Viral Isolation and Systemic Immune Responses After Intracameral Inoculation of Herpes Simplex Virus Type 1 in Igh-1-Disparate Congenic Murine Strains

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Igh-1-disparate congenic murine strains differ in their susceptibility to develop contralateral chorioretinitis after intracameral (AC) inoculation with Herpes simplex virus type 1 (HSV-1): 75% of BALB/cByJ (Igh-1a) and 5% of C.B-17 (Igh-1b) develop necrotizing chorioretinitis. To determine the mechanism of influence of host genetics on development of contralateral chorioretinitis, the authors did viral isolation studies in contralateral eyes, determined in vivo and in vitro T-cell responses, and HSV-antibody levels at various times after AC inoculation of BALB/cByJ and C.B-17 mice with HSV-1. Viral isolation was similar in both mouse strains \( P < 0.2 \). Similarities in systemic immune responses included suppressed delayed-type hypersensitivity responses 5 days, cytotoxic T-lymphocyte and lymphocyte proliferation responses 8 days, and viral neutralizing antibody titers 5 days postinoculation (PI). Differences in systemic immune responses included: (1) delayed-type hypersensitivity responses were not suppressed in C.B-17 mice \( P > 0.1 \) and were hyperactive in BALB/cByJ mice \( P < 0.025 \) 10 days PI and (2) HSV-neutralizing antibody production was higher in C.B-17 mice 10 days PI. These data suggest that the mere presence of HSV-1 in the uninoculated eye is insufficient for the development of chorioretinitis. Virus-specific delayed-type hypersensitivity reactions might be involved in the pathogenesis of retinitis in BALB/cByJ mice; and virus-neutralizing antibodies and suppressed HSV-specific delayed-type hypersensitivity reactions might be instrumental in the protection enjoyed by C.B-17 mice. Invest Ophthalmol Vis Sci 31:2335–2341, 1990

Intracameral (AC) inoculation of Herpes simplex virus type 1 (HSV-1) results in ipsilateral iridocyclitis, relative sparing of the ipsilateral choroid and retina, and contralateral necrotizing chorioretinitis in a murine model of HSV uveitis.1 Direct viral cytopathic effects and virus-induced host immune responses have been implicated in the pathogenesis of this interesting phenomenon.

Making use of genetically defined mouse strains differing only in a limited region associated with the Igh-1 locus on chromosome 12, we previously showed a profound influence of the Igh-1 locus on the development of HSV-1-induced necrotizing contralateral chorioretinitis: 75% of BALB/cByJ, 30% of C.AL-20, and 5% of C.B-17 mice developed destructive contralateral chorioretinitis.2

Igh-1 encoded gene products control both the constant and variable regions of immunoglobulin heavy chains and appear to influence T-cell activity and T-cell subset recruitment in immune responses; thus both cellular and humoral immune responses may influence the development of HSV-1 induced chorioretinitis.3-6 To determine if the Igh-1 locus exerted its influence through systemically detectable immune parameters and to understand better the kinetics of the immune response, we determined delayed-type hypersensitivity reactions (DTH), cytotoxic lymphocyte activity (CTL), lymphocyte proliferation activity (LPA), and herpetic antibody levels at various intervals after AC inoculation with HSV-1 in the Igh-1-disparate susceptible BALB/cByJ and resistant C.B-17 murine strains. In addition, viral isolation studies were done on contralateral uninjected eyes after ipsilateral AC inoculation to determine if histologically normal eyes of resistant mice harbored replicating HSV-1.

