Effect of Fas and Fas Ligand Deficiency in Resistance of C57BL/6 Mice to HSV-1 Keratitis and Chorioretinitis

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PURPOSE. To investigate the effect of Fas and Fas ligand (FasL) deficiency on the development of herpes stromal keratitis and on the von Szily model of herpes retinitis in C57BL/6 mice, which are ordinarily resistant to development of both of these herpetic diseases.

METHODS. Anterior chamber inoculation of the right eye of each mouse with various titer of HSV-1 (KOS strain) was performed. Both eyes of each mouse were enucleated on postinoculation day 15 and processed for histopathologic examination. HSV-1 was inoculated into one cornea of other mice, and the severity of stromal keratitis was scored.

RESULTS. Contralateral destructive chorioretinitis developed in susceptible Balb/cByj mice (19/23); ipsilateral chorioretinitis did not occur (0/23). Stromal keratitis developed in susceptible C.AL-20 mice (15/16). None of the C57BL/6 mice (0/10 for keratitis or 0/20 for retinitis) developed inflammation. Neither did B6.SMN.C3H.lpr mice (Fasl deficient; 0/12 or 0/28) or B6.MRL.lpr mice (Fas deficient; 0/11 or 0/34) develop keratitis or retinitis, respectively. Minimal scattering of inflammatory cells in the contralateral retina but not destructive chorioretinitis was observed in two C57BL/6 mice, three B6.SMN.C3H.lpr, and five B6.MRL.lpr mice. Few inflammatory cells were also found in the ipsilateral vitreous and vitreoretinal interface (but not destructive chorioretinitis) of all C57BL/6, two gld, and three lpr mice.

CONCLUSIONS. Immune dysregulation secondary to deficiency in Fas or Fasl system does not influence the resistance of the C57BL/6 mice to develop herpes simplex keratitis or destructive herpes simplex chorioretinitis. (Invest Ophthalmol Vis Sci. 2001;42:2505–2509)

Unocular anterior chamber inoculation of herpes simplex virus type 1 (HSV-1) into Balb/cByj mice results in ipsilateral inflammation of the anterior segment with relative choroidal sparing and contralateral herpes simplex retinitis (HSR). This was first described by von Szily in 1924 in rabbits, was adapted to mice by Whittum and colleagues in 1998, and is commonly known as the von Szily model. Investigators have demonstrated in mice and rabbits that HSV-1 may spread from the inoculated eye to the contralateral eye through neural pathways to the brain. Virus is necessary but not sufficient to produce the contralateral HSR. Several studies have implicated an immunologic process in ipsilateral retinal sparing and contralateral HSR. However, the precise role of virus and the host immune response in the disease pathogenesis of HSR remains unclear.

We have identified, using Igh-1 disparate congenic murine strains, a profound influence of gene products from the Igh-1 or closely associated loci on susceptibility to development of the destructive inflammatory response to HSV in von Szily retinitis. Thus, Balb/cByj mice (Igh-1) are susceptible, whereas their Igh-1 congenic relatives C.B-17 (1gh-1b) and C57BL/6 mice (Igh-1b) are resistant to contralateral retinitis. The Igh-1–associated gene products control both humoral and cellular aspects of the immune response, and we have demonstrated that T cells, B cells, and natural killer cells and macrophages play a role in the pathogenesis of or protection from contralateral HSR and ipsilateral retinal sparing. The kinetics of the immune response, virus specific delayed-type hypersensitivity reactions and virus neutralizing antibodies, has been shown to differ in the resistant and susceptible mice.

Resistance to a second HSV-1–induced ocular disease, herpes simplex keratitis (HSK), is also controlled by the Igh locus. Inbred mouse strains that carry the Igh alleles (e.g., CAL-20 (Igh1)) are susceptible to necrotizing stromal keratitis induced by infection with HSV-1, whereas congenic strains that carry the Igh allele (C.B-17) are highly resistant. The resistance to HSK in mice is associated with allotypic variation in immunoglobulin genes, possibly because circulating immunoglobulin-derived peptides can cross-tolerize T cells specific for corneal tissue autoantigens.

