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-I d) and moderately susceptible BALB/c (Igh-I a) mice, whereas HSV-1-resistant C.B-17 (Igh-I b) 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. Invest Ophthalmol Vis Sci 31:305–312, 1990

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-1 d), BALB/c (Igh-1 a), and C.B-17 (Igh-1 b) 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. 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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Titors were verified in triplicate.

**Animals**

BALB/cByJ (Igh-1a) mice were obtained from Jackson Laboratories (Bar Harbor, ME). C.AL-20 (Igh-1b) 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-1d) 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 × 10^6 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 solution overnight, and then embedded in LKB Historesin (LKB Produkter AB, Bromma, Sweden). Sections (2-μm thick) were cut with a Sorvall JB-4 microtome, and stained with hematoxylin and eosin (H&E) for histopathologic study.

The eyes were analyzed for the presence of neutrophils and 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 then 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% H2O2 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 Strep-tavidin (Jackson Immunoresearch Laboratories, Avondale, PA). The tissue was rinsed in PBS and overlayed with peroxidase substrate containing 3-amino-9-ethyl-carbazole and H2O2 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 Historesin-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 × 10⁴ 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 cornneas or limbi but could be found on mononuclear cells in the conjunctiva 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.

**Fig. 3**: Inflammatory cell types found in the central cornea of C.AL-20, BALB/c, and C.B-17 mice, pi day 11. Polymorphonuclear neutrophils (PMN) and mononuclear cells (MC) were counted in H&E-stained plastic sections. Macrophages (MAC) and T cells (Thy 1.2) were counted from immunoperoxidase-stained frozen tissue sections.

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 cornneas, limbi, and conjunctivae of C.AL-20 mice as

**Table 1. Lyt-1 to Lyt-2 Cell Ratios in Cornneas 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>
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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+).

**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.

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 pi 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 conjunctivae, 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 protection from HSK. Mice inoculated with HSV do not generate cytotoxic T cell reactivity as detected by standard cytotoxicity assays.\textsuperscript{16,17} HSV-1-specific cytotoxic T lymphocytes can be detected only after Cytoxan (Bristol-Myers, Evansville, IN) (cyclophosphamide) treatment in vivo or restimulation of immune cells in vitro.\textsuperscript{18,19} These experimental conditions suggest that HSV-specific T cytotoxic cells are allowed to function only in the absence of suppressor cell activity. 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 kinetics 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 stains travel to the spleen, effecting antigen specific suppression. Disparate immune effector cell populations would then migrate back to the cornea and effect either a local T cell mediated suppression of inflammation and protection 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 inflammatory 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.\textsuperscript{6} In fact, after corneal inoculation, virus-neutralizing antibody titers have been observed to be identical for the three murine strains (Raziman M, Ilhey T, and Foster CS; manuscript 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 demonstrate the importance of using congenic mice for immunogenetic analysis of viral keratitis and the power of immunohistochemical techniques for the determination of cell recruitment patterns. It appears that Igh-1-restricted HSK develops in mice when functionally 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 response 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 immunoregulatory feedback.

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

References


