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Ectopic Expression of Gamma Interferon in the Eye Protects Transgenic Mice from Intraocular Herpes Simplex Virus Type 1 Infections†

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Transgenic (rhoγ) mice provide a model for studying the influence of gamma interferon (IFN-γ) produced in the eye on ocular and cerebral viral infection. To establish this model, we injected BALB/c- and C57BL/6-derived transgenic and nontransgenic mice of different ages intravitreally with herpes simplex virus type 1 (HSV-1) strain F. Eye and brain tissues of these mice were assessed for pathological and immunocytochemical changes. HSV-1 infection induced severe retinitis of the injected eyes and infection of the brain in all mice. In transgenic mice inoculated with HSV-1, the left, nontreated eyes were protected from retinitis, whereas nontransgenic mice developed bilateral retinitis. Additional intravitreal injection of IFN-γ with the virus protected the noninoculated eyes of nontransgenic mice. Three-week-old nontransgenic mice died from HSV-1 infection, whereas transgenic mice of the same age and nontransgenic mice intravitreally treated with IFN-γ survived. Ocular IFN-γ production increased the extent of inflammation in transgenic mice but did not have a significant influence on the growth of HSV-1 until day 3 after inoculation and did not influence the neuroinvasion of this virus. Thus, the effects of IFN-γ were not caused by an early block of viral replication. Possible mechanisms of IFN-γ action include activation of the immune response, alteration of the properties of the virus, and direct protection of neurons.

Herpes simplex virus type 1 (HSV-1) infection of the human eye causes recurrent keratoconjunctivitis, frequently resulting in blindness (33a). HSV-1 can also induce acute necrotizing retinitis and encephalitis in immunocompetent and immunosuppressed patients (13, 29). However, immunodeficient individuals with HIV infection have a higher risk of developing herpetic infection of the retina and the brain. Severe forms of the AIDS-dementia complex might be associated with secondary infection of the brains with several viruses of the herpes family, mainly cytomegalovirus (12, 31, 41, 42). It is not known how these viruses enter the brain and cause chronic infection. The eye, including the retina, and the brain share an immune privilege featuring an efficient blood-tissue barrier and the absence of major histocompatibility complex (MHC) expression under normal conditions (21, 40, 48, 52, 53). However, it has been shown that most allografts are ultimately rejected in the brain (48), as opposed to the situation in the eye (55–57). Thus, the immune privilege might be more extended in the eye, since there is a strong active suppression of cellular infiltration and of delayed-type hypersensitivity to alloantigens presented in the eye, which is mediated by high amounts of immunosuppressive factors produced in the eye, such as transforming growth factor beta and α-melanocyte-stimulating hormone (54). In contrast to the brain (11), even peripherally induced delayed-type hypersensitivity is abrogated after intraocular exposure with the same antigen (51). Possibly, this impairment of the cellular immune response to viral infection influences the outcome of intraocular viral infection and facilitates the entrance of viruses to the brain via the eye as opposed to the peripheral route.

Gamma interferon (IFN-γ) is likely to be an important factor influencing the intraocular milieu. This cytokine probably reverses immunosuppression of the eye by antagonizing transforming growth factor beta, a major immunosuppressant present in the intraocular fluids (8), and thus is capable of influencing the intraocular microenvironment (14). Furthermore, IFN-γ has its own antiviral properties, among which is its impact on viral replication (18, 23, 37). Some studies suggested that the effects of IFN-γ might not be entirely favorable during corneal HSV-1 infection, since the cytokine seems to increase tissue damage (18).

We have developed a transgenic murine model in which IFN-γ expression overwhelms the immunosuppressive properties of the intraocular compartment. With apparently normal development of eyes and brain, these rhoγ transgenic mice express IFN-γ in the retinal photoreceptors from the first week of life to the age of 8 to 9 weeks. Rhoγ mice show alterations of the retina by 2 to 3 weeks of age, beginning with shortening of the photoreceptor outer segments, and subsequently lose their photoreceptors at the age of 8 to 9 weeks. Additionally, these animals develop cataracts and exhibit upregulation of both MHC class I and class II and increased infiltration of macrophages. Infiltration of T cells was not significant in these mice. No transgene expression was detected in the brain (14).

We infected 4- to 6-week-old transgenic mice, which still have photoreceptors producing IFN-γ, as well as nontransgenic mice of different genetic backgrounds with HSV-1 strain F (44). We used intravitreal injection because this route of infection facilitates the direct contact between virus and retina and promotes the spread of infection to the brain (3). To eliminate the effects of systemic IFN-γ production, we also infected 3-week-old mice which are reported to have a specific developmental lack of IFN-γ production (1). We found that

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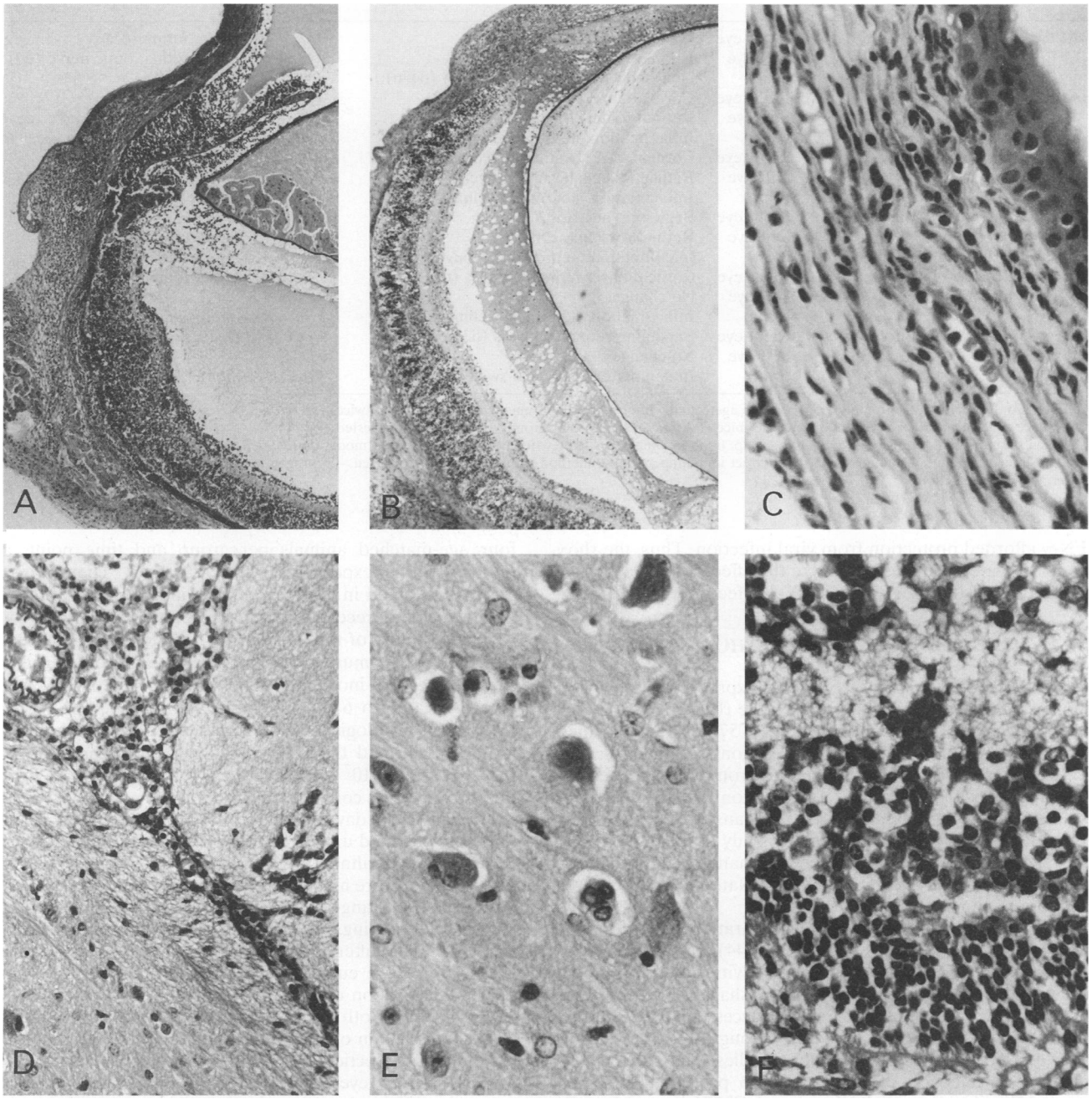