Materials and Methods

Experimental Design

Mice were inoculated AC with \( 1.5 \text{–} 2.0 \times 10^4 \) plaque-forming units (PFU) of HSV-1. Five or ten
days postinoculation (PI), DTH responses were measured by footpad swelling, or the mice were bled and unpooled serum processed to determine herpetic antibody titers using an enzyme-linked immunosorbent assay (ELISA), or virus-neutralizing assay (VNA). To determine suppressor T-cell-mediated suppression of HSV-specific DTH responses after subcutaneous (SC) immunization, mice were inoculated simultaneously AC and SC with HSV-1, and DTH responses were determined 5 and 10 days PI. These were compared with those in mice immunized SC only. The CTL activity was measured 8 days after AC inoculation by a chromium-release assay, and LPA responses were determined 9 days PI (results of pilot studies for CTL and LPA assays 5 days after HSV-1 inoculation in the AC were similar to the results obtained 8 and 9 days PI and were not repeated). Contralateral eyes of BALB/cByJ and C.B-17 mice were enucleated and processed for plaque assays 7 or 10 days PI. Separate mice were used for each arm of the experiment; all experiments were done in duplicate or triplicate. Data from the different runs of each experiment were pooled for data presentation and statistical analysis.

**Virus**

The HSV-1 strain KOS was obtained from Dr. David Knipe (Harvard Medical School, Boston, MA) and processed as described previously.7

**Animals**

BALB/cByJ (Igh-la) and C.B-17 (Igh-1b) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and Dr. Charles Sidman (Jackson Laboratories), respectively, and were bred in our animal facilities. Six- to 8-week-old age- and sex-matched mice of both strains were used in all experiments. Animals were housed in microisolators inside VR-1 laminar flow ventilated animal racks (Lab Products, Rochelle Park, NJ). They were handled in accordance with the ARVO Resolution on the Use of Animals in Research.

**Viral Inoculation**

The mice were anesthetized with ether before inoculating 1.5–2.0 PFU of HSV-1 in the AC as described previously.7 Mice for DTH assays were inoculated SC with 5 × 10⁶ PFU HSV-1 as described previously.2

**DTH and DTH-Suppression Assays**

Separate groups of mice (n = 8 each) received no HSV-1 (negative controls), HSV-1 only in the AC or simultaneously AC and SC (experimental groups), or HSV-1 SC only (positive controls). DTH was assayed 5 or 10 days later as follows. Mice were challenged in the right hind footpad with 1.3 × 10⁶ erstwhile PFU of ultraviolet (UV)-inactivated HSV-1 (UV-HSV) in 50 µl of medium using a 30-gauge needle on a Hamilton syringe (Reno, NV). The left hind footpads received an equal volume of uninfected supernate. Twenty-four hours later footpad swelling was measured using a Fowler engineer’s micrometer in a masked fashion. Experiments were done in triplicate. Tolerance was calculated as:

\[
\text{tolerance} = 100 \times \frac{[1 - (\text{experimental} - \text{negative control})]}{(\text{positive control} - \text{negative control})}
\]

**Cytotoxicity Assay**

**Effector cells:** Eight days after AC inoculation with HSV-1, spleens were removed, and single-cell suspensions were prepared. The cell culture medium was RPMI-1640 supplemented with 10% calf serum (CPSR-2; Sigma, St. Louis, MO), 200 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Effector spleen cells (ESC) were restimulated in mixed lymphocyte culture (MLC) as follows. A total of 25 × 10⁶ ESC were placed in microculture plates together with 4 × 10⁵, 3000-cGy x-irradiated, HSV-infected stimulator splenocytes from syngeneic naive animals with 5 ml of medium containing UV-HSV at a multiplicity of infection (MOI) of 5.

**Chromium release assay:** Anti-HSV cytotoxic activity was measured against uninfected and infected 3T3 fibroblasts. Infected targets were prepared by mixing 3T3 fibroblasts in 3 ml RPMI with 10% fetal calf serum (FCS) with HSV-1 at MOI 5, 37° C for 1 hr. Labeling was achieved by mixing target cells with 250 µCi of Na₂⁵¹CrO₄ in 1 ml of culture medium and incubating at 37° C for 1 hr. Targets were washed three times and counted. After 72 hr in MLC, titrated numbers of ESC were harvested, resuspended in medium, and placed in round-bottom microwell culture plates together with 2 × 10⁵ infected or uninfected ⁵¹Cr-labeled target cells in a total volume of 200 µl of medium per well. Each assay was done at effector-to-target ratios of 25:1, 50:1, and 100:1. After incubation at 37° C for 18 hr, radioactivity released from lysed cells was measured with 100 µl of each well’s supernatant in a gamma counter (LKB/Wallach Clinigamma counter, Finland). Percentages of specific chromium release were calculated as:

