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Cytokine-mediated survival from lethal herpes simplex virus infection: Role of programmed neuronal death

(interferon γ/transgenic mice/apoptosis/*Bcl2*)

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ABSTRACT **The mechanisms responsible for cytokine-mediated antiviral effects are not fully understood. We approached this problem by studying the outcome of intraocular herpes simplex (HSV) infection in transgenic mice that express interferon γ in the photoreceptor cells of the retina. These transgenic mice showed selective survival from lethal HSV-2 infection manifested in both eyes, the optic nerve, and the brain. Although transgenic mice developed greater inflammatory responses to the virus in the eyes, inflammation and viral titers in their brains were equivalent to nontransgenic mice. However, survival of transgenic mice correlated with markedly lower numbers of central neurons undergoing apoptosis. The protooncogene *Bcl2* was found to be induced in the HSV-2-infected brains of transgenic mice, allowing us to speculate on its role in fostering neuronal survival in this model. These observations imply a complex interaction between cytokine, virus, and host cellular factors. Our results suggest a cytokine-regulated salvage pathway that allows for survival of infected neurons.**

Immune responses to viruses in the central nervous system (CNS) are not fully understood but involve the action of several cytokines that contribute to the development of pathology. The pleiotropic cytokine interferon γ (IFN-γ) may have an important role in this process. IFN-γ has the capacity to activate macrophages, natural killer cells, as well as B and T lymphocytes (1). This cytokine also plays a crucial role in experimentally induced allergic encephalitis (2) and induces expression of major histocompatibility complex class I and class II antigens on astrocytes and microglia (3–5). The antiviral activities of IFN-γ have been attributed mainly to influences on the replication of viruses (6–10).

We have previously studied the effects of IFN-γ on herpes simplex virus type 1 (HSV-1) infection of the CNS using transgenic mice that express IFN-γ restricted to the photoreceptors of the retina (rhoγ mice) (11). We found that the ectopic expression of IFN-γ provided protection for mice infected with HSV-1 strain F. This effect was associated with increased inflammation in the eyes of infected mice but did not influence the neuroinvasion by the virus or viral replication in the eyes and the brain (9). In contrast to the known harmful effects of IFN-γ on the brain the apparently lesser extent of morphological damage to the brain led us to suspect other mechanisms of neuronal protection afforded by IFN-γ.

To investigate this possibility, we tested the influence of intraocular IFN-γ on the survival of mice after infection with the lethal HSV-2 strain 186 (12), which produced encephalitis in infected mice. The rhoγ transgenic mice and nontransgenic mice received intravitreal injection of HSV-2 or HSV-1 strain F (13) as a control. Intravitreal inoculation of the virus leads to rapid viral spread to the brain without mechanical disruption of the blood–brain barrier. To investigate the mechanisms involved in the possible protective effects of IFN-γ, we quantitated infectious virus recovered from tissues of infected animals and studied the occurrence of apoptosis in the brain. We found that the increased survival in transgenic mice correlated with markedly decreased apoptosis in the brain. We further studied the expression of the protooncogene *Bcl2*, which has been associated with the prevention of apoptosis (14) and the regulation of survival of developing neurons (15) in the brain. We detected the up-regulation of *Bcl2* expression in the HSV-2-infected brains of transgenic mice, providing a clue to the mechanism for the observed survival.

MATERIALS AND METHODS

Animals. We used BALB/c-derived rhoγ transgenic mice and nontransgenic littermates between 4 and 6 weeks of age, with three to four age-matched animals used per group and time point. All experiments were repeated at least once. Transgenic animals were routinely screened for the transgene using PCR amplification of tail DNA.

Virus and Infection Protocol. HSV-1 and HSV-2, strain 186 (provided by R. Schrier, University of California at San Diego) were grown on Vero cells, plaque-purified, and titrated by using the plaque-forming assay (16). One microliter containing 2×10^5 plaque-forming units (pfu) of HSV-1 or 2×10^4 pfu of HSV-2 was injected into the vitreous humor of each animal's right eye as described (9). One microliter of IFN-γ (Genentech; 1 mg/ml per 10^7 international units) containing 10^4 international units was coinjected with the virus or injected alone. The injection was repeated once at day 4 after the initial inoculation. Animals mock-injected with phosphate-buffered saline (PBS) served as controls. Sacrifice was done at one to five time points between day 1 and day 12 after infection. Infected mice were monitored daily for signs of disease. Animals with symptoms of severe encephalitis were euthanized. Both eyes and the brain were removed and fixed either in 10% (vol/vol) neutral buffered formalin or immediately frozen in OCT compound and sectioned in a cryostat (Reichert Jung). The mounted sections were fixed for 5 min in cold acetone before staining.