FIG. 1. Virus-induced pathology in eyes and brains of 5- to 6-week-old mice by day 6 after inoculation of HSV-1. (A) Rhoγ mouse, C57BL/6 derived, infected with HSV-1 strain F. The right eye displays an increase of cells in the cornea, the iris, the ciliary body, and the retina. Note free mononuclear cells in the anterior chamber and vitreous and exudate in both compartments. Remnants of the inoculum are recognizable in the subretinal space. PAS; magnification, ×48. (B) Nontransgenic mouse, BALB/c derived, infected with HSV-1. In the right eye, infiltration of all tissues and massive retinal destruction are clear. Note the relatively few infiltrating inflammatory cells compared with panel A. PAS; magnification, ×48. (C) Detail of the cornea from the nontransgenic mouse in panel B showing cellular infiltration of the whole cornea. PAS; magnification, ×480. (D) Detail of the brain from rhoγ mouse in panel A with mononuclear infiltration around the choroid plexus. PAS; magnification, ×240. (E) Detail of the brain from the same mouse showing swollen large neurons and cell ghosts. PAS; magnification, ×480. (F) Detail of the retina from the rhoγ mouse in panel A. Massive destruction of the retinal architecture is apparent, as is infiltration of the whole retina and underlying choroid with inflammatory cells. PAS; magnification, ×480.

TABLE 1. Pathology and course of disease in young mice infected with HSV-1 strain F

Mouse group[a] (age [wk])	Outcome	Organ	Pathology (time of detection)	Avg score of inflammatory cells[b]	Site of viral antigen[c] (time of detection)
BALB/c (3)	Death, d4–7[d]	Right eye	Massive retinal necrosis (d3)	+	Uvea, retina (d2)
		Left eye	Retinal folding, necrosis (d4)	0	Uvea, retina, optic nerve (d4)
		Brain	Neuronal swelling, inflammation (d4–6)	++	Diffuse (d4)
BALB/c (4)	50% death, d6–8	Right eye	Corneal haze, retinal necrosis (d3)	++	Uvea, retina (d2–3)
		Left eye	Retinal folding, necrosis (d5–6)	(+)	Uvea, retina, optic nerve (d5–6)
		Brain	Inflammation, neuronal swelling (d6)	++	Large suprachiasmatic areas (d6)
BALB/c × C57BL/6 (5–8)	Survival	Right eye	Corneal haze, beginning necrosis, (d3–4)	++	Uvea, retina (d3)
		Left eye	Retinal folding, beginning necrosis (d5–6)	+	Uvea, retina, optic nerve (d5–6)
		Brain	Inflammation, neuronal swelling (d6)	++	Suprachiasmatic area (d6)
BALB/c (3) + IFN-γ	Survival	Right eye	Beginning necrosis	++	Uvea, retina
		Left eye	Retinal folding, necrosis	+	Uvea, retina, optic nerve
		Brain	Inflammation, neuronal swelling	++	Suprachiasmatic area
Rhoγ, BALB/c derived (3)	Survival	Right eye	Corneal haze, retinal necrosis (d3)	++	Uvea, retina (d2–3)
		Left eye	No changes	0	—
		Brain	Inflammation, neuronal swelling (d5–6)	++	Suprachiasmatic area (d5–6)
Rhoγ, C57BL/6 derived (4–6)	Survival	Right eye	Beginning retinal necrosis (d3)	+++	Uvea, retina (d3)
		Left eye	No changes	0	—
		Brain	Inflammation, neuronal swelling (d6)	++	Suprachiasmatic area (d6)

[a] Each group consisted of three mice of the same age, usually littermates. Experiments were performed twice, except for those involving injection with anti IFN-γ (three mice) and injection of IFN-γ in 3-week-old mice (five mice). In all other groups, six animals were tested.
[b] Based on individual scores of all mice in a group. 0, no inflammation; +, few inflammatory cells; ++, moderate inflammation; +++, strong inflammation.
[c] Determined by immunostaining using the indirect immunoperoxidase method with DAB as a chromogen. —, antigen not detected.
[d] d*n*, day after infection.

IFN-γ afforded protection from viral infection. Thus, the rhoγ model provided a means to analyze the effects of ocular IFN-γ production on the progress of HSV-1 infection to the brain.