\[
\frac{([\text{experimental release} - \text{spontaneous release}]/(\text{total release} - \text{spontaneous release})) \times 100.}{100}
\]
Total releasable radioactivity was determined from lysate of targets mixed with 4% Triton X-100. Spontaneous release was determined from the supernatant of wells containing radiolabeled target cells alone. Means of values in six replicates were used in calculations. Data were expressed as lytic units (LU), defined as the number of effector cells required for 10% specific chromium release, calculated using a nonlinear least-squares fitting program as described previously.8 Assays were done in triplicate (n = 8 for either mouse strain, each run).

LPA

Mice were inoculated AC with HSV-1 and killed 9 days PI by cervical dislocation. Single-cell suspensions of splenocytes were prepared. Cells were separated over Lympholyte-M (Cedarlane Laboratories, Ontario, Canada). Lymphocytes, harvested from the buffy coat, were washed three times, and 2 x 10⁶ cells per well were plated onto microplates and incubated at 37°C in cell culture medium as described. Mitogens, 50 μg/ml of concanavalin-A, or 2 x 10⁶ erstwhile PFU of UV-HSV were added to the plates. Medium without mitogens was added to the control plates. After 72 hr of in vitro culture, wells stimulated with concanavalin-A and corresponding control wells were pulsed with 0.5 μCi of ³H-thymidine per well. After 16 hr, cells were harvested onto filter paper. The paper discs were dried for 24 hr, immersed in scintillation fluid, and counted in a beta scintillation counter. Wells stimulated with UV-HSV and their corresponding controls received the same 16-hr pulse of ³H-thymidine before harvesting. Stimulation indices were calculated as follows: mean cpm test cultures divided by mean cpm of the unstimulated cultures. Proliferation assays were done in triplicate using six mice of either strain for each run.

Serum

Mice were bled via the tail vein 5 or 10 days after AC inoculation with HSV-1. Whole blood was allowed to clot at 4°C. Twenty-four hours later the clot was gently broken and the samples centrifuged at 4°C and 1500 rpm for 15 min. Serum was pipetted and stored at -70°C for antibody assays. Control serum was similarly obtained from sham injected mice of both strains.

ELISA

Individual mouse serum samples were prepared in twofold serial dilutions (1:12.5-1:200) 5 or 10 days after AC inoculation of HSV-1. Serum was next added to HSV-1-coated and control-coated 96-well plates (Whittaker Bioproducts, Walkersville, MD). An indirect ELISA technique for detection of HSV-1-specific antibody was done using a 1:4000 dilution of secondary anti-mouse IgG urease-conjugated antibody (Boehringer Mannheim, Indianapolis, IN) and compatible urea substrate.9 Five hours after adding the substrate, the optical density (OD) of all HSV- and control-coated wells was measured at 590 nm by a Titertek Multiskan Spectrophotometer (Flow Labs, McLean, VA).

Herpetic antibody titers were determined for each sample as follows. First a linear-regression equation was generated by plotting serum dilutions versus corresponding OD values in HSV-coated wells only. Next a background value was determined for each serum sample using the following formula: (OD value of the 1:12.5 dilution of the control-coated well of the serum test sample) + (the OD difference between the HSV-coated well and the control-coated well of 1:12.5 serum dilution from an unimmunized mouse). Finally, the dilution of the test sample yielding the same OD as the background value (positive threshold) was determined using each test sample’s linear-regression equation (from HSV-coated wells) generated in the first step. The herpetic antibody titer was defined as the reciprocal of this dilution. All titers less than 12.5 were considered zero (ELISAs were done in duplicate).

Virus-Neutralizing Assay

Serially diluted sera from AC inoculated BALB/cByJ and C.B-17 mice were mixed with 8 x 10² PFU of HSV-1 and incubated for 30 min at 37°C. These were then adsorbed onto Vero cell monolayers for 60 min at 37°C before adding overlay medium consisting of Eagle's minimum essential medium with 5% FCS and 0.1% human IgG (165 mg/ml, Gamma; Armour,), and viral plaques were allowed to develop for 4 days at 37°C. The greatest serum dilutions which resulted in 50% reduction of the number of viral plaques compared with control values were reported as approximate titers of neutralizing antibody. All assays were done in triplicate.