Histology and Immunocytochemistry. Four-micrometer-thick paraffin-embedded sections and 6-μm-thick frozen sections were stained with hematoxylin/eosin or periodic acid/Schiff for morphological evaluation. Immunohistochemistry was done by using the indirect avidin–biotin–peroxidase com-

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Abbreviations: HSV, herpes simplex virus; IFN-γ, interferon γ; CNS, central nervous system; pfu, plaque-forming units; DAB, diaminobenzidine; GFAP, glial fibrillary acidic protein; NSE, neuron-specific enolase.

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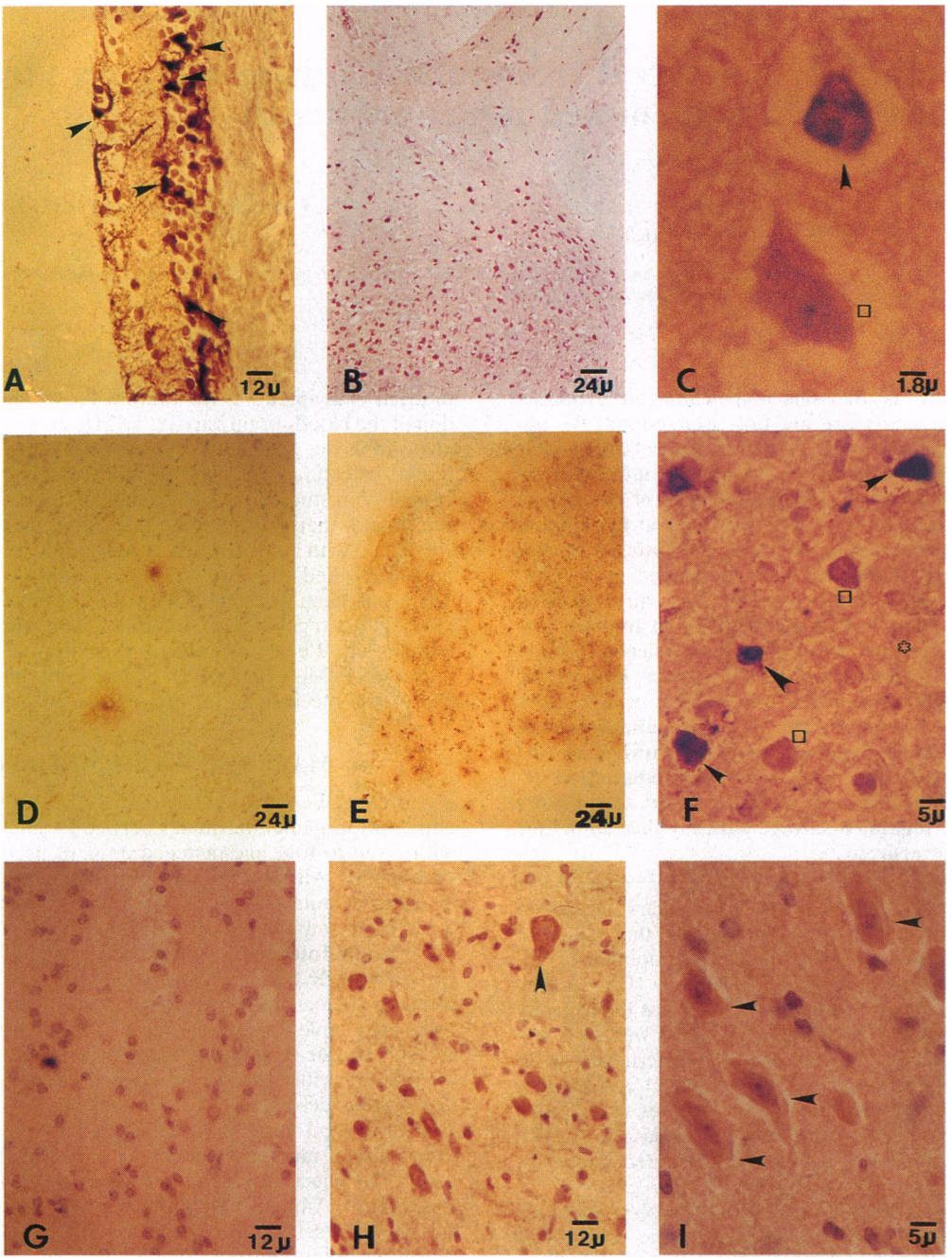