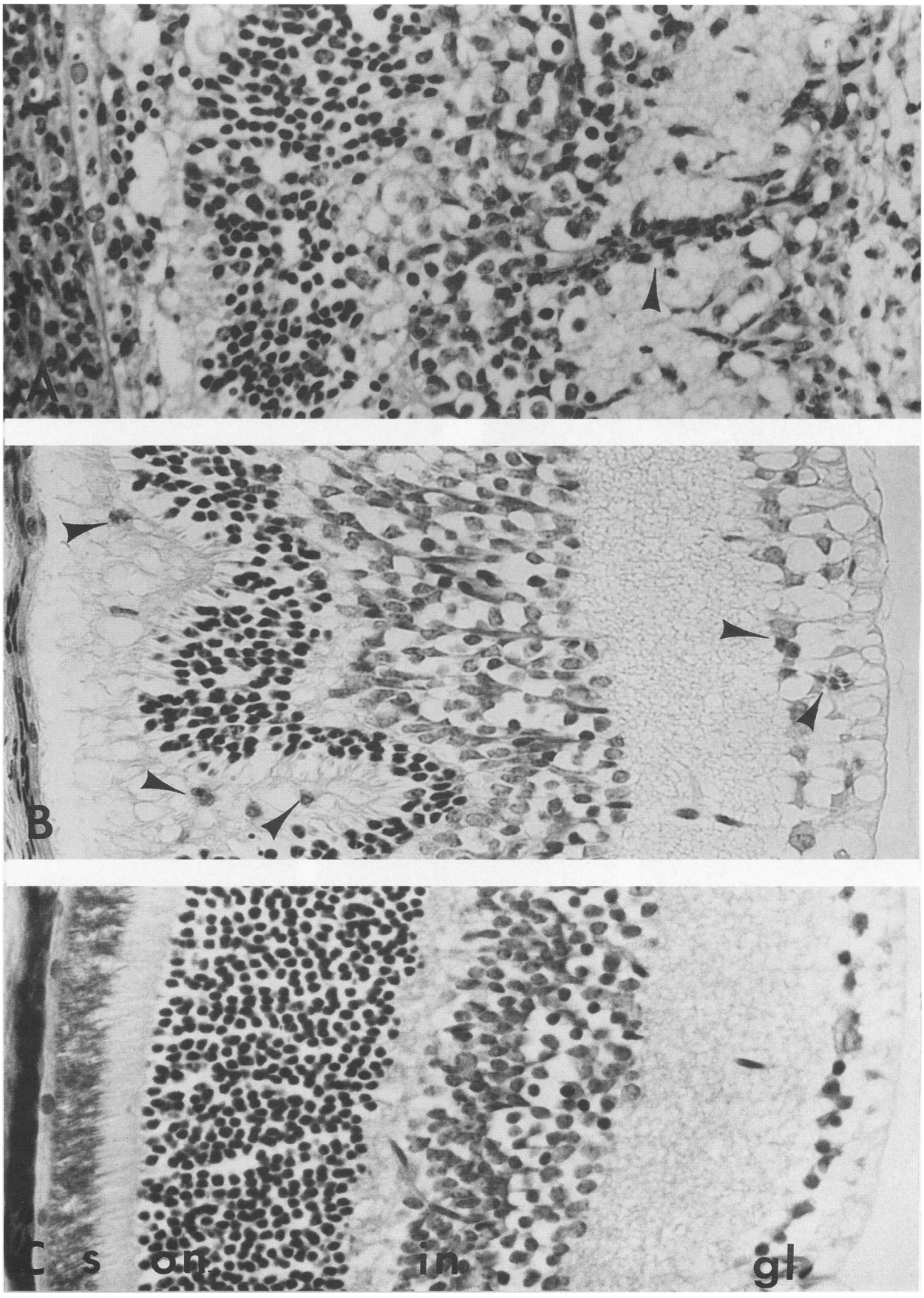
MATERIALS AND METHODS

Animals. Initially, to induce ectopic expression of IFN-γ in photoreceptors of the retina, we used the rhodopsin promoter to introduce the rhoγ transgene into C57BL76 mice, which were then crossed for four to six generations with BALB/c and C57BL/6 mice (14). These animals were routinely screened for the rhoγ transgene by PCR amplification of tail DNA and Southern blot hybridization, classed as transgenic or nontransgenic (littermates), and used for this study at 3 to 8 weeks of age. All animals were maintained and handled under veterinary supervision in accordance with National Institutes of Health guidelines.

Virus and injection protocols. A preparation (1 μl) containing 2×10^5 PFU of HSV-1 strain F (27, 44), passaged once by G. Lewandowski, was injected into the vitreous of each animal's right eye under visual control. A sharp puncture 2 mm behind the corneal limbus permitted access to the posterior chamber, where injection of virus through 10-μl Hamilton syringes with sterile, replaceable needles (26 gauge) was followed by injection of 2 μl of air to prevent reflux. We injected the virus into the right eyes of C57BL/6- and BALB/c-derived transgenic and nontransgenic animals, using three to four age-matched animals per group and time point, and performed the experiments at least twice. Therefore, the groups contained in total six animals unless stated otherwise. Mice which had received either a lower amount of virus or an accidental injury of the lens during injection, as confirmed by histology and immunocytochemistry, were removed from the study. Mice were inoculated with virus either at 3 and 4 weeks of age or at 5 to 6 weeks of age. Immunostaining for viral antigen on histological sections and the development of pathology confirmed the presence of infection. Either 1 μl of IFN-γ (1 mg/ml/10^7 IU; Genentech) or anti-IFN-γ (1 mg/ml; Pharmingen) was coinjected with the virus, and the procedure was repeated on day 4 after the initial injection. All animals' left eyes remained untreated. Animals mock infected by phosphate-buffered saline (PBS) inoculation served as controls. Infected mice were monitored daily for signs of disease, such as inflammatory changes of the skin and eyelids, rough coat, abnormal stretching, lethargy, ataxia, limb paralysis, and seizures. Animals suffering from seizures or other signs of severe encephalitis were euthanized. The other mice were killed by cervical dislocation or halothane overdose 3 to 10 days after viral injection. Both eyes and the brains were either fixed in 10% zinc formalin or immediately frozen in OCT compound.

To recover infectious virus, we homogenized (i) whole right eyes and (ii) left eyes and brains of at least three animals per group in 400 and 1,000 μl of PBS, respectively, with a mechanical homogenizer (Ultra-Turrax; IKA Laboratories).