Viral Isolation

Mice were killed 7 or 10 days after AC inoculation using ether overdose. Contralateral eyes were harvested and processed for plaque assays by previously described methods.7 Assays were done in duplicate.

Statistics

Student t-test was used for statistical analysis of significance of differences between means.
Results

Viral Isolation

Viral isolation studies (plaque assay) in contralateral uninjected eyes of C.B-17 and BALB/cByJ mice were done 7 and 10 days after ipsilateral AC inoculation of HSV-1. Eyes were examined biomicroscopically before enucleation; none of the contralateral, uninoculated eyes of C.B-17 mice had signs of inflammation. HSV-1 was isolated from four of six (67%) and five of seven (71%) contralateral eyes of C.B-17 mice 7 and 10 days PI, respectively, and five of six (83%) and six of seven (86%) contralateral eyes of BALB/cByJ mice 7 and 10 days PI, respectively. Mean PFU 7 days PI were $2.0 \times 10^4 \pm 1.9 \times 10^4$ in C.B-17 mice and $2.4 \times 10^3 \pm 1.0 \times 10^3$ in BALB/cByJ mice. The difference was not significant ($P < 0.2$). Mean PFU 10 days PI was $2.7 \times 10^3 \pm 1.5 \times 10^3$ in C.B-17 mice and $5.2 \times 10^3 \pm 1.3 \times 10^3$ in BALB/cByJ mice. The difference was not statistically significant ($P < 0.2$).

DTH and DTH Suppression

The DTH responses and suppression of DTH responses 5 days and 10 after AC inoculation of HSV-1 are shown in Figures 1 and 2, respectively.

The DTH responses in C.B-17 mice 5 days after AC inoculation of HSV-1 were not significant ($P > 0.1$) comparing columns 5 and 7 in Figure 1 (n = 8 and 10, respectively). In contrast, DTH responses 10 days PI were significant ($P < 0.005$) (Fig. 2, columns 5 and 7, n = 12 and 10, respectively). These responses in BALB/cByJ mice 5 days after AC inoculation of HSV-1 were significant ($P < 0.05$) (Fig. 1, columns 1 and 3, n = 10) as were DTH responses 10 days PI ($P < 0.005$) (Fig. 2, columns 1 and 3, n = 10).

The DTH responses in C.B-17 mice 5 days after simultaneous SC and AC challenge with HSV-1 were significantly suppressed ($P < 0.005$) comparing columns 6 and 8 in Figure 1 (n = 14); there was 47% suppression. In contrast, comparing columns 6 and 8 in Figure 2, DTH responses were not significantly suppressed (14.2%, $P > 0.1$) 10 days after HSV-1 inoculation (n = 14). The DTH responses in BALB/cByJ mice were significantly suppressed ($P = 0.005$) 5 days after simultaneous SC and AC challenge with HSV-1 (Fig. 1, columns 2 and 4, n = 8); there was 66% suppression. After 10 days, DTH responses were, in contrast, hyperactive ($P < 0.05$) (Fig. 2, columns 2 and 4, n = 14); there was −35% suppression.

Cytotoxicity

Cytotoxic activity was measured in BALB/cByJ and C.B-17 congenic mice 8 days after AC inoculation with HSV-1 (pilot CTL studies 5 days PI were similar to results obtained 8 days PI and were not repeated). No significant difference between the two mouse strains was detected (Table 1).

Lymphocyte Proliferation

Results of LPA in BALB/cByJ and C.B-17 mice are shown in Table 2. Lymphocyte proliferation was sim-
ilar in both mouse strains 9 days after AC inoculation with HSV-1. Mitogenic stimulation with concanavalin-A (positive control) showed proliferation in both mouse strains (pilot LPA studies 5 days PI were similar to results obtained 9 days PI and were not repeated).