FIG. 1. (*A*) Detection of viral antigen in the retina of the left noninoculated eye at day 6 after inoculation with HSV-2 (transgenic mouse, 6 weeks old). Typically for a transgenic mouse, the photoreceptors of the retina are diminished. Cells positive for viral antigen (arrowheads) are located in all layers of the retina, which shows only slight destructive changes (paraffin-embedded tissue, indirect immunoperoxidase technique with DAB as a chromogen, and hematoxylin counterstaining). (×280.) (*B*) Viral antigen in the trigeminal root zone of a transgenic mouse day 9 after infection (paraffin-embedded tissue, indirect immunoperoxidase technique with DAB as a chromogen, and hematoxylin counterstaining). (×130.) (*C*) Apoptosis in the brain of an infected day-8 nontransgenic mouse after infection with HSV-2. The apoptotic neuron shown (arrowhead) is smaller in size than a neighboring normal looking neuron (square) and has a hyperdense fragmented nucleus (paraffin-embedded tissue, hematoxylin/eosin staining). (×1650.) (*D* and *E*) Apoptosis in the brain at day 8 or 9 after infection with HSV-2 (immunoperoxidase detection of digoxigenin-labeled DNA fragments with DAB as a chromogen, in fresh-frozen tissue, counterstained in methyl green). (×130.) (*D*) Transgenic mouse (day 9) demonstrating comparatively few apoptotic cells. (*E*) Nontransgenic mouse (day 8) with large clusters of cells undergoing apoptosis in an anatomically similar location. (*F*) Colocalization of apoptosis and HSV in the brain of 4- to 6-week-old nontransgenic mice after HSV-2 infection. Dark staining (arrowheads) illustrates virally infected cells undergoing apoptosis. Lighter purple staining is seen in virally infected cells without apoptosis (squares). Uninfected cells appear almost unstained (*) (ApopTag, digoxigenin–peroxidase with DAB as a chromogen, double-labeling with HSV using the indirect peroxidase technique and Vector VIP as a chromogen in paraffin-embedded tissue, and counterstaining with hematoxylin). (*G–I*) Immunostaining for *Bcl2* in the brains of 5- to 6-week-old transgenic and nontransgenic mice at day 8 or 9 after infection with HSV-2 (paraffin-embedded tissue, indirect immunoperoxidase technique with Vector VIP as a chromogen and hematoxylin counterstaining). (*G*) Cortex of an infected nontransgenic mouse without positive cells. (*H*) Anatomically similar area of an infected transgenic mouse with clusters of positive cells, showing positive staining (purple) within the cytoplasmatic area (arrowhead). (*I*) Detail of the cortex of the mouse in *H*, identifying the *Bcl2*-expressing cells mostly as neurons (arrowheads). μ = μm. (*G* and *H*, ×280; *I*, ×800.)