FIG. 2. Pathology of the retina in HSV-infected mice. PAS; magnification, ×560. (A) Five-week-old rhoγ transgenic mouse, right eye, 8 days after intravitreal infection with HSV-1. Note beginning destruction of the retinal architecture and inflammatory cells in all layers of the retina, especially along a retinal vessel (arrowhead), and in the choroid. (B) Retina of an uninfected rhoγ mouse at 5 weeks of age, with folding of the photoreceptor layer, loss of the outer photoreceptor segments, and inflammatory cells in the photoreceptor layer between retinal folds and in the ganglion cell layer (arrowheads). (C) Normal retina of a 5-week-old BALB/c mouse without infection for comparison. s, photoreceptor segments consisting of inner and outersegments (darker area); on, outer nuclear layer containing the nuclei of the photoreceptors and the radial glia (Müller cells); in, inner nuclear layer containing the nuclei of amacrine and bipolar cells; gl, ganglion cell layer containing the nuclei of the ganglion cells and most of the retinal vessels. Note (i) the broad photoreceptor layer without folding or inflammatory cells and (ii) the intact segments of the photoreceptors.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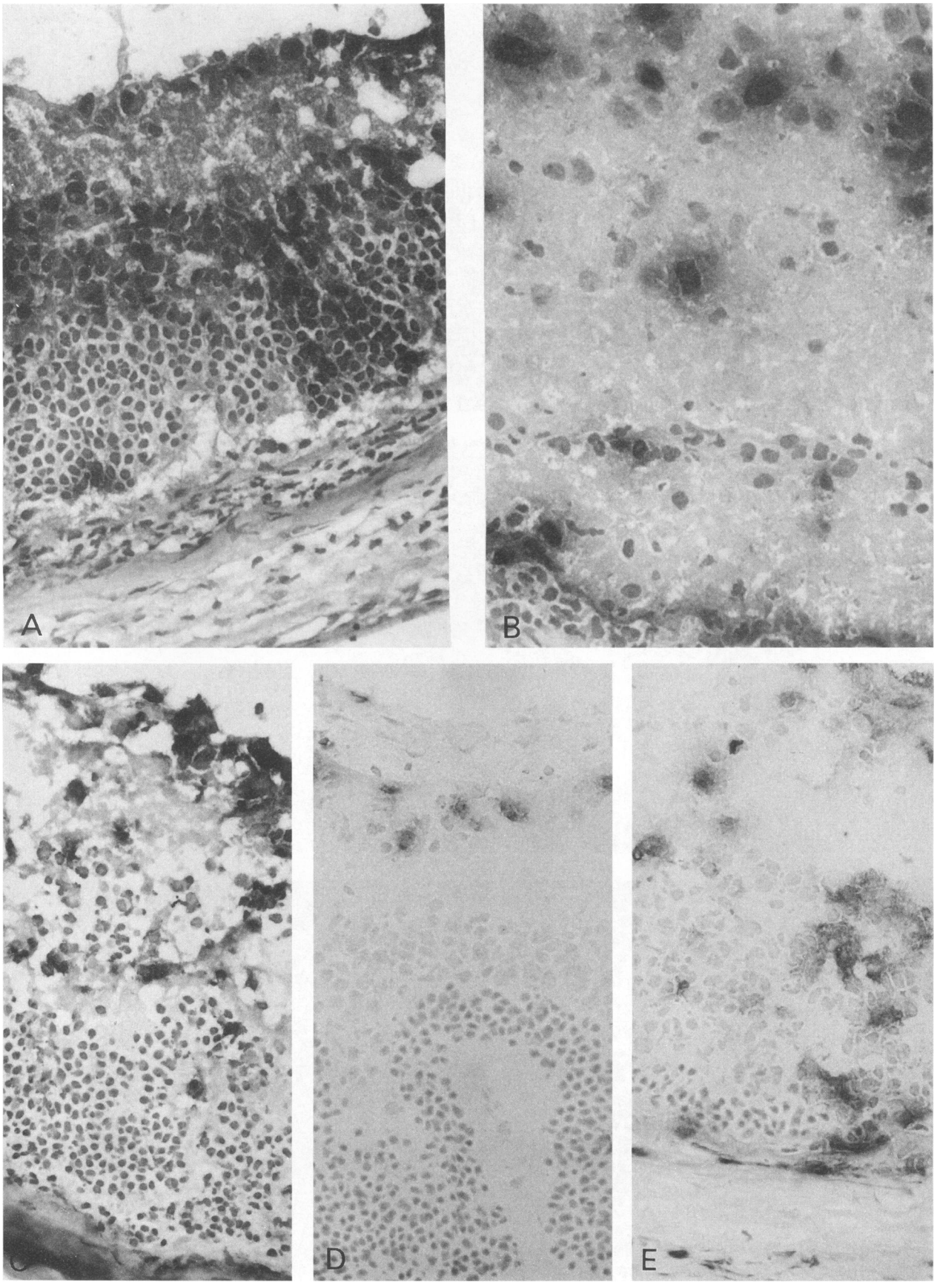
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All right eyes of a group were pooled together. The same procedure was used for left eyes and brains. After removing the cell debris by centrifugation at 2,000 rpm (Sorvall RT 6000B), we performed plaque assays on Vero cells with an incubation time of 60 min at 37°C, subsequent Dulbecco modified Eagle medium-agarose overlay, and development for 2 days. Viral yield was calculated from serial dilutions on plates yielding 10 to 100 plaques (7).

Histology and immunocytochemistry. Paraffin-embedded sections of brain and eye tissues were stained with hematoxylin-eosin or periodic acid-Schiff (PAS). Frozen serial sections, 6 μm thick, were cut and stained with hematoxylin or analyzed by immunocytochemistry. Immunostaining was performed by the indirect avidin-biotin-peroxidase complex method (Vector Laboratories) on frozen sections after fixation in cold acetone for 5 min. Primary antibodies (polyclonal anti-HSV-1 [Dako], monoclonal MAC-1 [Boehringer Mannheim], monoclonal Ly2, L3T4, and NK [Pharmingen], and monoclonal F4/80 [Serotec]) were applied at a concentration of 5 μg/ml; 0.05% diaminobenzidine (DAB)–0.04% nickel sulfate–0.02% hydrogen peroxide served as a chromogen. Counterstaining was performed either in hematoxylin (2 g/ml) or in 1.4% methyl green–PBS.

Ly2 (CD8)- and L3T4 (CD4)-positive cells were counted in a light microscope at ×20 magnification on 6-μm-thick sections of fresh-frozen whole eyes and counterstained with methyl green, using an underlying 1-mm grid as a reference. The counted areas included the corneal limbus but excluded the sclera, extraocular tissue, and optic nerve outside the eye.

RESULTS

Manifestation of disease. All mice inoculated with HSV-1 strain F developed ocular, neuronal, and systemic signs of infection (Fig. 1). Histological changes of the eyes included necrotizing retinitis similar to the acute retinal necrosis syndrome associated with HSV-1 infection of humans (13). However, the course of disease varied depending on both the age of the infected mice and the presence of IFN-γ in the inoculated eye.

Survival and protection from neurologic disease in young mice. The eyes and brains of all HSV-1-inoculated animals became infected (Fig. 1 to 3).[5] All 3-week-old and about 50% of the 4-week-old nontransgenic animals developed signs of encephalitis, such as hunched posture, rough coat, lethargy, anorexia, and ataxia, and died within 4 to 8 days, whereas adult BALB/c mice (5 to 6 weeks old) and rhoγ mice of all studied ages survived without clinical signs of encephalitis. Three-week-old nontransgenic mice which had received additional intraocular injection of IFN-γ together with the virus survived as well (Table 1).