Antibody

Mean herpetic antibody titers (ELISA) 5 days after AC inoculation of HSV-1 were remarkably similar in C.B-17 and BALB/cByJ mice at 1:14.30 (n = 7). Ten days PI, mean titers (ELISA) were significantly higher in BALB/cByJ mice at 1:37.5 ± 24.6 compared with C.B-17 mice at 1:37.5 ± 17.5 (P < 0.007) (n = 8 and 7, respectively). In contrast, neutralizing herpetic antibody (determined by VNA) 10 days PI were higher in C.B-17 mice with a mean titer of 20 ± 3.0 (n = 5) than in BALB/cByJ mice (mean titer of 12.5 ± 4.0, n = 5). The difference was not significant (0.05 < P < 0.1).

Discussion

Different strains of mice vary in their susceptibility to develop contralateral necrotizing chorioretinitis after ipsilateral AC inoculation with HSV-1. Several groups of investigators, often using inbred mouse strains, have studied this phenomenon. The relative roles of direct viral cytopathic effect versus a rampant immune response in the pathogenesis of contralateral chorioretinitis remains, however, unclear. Furthermore, use of inbred mouse strains does not permit exclusion of the influence of multiple gene loci on the disease pattern.

We had the opportunity to study the chorioretinitis patterns in congenic strains of mice differing only in a limited region localized around the Igh-1 locus on chromosome 12. Seventy-five percent of BALB/cByJ (Igh-1a) and only 5% of C.B-17 (Igh-1b) mice developed contralateral chorioretinitis.2

We now show that HSV-1 spreads to, and can be cultured from, clinically and histologically normal contralateral eyes of resistant C.B-17 mice. Isolation of HSV from normal contralateral eyes without retinitis was previously reported. Atherton et al10,11 were able to isolate HSV-1 and HSV-2 from normal contralateral eyes after ipsilateral inoculation in the AC. In a more recent report, the same authors showed the presence of HSV-1 in relatively normal uninjected eyes of athymic mice after unilateral AC inoculation.12 We previously reported the presence of glycoprotein-C-deficient, HSV-1 mutants in normal, contralateral, uninjected eyes of BALB/cByJ mice after ipsilateral inoculation.7 We therefore concluded that the mere presence of HSV in the contralateral eye was insufficient for the development of necrotizing chorioretinitis. Some authors suggest that the amount of virus reaching the uninjected eye might be critical to the development of retinitis.10,11,12 Our data in the congenic mice do not support this. Viral titers were not significantly different between the susceptible and resistant mouse strains.

Anterior chamber-associated immunodeviation (ACAID) developed in mice after injecting HSV-1 into the AC. This phenomenon was first described by Whittum et al14 in 1983 and was characterized by suppression of virus-specific DTH reactions and normal/enhanced antiviral humoral responses. Several investigators studied this phenomenon to explain the development of, or protection from, HSV-induced chorioretinitis. Atherton and Streilein10 showed that inoculation of HSV-2 into the AC of mice did not induce contralateral destructive retinitis. These mice (infected AC with HSV-2) also developed vigorous HSV-2-specific DTH reactions (absence of suppression). In addition, the same authors showed that bilateral inoculation of HSV-1 into the AC abrogated the development of retinitis and that contralateral retinitis did not occur in mice whose HSV-1-inoculated eyes were enucleated within 3 days of inoculation.15 Vigorous HSV-specific DTH reactions developed in these mice. Also, significantly milder contralateral retinitis developed in T-cell-deficient, athymic mice after ipsilateral inoculation of HSV in the AC.12 Kielty et al13 studied two inbred mouse strains for the development of HSV-1-induced retinitis and the possible relation to suppression of viral DTH responses. Mice without contralateral retinitis did not suppress HSV-1-specific DTH responses, while retinitis-susceptible mice developed significant DTH suppression. Hayashi et al16 reported a marked decrease in HSV-1-induced contralateral retinitis in mice bred in the dark. These mice did not develop ACAID. Combined, these reports support the theory that susceptibility to contralateral chorioretinitis is linked to the development of HSV-specific suppression of DTH responses (ACAID).