Fas (CD95) is a 36-kDa cell surface protein that induces apoptosis in appropriate target cells. Fas is expressed on a variety of cell types, including hemopoietic, epithelial, and other cells. The ligand for Fas (FasL) has a more restricted pattern of expression. Expression of FasL is induced on mature T lymphocytes after their activation, but it is not expressed on any other hemopoietic cells. Fas–FasL interactions have been shown to limit the magnitude of helper T-cell–dependent B-cell activation. The constitutive expression of FasL in ocular tissue has been reported to play an important role in curtailing HSV-induced inflammatory response. However, these studies did not establish a role of Fas–FasL-dependent apoptosis in corneal and retinal destruction after HSV-1 infection of resistant mouse strains.

In this study, we ask if Fas–FasL interactions are responsible for prevention of ocular inflammatory damage after corneal or anterior chamber inoculation of HSV in HSK- and HSR-resistant mice. Therefore, we studied the effects of immune dysregulation...
tion secondary to Fas or Fasl deficiency on lpr (Fas deficient) and in gld (FasL deficient) mice, on the development of von Szily HSR and HSK in resistant C57BL/6 mice.

**METHODS**

**Virus**

HSV-1 (strain KOS) was cultured in VERO cells derived from African Green Monkey kidneys (CCL; American Type Culture Collection, Manassas, VA). Plaque assays were performed in the same cell line. Viral and cell cultures were grown according to previously described methods.10,24

**Animals**

Male and female HSV-1–susceptible Balb/cByJ (Igh-1a), C.AL-20 (Igh-1b), HSV-1–resistant C57BL/6 (Igh-1a), B6.SMN.C3H.gld (Fas-ligand deficient), and B6.MRL.lpr (Fas deficient) mice, aged 7 to 9 weeks, were housed in microisolators mounted in ventilated animal racks (VR-1; Laboratory Products, Inc., Rochelle Park, NJ). All mice were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**HSK Model**

**Inoculation.** Animals were anesthetized with a 0.1 ml intraperitoneal injection of a 1:1:2 mixture of 100 mg/ml ketamine hydrochloride, 20 mg/ml xylazine, and sterile water. The right eye of each mouse was scratched with a 23-gauge needle, and 5 μl 4 × 10^5 plaque-forming units (PFU) of HSV-1 (strain KOS) in minimum essential media (MEM; GIBCO Laboratories, Grand Island, NY) was deposited onto the scarified cornea. Ten C57BL/6, 16 C.AL-20, 11 B6.SMN.C3H.gld, and 12 B6.MRL.lpr mice per group were used.

**Clinical Observation and Grading.** The severity of clinical stromal keratitis was graded at day 14 using a Zeiss binocular surgical microscope (Thornwood, NY) and a scoring system, as previously described.25,53 Briefly, the geographic area of the active stromal keratitis was scored from 0 to 4+. Active stromal keratitis was defined as a stromal white blood cell infiltration of sufficient density to obscure the underlying intraocular structures and/or definite stromal ulceration. A clinical score of 1+ was consistent with a corneal involvement of < 25%; 2+ between 25% and 50%; 3+ between 50% and 75%; and 4+ > 75% of the cornea.

**HSR Model**

**Inoculation.** Animals were anesthetized with a 0.1 ml intraperitoneal injection of a 1:1:2 mixture of 100 mg/ml ketamine hydrochloride, 20 mg/ml xylazine, and sterile water. An anterior chamber paracentesis was performed in the right eye using a glass micropipette, and aqueous was drained to decompress the globe. The anterior chamber of the right eye was then inoculated with HSV using a 33-gauge needle or a 50-μL Hamilton syringe under binocular microscopy. The mice were divided into two groups. The first group consisted of 18 Balb/cByJ, 15 C57BL/6, 13 B6.SMN.C3H.gld, and 19 B6.MRL.lpr mice; each received an anterior chamber inoculum of 4 × 10^5 PFU of HSV-1 in 5 μl of MEM. The second group consisted of 5 Balb/cByJ, 5 C57BL/6, 15 B6.SMN.C3H.gld, and 15 B6.MRL.lpr mice; each received 6.2 × 10^5 PFU of HSV-1 in 5 μl of MEM.