Protection from HSV-1-induced infection of the contralateral eye. In rhoγ transgenic mice, the nontreated left eye was protected from retinitis. In contrast, all nontransgenic animals developed viral infection and retinitis in both eyes. Intravitreal coinjection of exogenous IFN-γ with the virus prevented the development of retinitis in the left, nontreated eye (Tables 1 and 2) in adult, but not 3-week-old, nontransgenic mice. Anti-IFN-γ coinjected with the virus did not alter the outcome of infection (Table 2). C57BL/6 and Balb/c mice proved equally susceptible to infection and to retinal damage.

Morphology and viral antigen in infected tissues. Viral antigen was found in all tissues of the inoculated eye (Fig. 3A), the optic nerve (Fig. 3B), the brain, and in most cases the trigeminal nerve, as determined by immunostaining. C57BL/6 mice had significantly less viral antigen in the cornea than did BALB/c mice, although they showed equal involvement of the uvea and the retina (not shown). Additionally, the noninoculated eyes of nontransgenic mice yielded HSV antigen by day 6, whereas no antigen could be found by immunostaining in the nontreated eyes of transgenic mice.

Viral antigen and pathological changes of the brain were located mostly in the optic and trigeminal projection, including the geniculate and the colliculus superior. The infection was most pronounced in the suprachiasmatic area. Aggregates of virus-infected cells and mononuclear cells, mostly small lymphocytes, were often located in the vicinity of blood vessels (Fig. 1D). Infected neuronal areas, especially the inner retina and the suprachiasmatic area of the brain (Fig. 1E and F), contained rounded, swollen cells. No multinucleated giant cells were visible. Viral antigen seemed to disappear faster from the brains of transgenic mice compared with nontransgenic mice, which by immunocytochemistry still had vast quantities of antigen in their brains at day 10 (not shown).

Stronger inflammatory response to viral infection in transgenic mice. The inflammatory response to HSV-1 comprised natural killer cells, macrophages (Fig. 3C), and T cells (Fig. 3D and E) in the eyes and brain, as confirmed by immunostaining for the antibodies MAC-1, NK, LFA-1, Ly2, and L3T4. Viral antigen and polymorphonuclear infiltration appeared simultaneously in the injected eyes. Both the extent and the numbers of infiltrating cells were greater in the oculated eyes of transgenic mice than in those of nontransgenic animals (Fig. 1F). In nontransgenic animals, inflammation was confined mainly to the uvea and cornea at early stages of infection (days 3 to 4); later, the retina was infiltrated. These infiltrates contained a relatively high number of natural killer cells and macrophages (Fig. 3C), whereas the inoculated eyes of transgenic mice contained more lymphocytes than did the eyes of nontransgenic animals (Fig. 3D and E).

Subsequently, the noninjected eyes of nontransgenic animals became infected, and inflammatory cells appeared around virally infected cells in the retina and the underlying choroid. However, the number of inflammatory cells in these eyes remained extremely low, in contrast with the high amount of viral antigen and ocular destruction seen in these mice, while transgenic mice were protected and showed no inflammation of the noninoculated eye above the background usually observed in our transgenic mice (14).

At the same time, viral antigen and inflammatory cells appeared in the brains of all animals. However, in the brain,

FIG. 3. Viral antigen and inflammation in infected mice at day 6 after intravitreal injection. Immunostaining was done by the indirect avidin-biotin-peroxidase complex method with DAB as the chromogen. (A) Rhoγ transgenic mouse infected with HSV-1 showing viral antigen in the choroid and all layers of the retina. Counterstained with hematoxylin; magnification, ×340. (B) Viral antigen in the optic nerve of the same mouse. Counterstained with hematoxylin; magnification, ×480. (C) Nontransgenic mouse infected with HSV-1. Staining with the antibody MAC-1 indicates macrophages and natural killer cells in the peripheral retina. Note positive cells in all layers of the retina, the choroid, and the sclera. Counterstained with hematoxylin; magnification, ×340. (D) Nontransgenic mouse infected with HSV-1. Staining was with antibody L3T4 (CD4). Positive cells are located mainly in the ganglion cell layer. Counterstained with methyl green; magnification, ×340. (E) Rhoγ transgenic mouse infected with HSV-1. Staining was with antibody L3T4 (CD4). Positive cells appear in all layers of the retina. Counterstained with methyl green; magnification, ×340.

TABLE 2. Pathology and course of disease in adult mice treated with IFN-γ or anti-IFN-γ and infected with HSV-1 strain F

Mouse group[a] (age [wk])	Organ	Pathology (time of detection)	Avg score of inflammatory cells[b]	Viral antigen[c] (time of detection)
BALB/c (5–6)	Right eye	Corneal haze, beginning retinal necrosis (d3–4[d])	++	Uvea, retina (d3)
	Left eye	Retinal folding, beginning retinal necrosis (d5–6)	+	Uvea, retina, optic nerve (d5–6)
	Brain	Inflammation, neuronal swelling (d6)	++	Suprachiasmatic area (d6)
Rhoγ, BALB/c derived (5–6)	Right eye	Corneal haze, beginning retinal necrosis (d3)	+++	Uvea, retina (d3)
	Left eye	No changes	0	—
	Brain	Inflammation, neuronal swelling (d6)	++	Suprachiasmatic area (d6)
BALB/c (5–6) + IFN-γ	Right eye	Corneal haze, beginning retinal necrosis (d3)	+++	Uvea, retina (d3)
	Left eye	No changes	0	—
	Brain	Inflammation, neuronal swelling (d6)	++	Suprachiasmatic area (d6)
Rhoγ, BALB/c derived (5–6), + IFN-γ	Right eye	Corneal haze, beginning retinal necrosis (d3)	++++	Uvea, retina (d3)
	Left eye	No changes	0	—
	Brain	Inflammation, neuronal swelling (d6)	++	Suprachiasmatic area (d6)
BALB/c (5–6) + anti-IFN-γ	Right eye	Corneal haze, beginning retinal necrosis (d3–4)	+++	Uvea, retina (d3)
	Left eye	Retinal folding, beginning retinal necrosis (d5–6)	+	Uvea, retina, optic nerve (d5–6)
	Brain	Inflammation, neuronal swelling (d6)	++	Suprachiasmatic area (d6)
Rhoγ, BALB/c derived (5–6), + anti-IFN-γ	Right eye	Corneal haze, beginning retinal necrosis (d3)	++++	Uvea, retina (d3)
	Left eye	No changes	0	—
	Brain	Inflammation, neuronal swelling (d6)	++	Suprachiasmatic area (d6)

[a] For compositions of groups, see Table 1, footnote *a*. +IFN-γ, additional intraocular injection of IFN-γ; +Anti-IFN-γ, additional intravitreal injection of anti-IFN-γ. All mice survived.