Table 2. Lymphocyte proliferation

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Con-A* stimulation</th>
<th>HSV-1* stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive BALB/cByJ</td>
<td>32.0 ± 11.0</td>
<td>2.3 ± 0.6*</td>
</tr>
<tr>
<td>Naive C.B-17</td>
<td>114.7 ± 17.0</td>
<td>4.0 ± 0.2*</td>
</tr>
<tr>
<td>Infected BALB/cB</td>
<td>78.3 ± 1.0</td>
<td>2.1 ± 0.7*</td>
</tr>
<tr>
<td>Infected C.B-17</td>
<td>79.6 ± 9.0</td>
<td>1.8 ± 0.6*</td>
</tr>
</tbody>
</table>

* Stimulation index.

<sup>a</sup> No statistically significant difference between these values.
Metzger et al\textsuperscript{17} injected HSV-1 into mouse eyes by various routes and showed that the development of contralateral retinitis did not necessarily correlate with systemic immunity (DTH suppression). Whittum-Hudson et al\textsuperscript{18} reported that athymic or irradiated mice developed bilateral necrotizing retinitis after unilateral inoculation with HSV-1 (contrary to a more recent report by Atherton et al\textsuperscript{12} where significant contralateral retinitis did not develop in athymic mice PI with HSV in the AC). Reconstitution with immune cells from mice challenged with HSV-1 in the AC restored ipsilateral retinal protection. These same authors\textsuperscript{19} also showed that HSV-1 induced ACAID (and thus HSV-specific DTH suppression) in mice that did not develop contralateral retinitis. These reports suggest that resistance to contralateral retinitis does not correlate with systemic immune responses, specifically the development of ACAID.

Thus although there is ample evidence supporting a role for immunopathogenic processes in the development of HSV-1-induced chorioretinitis, a definite role for ACAID is uncertain. Furthermore, the role of other systemic immune parameters has not been defined.

Our data show several differences in systemic immune responses between retinitis-resistant C.B-17 and retinitis-susceptible BALB/cByJ mice after inoculating HSV-1 in the AC. Interestingly, neutralizing HSV-antibody titers were higher in C.B-17 mice 10 days PI. This was in contrast to antibody determination by ELISA where levels were markedly higher in BALB/cByJ mice. These data suggest a possible role for viral neutralizing antibodies and/or other B-cell dependent functions in resistance to HSV-1 retinitis in C.B-17 mice.

BALB/cByJ mice had more active DTH responses. Specifically, despite documentation of DTH suppression in both mouse strains 5 days after simultaneous SC and AC inoculations with HSV-1, this suppression was transient in BALB/cByJ mice and was overcome 10 days PI; these mice developed vigorous HSV-1-specific DTH reactions. This might be significant as destructive retinitis, and peak levels of infectious virus were detected in uninjected eyes 10 days PI. Niederkorn et al\textsuperscript{20} and later Streilein\textsuperscript{21} described a similar reversal of DTH suppression after injecting tumor cells into the AC of mice. High tumor immunogenicity possibly contributed to the reversal of DTH suppression in this tumor model. Similar mechanisms might explain our findings.

Ksander and Hendricks\textsuperscript{22} previously showed suppressed in vitro LPA and CTL responses after inoculating HSV-1 in the AC of mice. We studied these responses (LPA and CTL) in the resistant C.B-17 and susceptible BALB/cByJ congenic mice and found them to be similar. Therefore, LPA and CTL responses do not predict susceptibility to HSV-1-induced retinitis in this model.

In conclusion, HSV-1 spreads to the contralateral eyes of retinitis-resistant C.B-17 and retinitis-susceptible BALB/cByJ mice equally. Higher HSV-1 neutralizing antibody production in C.B-17 mice, and absence of suppression of HSV-specific DTH responses 10 days PI in BALB/cByJ mice, possibly led to the distinctive HSV-1-induced retinitis patterns observed in these mouse strains.

Key words: HSV-1, chorioretinitis, IgH-1 gene locus, murine, immune parameters

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References