**Histopathology.** Mice were killed by overdose with the anesthetic solution described above on the 15th day postinoculation. Both eyes were enucleated and processed for histopathologic examination. The globes were fixed in Karnovsky’s fixative (1% paraformaldehyde, 1.25% glutaraldehyde in 0.2 M sodium cacodylate buffer) for 24 hours at 4°C, rinsed in buffer solution, and dehydrated using serial ascending concentrations of ethanol. The globes were then infiltrated with glycol methacrylate solution overnight and then embedded in LKB Historesin (LKB Producker AB, Bromma, Sweden). Five-micrometer sections were prepared using a JR-4 microtome (Sorval; E. I. DuPont, Wilmington, DE) and stained with hematoxylin-eosin. Ipsilateral chorioretinitis was defined as the presence of focal or diffuse chorioretinal inflammation or destruction; contralateral chorioretinitis was defined as the presence of chorioretinal necrosis, vitreous cellular infiltration, or retinal edema.

All experiments were replicated, and all evaluations were conducted in a masked manner.

**RESULTS**

**Herpes Simplex Keratitis**

Ninety-four percent (15/16) of the susceptible C.AL-20 mice developed grade 4+ ipsilateral keratitis; one mouse (1/16) developed grade 2+ keratitis. None of the C57BL/6 (0/10), B6.SMN.C3H.gld (0/11), or B6.MRL.lpr (0/12) mice developed any degree of keratitis (Table 1).

**Herpes Simplex Retinitis**

Contralateral chorioretinitis developed in 83% (19/23) of HSV-susceptible Balb/cByJ mice: 16 of 18 mice inoculated with 4.0 × 10^5 PFU of HSV-1 and 3 of 5 mice inoculated with 6.2 × 10^5 PFU of HSV-1 (Fig. 1). Ipsilateral chorioretinitis with disorganization of the retinal architecture did not occur in any of the Balb/cByJ mice.

None of the C57BL/6, inoculated with either titer of HSV, developed contralateral chorioretinitis (Fig. 2). Few inflammatory cells were present in the vitreous and on the vitreoretinal interface in the ipsilateral eyes of all C57BL/6 mice, but the retinal architecture was preserved in 19 of 20 mice. One of the 15 C57BL/6 mice inoculated with 4.0 × 10^5 PFU of HSV-1 developed ipsilateral retinitis. Two of five C57BL/6 mice inoculated with 6.2 × 10^5 PFU of HSV-1 showed scattered inflammatory cells in contralateral retina, but the architecture was preserved in the contralateral retina of all 20 mice.

None of the B6.SMN.C3H.gld mice (0/28) or B6.MRL.lpr (0/34) mice developed contralateral chorioretinitis, after inoculation with both low and high titers of HSV (Fig. 3). Three of 15 B6.SMN.C3H.gld mice and five of 15 B6.MRL.lpr mice inoculated with 6.2 × 10^5 PFU of HSV-1 showed scattering of inflammatory cells in the contralateral retina, but the architecture was preserved in the contralateral retina in all gld and lpr mice. Inflammatory cells were present in the vitreous and on the vitreoretinal interface in the ipsilateral eyes of two gld and three lpr mice inoculated with 6.2 × 10^5 PFU of HSV-1, but the retinal architecture was preserved in 30 of 34 lpr and all gld mice. Three of the 19 B6.MRL.lpr mice inoculated with 4.0 × 10^5 PFU of HSV-1 and 1 of the 15 mice inoculated with 6.2 × 10^5 PFU of HSV-1 developed retinitis in the ipsilateral eye (Table 2).