[b] Based on individual scores of all mice in a group. 0, no inflammation or no inflammation above background in transgenic mice; +, few inflammatory cells; ++, moderate inflammation; +++, strong inflammation; ++++, whole organ infiltrated with inflammatory cells.

[c,d] See Table 1, footnotes *c* and *d*.

these differences were less drastic; both transgenic and nontransgenic mice developed nearly the same amount of inflammation.

The effect of adding IFN-γ to the viral inoculum was notable in that injected eyes of nontransgenic animals given the mixture showed nearly the same morphology and immune response as did their transgenic littermates.

Increase of CD4 and CD8 cells in infected tissues of transgenic mice. To ascertain the type of inflammatory cells observed, we quantified CD4 (L3T4) and CD8 (Ly2) cells. The difference between transgenic mice, which showed three- to five-times-higher total cell counts and relatively more CD8 cells from the beginning of infection (Fig. 4), and nontransgenic animals was quite striking. In the noninjected eyes of nontransgenic mice, these T-cell subsets were still enhanced (Fig. 4), but their numbers did not match the amount of herpetic pathology. The cell counts and the CD8/CD4 ratio were scarcely influenced by administration of anti-IFN-γ and were not significantly different in the brains of transgenic and nontransgenic mice at 5 weeks of age (not shown). Additional intraocular treatment with IFN-γ produced an increase of L3T4- and Ly2-positive cells in the inoculated eyes of nontransgenic mice.

Enhanced MHC expression in infected tissues of transgenic mice. In both transgenic and nontransgenic animals, all affected tissues expressed an overall increase of MHC class I and class II antigens on activated glial cells and infiltrating inflammatory cells. Both MHC classes were expressed more abundantly in transgenic animals, which already express MHC class I and class II in the retina without viral infection (14), than in the nontransgenic group (Fig. 5).

No early viral replication block in transgenic mice. To determine whether the survival of transgenic mice after HSV-1 infection resulted from a block of viral replication induced by IFN-γ, we attempted to recover infectious virus from infected tissue homogenates. Virus was recoverable by day 1 from the injected (right) eyes of all animals studied and from the brains and the noninjected (left) eyes on day 2, a time when viral antigen was not yet detectable by immunostaining, which is a considerably less sensitive method for the detection of virus. No virus was recovered from noninfected control animals or from animals infected for more than 3 weeks. At days 1 to 2, tissues of transgenic and nontransgenic mice infected with HSV-1 contained similar amounts of virus (Fig. 6). Subsequently, the relative amount of viral yield decreased in the transgenic mice. By day 4, no virus could be recovered from the noninoculated left eyes of these mice.

DISCUSSION

Intraocular production of IFN-γ rendered mice resistant to intraocular infection with HSV-1. After transgenic mice received HSV-1 injections in one eye, their ectopic expression of IFN-γ protected the other eye from virus-induced pathology, although it briefly contained infectious virus. The same effect was achieved in adult nontransgenic mice by coinjection of IFN-γ with the virus. However, the cytokine did not prevent the virus from spreading to the brain.

The lethal susceptibility to HSV-1 strain F of 3-week-old BALB/c mice could be related to the selective defect of IFN-γ production in mice of this age (1). This defect is correlated with the lack of IFN-γ production in response to viral infection in macrophages (28) and the unresponsiveness of natural killer cells to stimulation with IFN-γ (43). Adult-level responsiveness to stimulation with IFN-γ is reached at 4 to 5 weeks of age (43), whereas the full capacity to produce this cytokine follows much later (1). Protection afforded in 3-week-old transgenic mice could be due to the local production of IFN-γ. Consistently, 50% of the 4-week-old nontransgenic animals which are reported to have systemic IFN-γ production, although considerably below adult levels (1), survived. However, the amount of additionally given IFN-γ was apparently insufficient to protect the second eyes of young nontransgenic mice. Thus, we do not know whether other factors, such as induction of resistance to virus-induced damage on a cellular level or an age-related

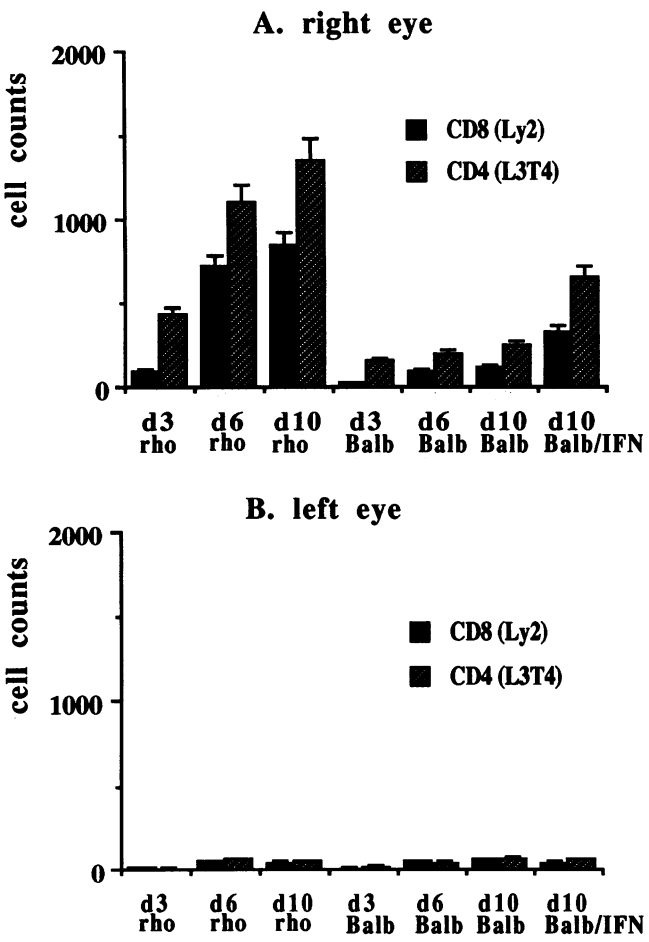

FIG. 4. Counts of CD4 (L3T4) and CD8 (Ly2) cells in eyes of 5- to 6-week-old mice infected with HSV-1. Sections of 6 μm were analyzed; immunostaining was with DAB as the chromogen, and counterstaining was with methyl green. Ly2- and L3T4-positive cells were counted in the light microscope at ×20 magnification of whole eyes. The counted areas included the corneal limbus but excluded the sclera, extraocular tissue, and optic nerve outside the eye. The plot uses mean values of groups of three animals (5- to 6-week old BALB/c-derived mice). The variance of recounts the same eye stayed below 5%; however, individual mice varied as much as 10% within a group. Error bars represent the standard deviation of each group ($x\sigma n - 1$). d*n*, day after infection.

increased susceptibility of neurons to HSV-1 (32), play an additional role.

