Such activity would appear to be a local or regional immunologic event, since the Igh-1-disparate murine strains develop equivalent systemic delayed-type hypersensitivity (DTH) responsiveness after corneal inoculation with HSV-1 (Raizman M, unpublished data).

Relatively higher numbers of Lyt-2-positive T suppressor/cytotoxic cells were found in the conjunctiva, limbi, and corneas of resistant C.B-17 mice as compared to the other BALB/c congenic mouse strains, a finding that suggests a role for the T suppressor/cytotoxic cell subset in mediating protection from HSK. Other work in our laboratory has shown a similar relationship demonstrating a protective effect, seen with adoptive transfer of immune Lyt-2-positive lymph node cells in an A/J mouse model. It is not possible to discriminate suppressor from cytotoxic lymphocyte activity by means of cell surface markers. The relative role of these distinct cell functions could not be determined in the current study. There is con-
siderable evidence, however, supporting a role for T
cell suppression over cytotoxic functions in protec-
tion from HSK. Mice inoculated with HSV do not
generate cytotoxic T cell reactivity as detected by
standard cytotoxicity assays. HSV-1-specific cy-
totoxic T lymphocytes can be detected only after Cy-
toxan (Bristol-Myers, Evansville, IN) (cyclophospha-
side) treatment in vivo or restimulation of immune
cells in vitro. These experimental conditions sug-
gest that HSV-specific T cytotoxic cells are allowed to
function only in the absence of suppressor cell activ-
ity. Furthermore, we were unable to detect significant
numbers of Lyt-2 cells early in the cornea during a
period of active viral replication, when destruction of
infected cells by cytotoxic T lymphocytes would be
advantageous.

It is noteworthy that all BALB/c congenic mouse
strains demonstrated relatively fewer T cells and IA-
positive cells in the conjunctiva on pi days 8 and 11 as
compared to pi day 4. By pi day 21, the numbers of
IA- and Thy-1.2-positive conjunctival cells paralleled
Igh-1-influenced disease severity, with the highest
numbers of these cells found in C.AL-20 mice. Small
numbers of IA-positive, antigen-presenting cells
could be seen migrating into the central corneas of
the three BALB/c congenic mice at pi days 8 and 11,
but could not account for the majority of the cell loss.
Precise lymphocyte trafficking and recruitment ki-
etics after challenge with HSV cannot be completely
addressed by this type of histologic study; however,
this local reduction in T cells and antigen presenting
cells may represent lymphocyte migration to regional
lymph nodes or alternatively to the spleen, for antigen
processing. It may be postulated that lymphocytes
from susceptible mice travel to regional lymph nodes
and result in sensitization, whereas IA- and Thy-1.2-
positive cells from resistant strains travel to the spleen,
effecting antigen specific suppression. Disparate im-
mune effector cell populations would then migrate
back to the cornea and effect either a local T cell
mediated suppression of inflammation and protec-
tion from keratopathy, or alternatively, in keratitis-
susceptible strains, mediate a strong recruitment of
inflammatory cells through T helper/DTH activity
with resultant HSK.

In the current study, different staining patterns for
immunoglobulin were noted among the congenic
mice strains. As expected, this staining was strongest
in the HSK-susceptible C.AL-20 and BALB/c mice.
These patterns corresponded to the degree of inflam-
matory cell infiltration, and were interpreted as non-
specific, secondary responses. One could postulate
that in this model, the Igh-1 locus modulates disease
expression through a humoral mechanism. While this
possibility currently is being investigated, evidence to
date does not support this premise. In fact, after cor-
neal inoculation, virus-neutralizing antibody titers
have been observed to be identical for the three mu-
rine strains (Raizman M, Ilhey T, and Foster CS: manu-
script submitted for publication). Furthermore, adoptive
transfer of immune serum between the Igh-1
congenic strains has protected animals equally from
HSK. These data do not support a dominant role for
humoral mechanisms in Igh-1-restricted HSK.

The data presented in this communication demon-
strate the importance of using congenic mice for im-
munogenetic analysis of viral keratitis and the power of
immunohistochemical techniques for the determi-
nation of cell recruitment patterns. It appears that
Igh-1-restricted HSK develops in mice when func-
tionally active T helper/DTH cells are present in the
local ocular milieu with subsequent recruitment of a
nonspecific inflammatory response. In contrast,
HSK-resistant mice appear to down-regulate this re-
sponse by means of enhanced or more efficient T
suppressor/cytotoxic cell activity. C.AL-20 and to a
lesser extent BALB/c mice appear to lack this im-
munoregulatory feedback.

Key words: Igh-1 locus, mouse, cornea, infection, herpes
simplex virus (HSV-1)

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