**TABLE 1. Development of Grade 4+ Ipsilateral Keratitis after HSV Corneal Inoculation in Various Strains of Mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Cumulative</th>
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<tbody>
<tr>
<td>C.AL-20</td>
<td>9/9 (100)</td>
<td>6/7 (85)</td>
<td>15/16 (94)*</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>B6.SMN.C3H.gld</td>
<td>0/5 (0)</td>
<td>0/7 (0)</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td>B6.MRL.lpr</td>
<td>0/5 (0)</td>
<td>0/6 (0)</td>
<td>0/11 (0)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

* Fifteen C.AL-20 mice developed grade 4+ ipsilateral keratitis; one developed only grade 2+ keratitis. None of the mice in the other three strains developed any degree of keratitis.
DISCUSSION

There is currently no clear explanation for the exact mechanism of the pathogenesis of contralateral retinitis and relative sparing of ipsilateral retina after uniocular anterior chamber HSV inoculation in the von Szily mouse model of HSR. Evidence suggests that the presence of live virus in the posterior segment of the contralateral eye is necessary, although not sufficient for the development of HSR. Tracing studies have demonstrated in euthymic mice that the virus spreads from the injected eye to the contralateral optic nerve and retina via synaptically related neurons but does not spread to the ipsilateral optic nerve and the retina.

HSK resulting from HSV-1 (KOS) infection of C.AL-20 mice represents a virally induced autoimmune reaction against corneal tissues initiated by T cells. HSK can be mediated by T-cell clones specific for corneal self-antigens, which also recognizes an allotype-bearing peptide derived from IgG2a, and exposure of HSK-susceptible mice to a soluble form of this peptide (tolerization to it) confers resistance to HSK. Most recently, UL6, a coat protein of HSV-1 (KOS), has been shown to be recognized by these autoreactive T cells, which target corneal antigens, suggesting that this model of autoimmune disease may be exacerbated by viral mimicry. No such virion-associated protein has been identified in the HSR model.

Several studies have implicated immunologic processes in the pathogenesis of ipsilateral retinal sparing and the contralateral HSR after uniocular anterior chamber HSV-1 inoculation. The contributions by various participants of the immune system, such as T cells, B cells, and natural killer cells and macrophages, have been reported. The role of T cells in this interesting model has been elucidated by studies involving T-cell depletion in euthymic BALB/c mice using anti-CD4

FIGURE 1. Contralateral (left) chorioretinitis developed in HSR-susceptible Balb/cByj mice (magnification, ×100). The mice were anesthetized with intraperitoneal injection of a mixture of ketamine hydrochloride, xylazine, and sterile water. An anterior chamber paracentesis was performed in the right eye using a glass micropipette, and aqueous was drained to decompress the globe. Five microliters containing $6.2 \times 10^8$ PFU of HSV-1 was injected into the ipsilateral (right) anterior chambers of the mice. The mice were killed on the 15th day postinoculation. Both eyes were enucleated and processed for histopathologic examination. Five-micrometer sections were prepared using a JB-4 microtome and stained with hematoxylin-cosin.

FIGURE 2. The chorioretinal structure is preserved in the contralateral eye of the HSR-resistant C57BL/6 mice (magnification, ×100). The mice were anesthetized with intraperitoneal injection of a mixture of ketamine hydrochloride, xylazine, and sterile water. An anterior chamber paracentesis was performed in the right eye using a glass micropipette, and aqueous was drained to decompress the globe. Five microliters containing $6.2 \times 10^8$ PFU of HSV-1 was injected into the ipsilateral (right) anterior chambers of the mice. The mice were killed on the 15th day postinoculation. Both eyes were enucleated and processed for histopathologic examination. Five-micron sections were prepared using a JB-4 microtome and stained with hematoxylin-cosin.
(helper/inducer) and -CD8 (cytotoxic/suppressor) antibodies and adoptive transfer of selective T-cell subsets in athymic BALB/c and SCID mice. In athymic BALB/c (nude) mice, bilateral necrotizing retinitis develops after unilateral anterior chamber injection with HSV. Adoptive transfer studies have revealed that CD4\(^+\) cells can mediate destruction of the contralateral retina, whereas CD8 cells have been implicated in preventing contralateral retinitis.