plex method (Vector Laboratories). Primary antibodies were applied at a concentration of 5 μg/ml. These antibodies were polyclonal HSV-2 and antiglial fibrillary acidic protein (GFAP) (Dako), polyclonal antineuron-specific enolase (NSE) (BioGenex Laboratories, San Ramon, CA) monoclonal MAC-1, H-2, Ia, LFA-1 (Boehringer Mannheim), monoclonal Ly-2, L3T4, natural killer cells (PharMingen), and monoclonal F4/80 (Serotec). Diaminobenzidine (DAB; Sigma) at 0.05% in 0.02% hydrogen peroxide/phosphate-buffered saline and Vector VIP served as chromogens. Counterstaining was done in hematoxylin at 2 g/ml of PBS or 1.4% methyl green/PBS. Apoptosis was tested on paraffin-embedded frozen tissue using the ApopTag Kit (Oncor), based on *in situ* labeling of fragmented 3′-OH DNA ends, with a digoxigenin–peroxidase-labeled secondary antibody and DAB as a chromogen. For double-labeling with polyclonal HSV-1/2 (Dako), NSE (BioGenex Laboratories), and GFAP (Dako), Vector VIP was used as substrate. Only cells fulfilling the morphological criteria of apoptosis were counted and compared in sections from similar anatomical locations, at a magnification of ×20 in the light microscope with an underlying 1-mm grid for reference. Polyclonal anti-mouse *Bcl2* (1634, La Jolla Cancer Foundation) (17) was used on paraffin-embedded sections after microwaving twice for 5 min in 10 mM sodium citrate, pH 6.2. Enzyme-linked immunosorbent assay (ELISA) for IFN-γ on homogenized brain extracts of infected mice and transgenic and nontransgenic uninfected controls was done by using a two-antibody sandwich assay (PharMingen). Three brains per group were homogenized in 2 ml of PBS for 15 sec using a mechanical homogenizer (Ultra-Turrax; IKA). Cell debris was removed by 10-min centrifugation with 2000 rpm (Sorvall RT 6000B). The supernatant was used for ELISA.

To recover infectious virus, we homogenized tissues of eyes and brains of five mice per group separately in 400 or 1000 μl of PBS, respectively, as described above. Plaque assays were done on Vero cells with an incubation time of 60 min at 37°C, subsequent Dulbecco's modified Eagle medium (DMEM)/agarose overlay, and development for 2 days. Viral yield was calculated from serial dilutions on plates yielding 10–100 plaques (9, 16).

RESULTS

Manifestations of Disease. All mice infected with HSV-1 survived the infection and developed pathology in eyes and brain, as described (9). All mice infected with HSV-2 became sick by day 5 or 6, displaying a rough coat, lethargy, abnormal stretching, and beginning ataxia as scored visually and by the capacity of limb usage. However, only the nontransgenic mice progressed to forelimb paralysis and seizures and died by day 7 or 8 from encephalitis. In contrast, infected transgenic mice survived and recovered completely from their symptoms by days 12–14. Nontransgenic mice treated with IFN-γ developed the same amount of macroscopic pathology as untreated animals but survived until day 9.

Histological and Immunohistochemical Assessment of the Eyes and Brains from Mice Infected with HSV-2. Morphological changes and viral antigen detected by immunostaining were evident in all inoculated eyes at day 3 after infection with HSV-2, including moderate cellular infiltration of the whole eye but only minor retinal destruction. By days 5–7 pathology and expression of viral antigen were seen in the brain, in both optic nerves with their enveloping sheaths, and in the uninfected eye of all intravitreally inoculated mice (Fig. 1 *A* and *B*). These changes correlated with considerable damage of the inner retina, choroidal swelling of the injected eye, and beginning destruction of the retina in the contralateral, uninjected eye. Eyes of transgenic mice showed the same amount of retinal damage but more inflammatory cells when compared with the eyes of control animals.

Virus-induced pathology in the brains consisted of a moderate increase of mononuclear cells, especially around vessels, expression of viral antigen, and neuronal damage. Some affected neurons had a swollen appearance, but shrunken cells with chromatin condensation and nuclear fragmentation, indicating apoptosis, were present as well. Pathological changes in the brain were correlated with the presence of viral antigen and were apparent in the lateral geniculate, the superior colliculus, and in the suprachiasmatic area (18). In the course of disease, the observed damage and the expression of viral antigen extended to the cortex, mostly associated with the visual pathway and the ventricular system. Nontransgenic animals appeared to have a higher degree of neuronal damage than transgenic mice.

The inflammatory response to infection with HSV-2 consisted of natural killer cells, macrophages, and T cells in the eyes and brains, as confirmed by immunostaining similar to earlier findings in HSV-1 infection (9). Rhoγ mice showed a markedly stronger inflammation, involving all structures of the eyes, than did nontransgenic mice. Cell counts for L3T4- (CD4) and Ly2-positive cells (CD8) confirmed considerably higher numbers of both T-cell subsets in the eyes of transgenic mice than in control mice. However, the brains of both groups had virtually the same content of T cells (Fig. 2). IFN-γ could not be detected in the brains of HSV-2-infected transgenic or nontransgenic mice by ELISA.