The properties of IFN-γ that could be responsible for the observed effects include block of viral replication, activation of macrophages and natural killer cells, and enhancement of cytotoxicity. Alternatively, IFN-γ could act indirectly via the increased MHC expression in transgenic mice (5, 6, 10, 17, 19, 36, 39, 46, 54). Because we found nearly equal amounts of infectious virus in transgenic and nontransgenic mice until day 2 after infection, a time when the virus was already present in the second eye, any block of viral replication would occur too late to affect its invasion of the brain. This result fits well with earlier reports describing an 18-h delay between the presence of IFN-γ and the beginning of effects on viral replication in culture (2a, 23). Although we cannot exclude the possibility that a small pool of replicating virus can influence the outcome of infection at a late stage, our results do explain the failure of IFN-γ to prevent the neuroinvasion of HSV-1. Since we observed an influence of IFN-γ in our model, which was similar to that observed on cultured cells, it is not likely that retinal changes present in uninfected transgenic mice have an influence on the behavior of the virus. Thus, it is possible that a disturbance of the blood-retina barrier in transgenic mice has an influence on the intraocular immune privilege (35, 52). Exactly why the second eyes of transgenic mice do not develop pathology, despite the small amounts of virus recovered at day 2, why these mice show no clinical signs of encephalitis, and why they survive an otherwise lethal infection when infected at 3 weeks of age remain conjectural. Once in the brain, the virus should replicate with equal speed in transgenic and nontransgenic mice, unless the cytokine has either directly influenced the propagation characteristics of the virus during its passage through the eye or sent a systemic signal protecting infected neurons from death. IFN-γ might similarly send a systemic signal to boost an immune response in the brain, although increased inflammation in the brain would not necessarily have a positive effect. The amounts of inflammation in the brains of transgenic and nontransgenic mice did not differ greatly. Instead, the lack of neurologic symptoms in young transgenic mice could indicate its protective effect. Since HSV-1 strain F has been shown to develop latency (33, 34), it is not likely that the virus is ever cleared from surviving infected cells. Therefore, the more rapid loss of virus detection from the brains of transgenic mice could mean that IFN-γ favors the pathway toward latency. Additional mechanisms, including synergistic action with antibodies (49, 50) and other cytokines such as tumor necrosis factor alpha (45) or protection of infected neurons from being killed by cytotoxic T cells (30), should be considered. This might involve a prevention of apoptosis as demonstrated for antibody action in Sindbis virus (16, 26). Furthermore, we do not know the role of IFN-γ-induced production of nitric oxide in the development of disease (9, 15, 22).

We detected a greater infiltration of leukocytes in the eyes of the transgenic mice than in those of nontransgenic animals, possibly reflecting a faster and more adequate immune response in the rhoγ animals. Since the infiltrates contained natural killer cells, macrophages, and cytotoxic T cells, multiple mechanisms could be responsible. The already present MHC class I expression on retinal cells in transgenic mice (14) could play an important role in this process by presenting HSV antigen to CD8 cells.

The finding that C57BL/6 and BALB/c mice were equally susceptible to infection with HSV-1 was unexpected. C57BL/6 mice, whose MHC class I haplotype has been recognized as a major factor for genetically determined protection (38, 47), are regarded as comparatively resistant to HSV-1 infection. However, the intraocular mode of infection and the viral strains that we used apparently overrode possible genetic protection, although this does not necessarily support the conclusion that MHC class I might be less important than other factors in accounting for the activities of this virus, since MHC class I is not likely to be expressed on neurons under normal conditions. Apart from infiltrating cells, the MHC class I expression that we found in the retinas and brains of nontransgenic mice is probably located on activated glial cells, consistent with earlier studies of HSV-1 strain F infection in rats (60).

The substantial number of CD4 and CD8 cells present in this model confirms the importance of cytotoxic T cells, which are not present in uninfected rhoγ mice (14), in intraocular viral infection (4, 20, 24, 25, 61, 62) and suggests that CD8 cells, the amounts of which are low in the left untreated eyes of nontransgenic mice, might have a crucial role in the outcome

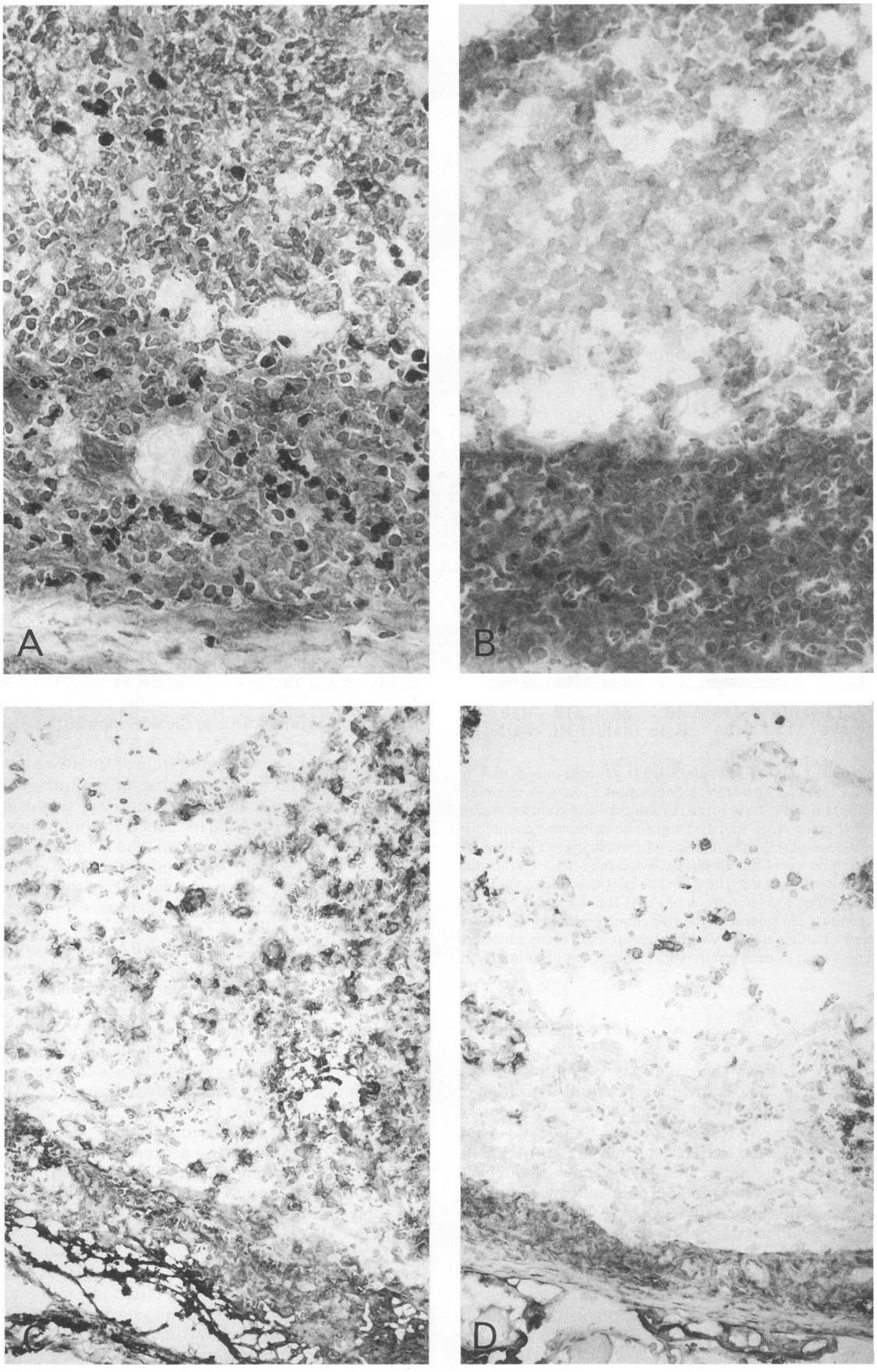
A
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FIG. 5. Immunostaining for MHC class I in right eyes of transgenic and nontransgenic mice infected with HSV-1 at day 6. Indirect immunoperoxidase technique with DAB as the chromogen, counterstaining with hematoxylin; magnification, ×400. (A) Rhoγ transgenic mouse. Staining of the retina with MHC class I antibody (H-2) shows positive cells through all layers of the retina. (B) Nontransgenic mouse with only a few positive cells in the inner retina and in the choroid. (C) Rhoγ transgenic mouse. Staining of the retina with MHC class I antibody (H-2) shows positive cells in all layers of the retina. (D) Nontransgenic mouse with few positive cells in the inner retina and in the choroid.