Because depletion of B cells in resistant CB-17 mice results in bilateral chorioretinitis, B cells may indirectly contribute to inhibition of contralateral retinitis. We have also shown that the contralateral retina is protected in susceptible mice after intravitreal injection of anti-CD11, an antibody against immune effector cells, macrophages, and natural killer cells. Together, these studies suggest a role for immune-mediated protection of the ipsilateral retina and for immune-mediated destruction of the contralateral retina.

Recently, the Fas–FasL system has been demonstrated to limit the magnitude of helper T-cell-dependent B-cell activation. FasL is expressed constitutively on ocular tissue and has been suggested to play an important role in curtailing the virus-induced inflammatory response. We therefore studied the effect of immune dysregulation in Fas- and FasL-deficient mice on the development of retinitis in resistant C57BL/6 mice.

Griffith and colleagues reported the results of their studies in FasL-deficient congenics. In the C57BL/6 mice, the response was contained within the anterior segment, with only minimal spread of inflammatory cells into the vitreous cavity and no invasion of the retina by inflammatory cells or virus. In contrast, in gld mice (FasL deficient), there were numerous inflammatory cells in the vitreous cavity of ipsilateral eyes, with cells attached to and invading the retina and the optic nerve. In addition, there were numerous inflammatory cells attaching to and invading the cornea, inducing keratitis. The authors, on the basis of these results, concluded that the induction of apoptosis by Fas-FasL interactions is a potent mechanism of “immune privilege” and that the consequences of defective Fas-FasL interactions for the eye can be the spread of tissue-destructive inflammatory responses.

The results of our studies indicate that defects in the Fas or FasL system do not influence the resistance of C57BL/6 to von Szily HSR nor to the development of HSK. Despite an enhanced inflammatory response in the ipsilateral vitreoretinal interface and the infiltration of inflammatory cells in the inner retinal layers of the lpr and gld mice, compared with the wild-type C57BL/6 mice, there was no evidence of retinal architectural destruction.

Griffith et al. did not indicate whether they had examined the contralateral retina in their gld and lpr mice. Our definition of retinitis is based on the von Szily model, which requires destruction of retinal architecture, in addition to the presence of inflammatory cells on the retinal surface (which is a minor criterion in our description, but is used by Griffith et al. as

### Table 2. Development of Ipsilateral and Contralateral Retinitis after HSV-1 Anterior Chamber Inoculation in Various Strains of Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ipsilateral Eye</th>
<th>Contralateral Eye</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Group 1 (4.0 × 10⁵ PFU of HSV-1)</td>
<td>Group 2 (6.2 × 10⁶ PFU of HSV-1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6C3F1</td>
<td>0/18</td>
<td>0/5</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>1/15</td>
<td>0/5</td>
</tr>
<tr>
<td>B6.MRL.lpr</td>
<td>0/15</td>
<td>0/15</td>
</tr>
<tr>
<td>B6.MRL.gld</td>
<td>1/15</td>
<td>1/15</td>
</tr>
</tbody>
</table>

Values are number of mice, with percentage in parentheses.

* PFU, plaque-forming units.
evidence to show the role of Fas and FasL). A very small percentage of our gld and Ipr mice showed scattered vitreous inflammatory cells, which may be similar to the findings that Griffith et al. reported. However, ipsilateral or contralateral destructive retinitis was not seen. Thus, we cannot conclude that Fas and/or Fasl system influences the resistance (to the full extent of keratitis or retinitis) of C57BL/6 to von Szily HSR or to the development of HSK, as suggested by Griffith and colleagues. It has been shown that in the von Szily model, herpes virus is transmitted from the central nervous system to the retina of the contralateral eye by retrograde axonal transport through the optic nerve along the endocrine–optic pathway between the retina and the suprachiasmatic nucleus of the hypothalamus.5,6 The Fas or Fasl system may play a partial role in the resistance of certain murine strains (e.g., C57BL/6) to keratitis or retinitis. However, there may be other more dominant factors along the optic nerve transmission route, which maintain most of the HSV-1 resistance, even in the absence of the Fas or Fasl system. Although resistance to HSK and herpes retinitis in mice may, to some degree, involve activation-induced cell death, the presence or absence of Fas and Fasl does not play a significant role in genetic resistance to HSK and necrotizing retinitis.

References