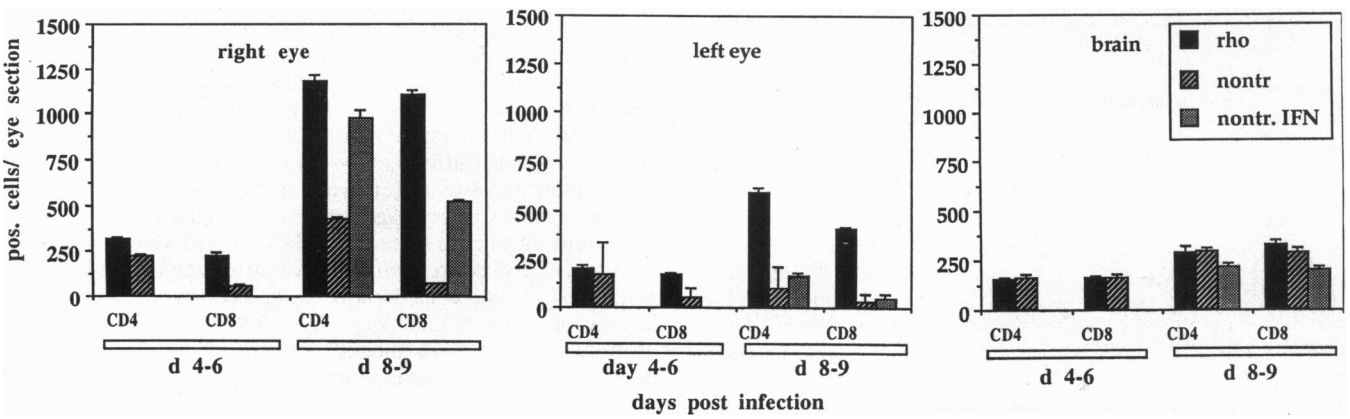

FIG. 2. Counts of CD4 (L3T4) and CD8 (Ly2) cells in the eyes of 5- to 6-week-old mice infected intravitreally with 2×10^4 pfu of HSV-2 (6-μm sections, indirect immunoperoxidase immunostaining with DAB as chromogen, and counterstaining with methyl green). Ly2- and L3T4-positive (pos.) cells were counted in the light microscope at ×20 magnification of whole eyes. The counted areas included the corneal limbus but excluded the sclera, extraocular tissue, and the optic nerve outside the eye. The plot uses mean values of groups of three animals (5- to 6-week-old BALB/c-based mice). Error bars represent the SD of each group ($\sigma n - 1$). d, Day; nontr, nontransgenic.

Virus Recovery in HSV-2-Infected Mice. To assess the effects of IFN-γ on viral replication we quantitated infectious virus in the tissues of infected mice by cytopathic effects on Vero cells. Both eyes and the brains of all animals yielded infectious virus 24 hr after infection. In transgenic mice the amount of virus was 10-fold lower in the eyes, displaying a temporary drop in the noninjected eye. However, in the brains there was no significant difference between transgenic and nontransgenic animals (Fig. 3).

Apoptosis in the Brains of Mice Intravitreally Infected with HSV-1 and HSV-2. We used immunostaining to look for apoptosis in the brains of mice infected with HSV-1 or HSV-2 in search of a mechanism that could be responsible for the increased survival of transgenic mice. Apart from cells undergoing necrosis, which displayed diffuse DAB staining of the whole cell, we found apoptotic cells with strong staining of the fragmented nucleus (Fig. 1*C*) and cells with labeling of small viral inclusion bodies. The brains of transgenic mice inoculated with either virus contained significantly fewer cells undergoing apoptosis than the brains of nontransgenic mice (Fig. 4). The difference was more pronounced in animals infected with the lethal HSV-2 (Figs. 1 *D* and *E* and 4), in which case the amount of apoptosis coincided with the severity of neurological symptoms and the survival of transgenic mice. By double-labeling with antibodies to GFAP or NSE and HSV, apoptotic cells were identified as mostly neurons (Fig. 1 *C* and *F*), few astrocytes (data not shown), and some cells that were negative for either GFAP or NSE, presumably microglial cells. Although apoptosis occurred in areas containing infected cells, only ≈25% of the apoptotic cells showed double-staining for viral antigen (Fig. 1*F*).