of disease. If so, the MHC hyperexpression induced by ectopic IFN-γ could be capable of overriding a possibly subtle interference with antigen expression (58, 59) by the virus.

Our data show that IFN-γ can protect animals from HSV-1 infection of the eye without influencing its neuroinvasion. That this occurs without a significant increase in retinal damage suggests either involvement of synergistic factors or counterregulation by the intraocular microenvironment. Possible changes in the immunosuppressive properties of the intraocular microenvironment might be responsible for the protection of the second eyes in transgenic mice (2). The intraocular immune privilege could be influenced either by IFN-γ directly or by a cytokine-induced disturbance of the blood-retina barrier. Although development of rhoγ mice is apparently normal (14), IFN-γ could have a subtle effect on the neuronal development which could influence the susceptibility of these neurons to virus infection. However, it is not likely that the observed alterations of the neuronal morphology in rhoγ mice are to influence the neuronal spread of HSV-1. These alterations are mainly present in the photoreceptors of the retina, and the spread of HSV-1 to the brain is not dependent on the function of the photoreceptors. In fact, the earliest appearance of viral antigen by staining occurred in the ganglion cell layer of rhoγ and nontransgenic mice without a significant difference. Therefore, the virus could travel directly to the brain via the ganglion cells. A further pathway, using the iris and the ciliary ganglion, is also not likely to be anatomically influenced by the presence of IFN-γ in the eye.

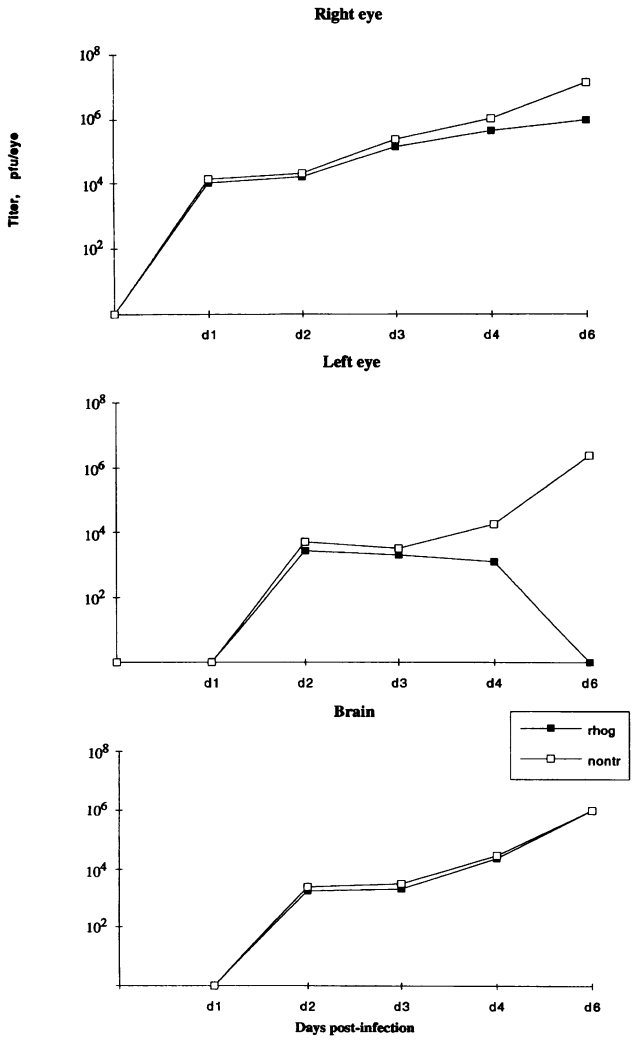

FIG. 6. Recovery of infectious virus from eyes and brains of 5- to 6-week-old mice infected with HSV-1 strain F. Each group per experiment consisted of three mice. Whole right eyes (three per group) and left eyes and brains were pooled separately, homogenized in a fixed volume as described in Materials and Methods, and processed for plaque assay on Vero cells. The values shown are based on the means of two identical experiments, the results of which had a variance below a factor of 3. rhog, rhoγ; nontr, nontransformed.

The mechanisms involved in protective effects of IFN-γ remain speculative. Thus, they are likely to induce a more chronic course of disease, including the possibility of local reactivation and slowly developing dysfunction of infected cells, parallel to the phenomena observed in AIDS dementia (31). The rhoγ mouse has proved to be a suitable tool for manipulating IFN-γ function in the eye, particularly in terms of viral infection.

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