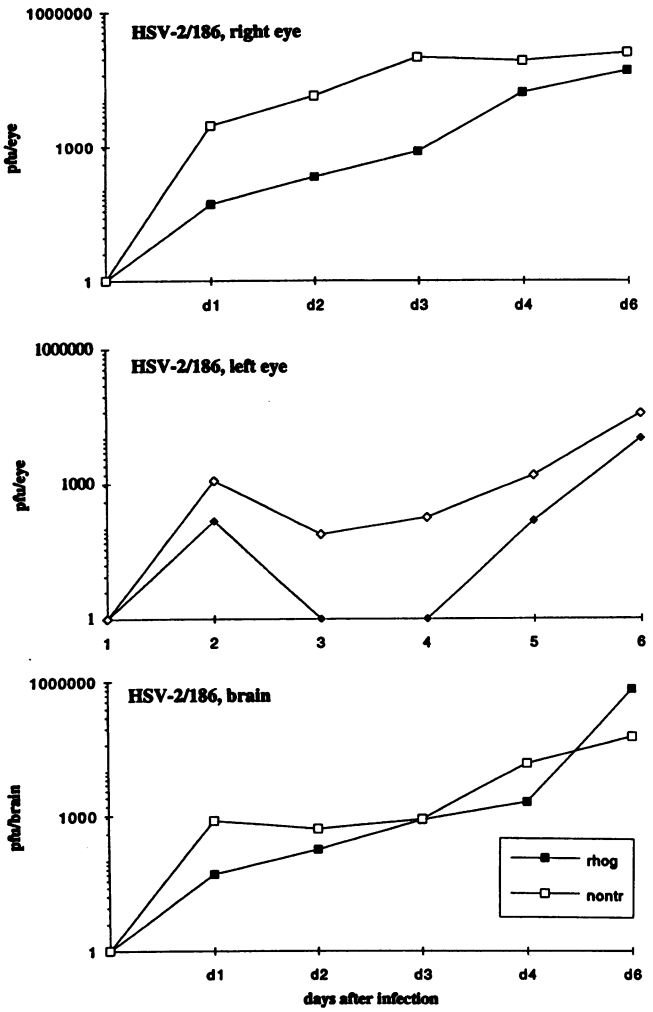

FIG. 3. Recovery of infectious virus from eyes and brains of 5- to 6-week-old mice infected with HSV-2, strain 186. Each group per experiment consisted of five mice. Whole right eyes (five per group), left eyes, and brains were pooled separately, homogenized, and processed for plaque assay on Vero cells, as described. nontr, Nontransgenic.

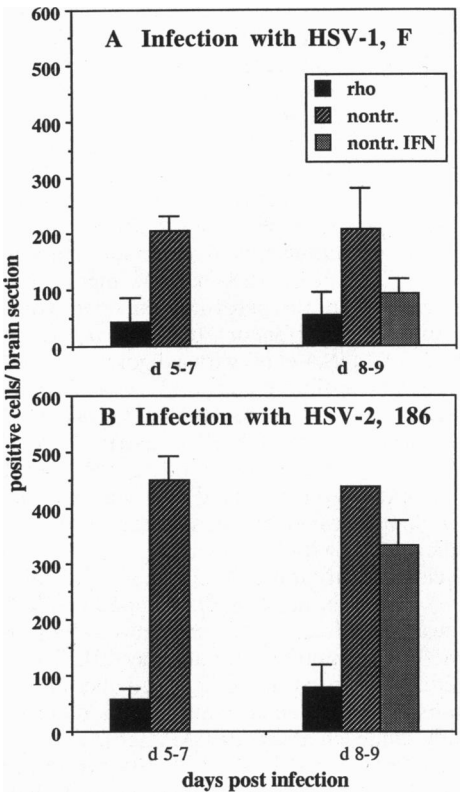

FIG. 4. Apoptosis in the brains of 5- to 6-week-old mice intravitreally infected with 2×10^5 pfu of HSV-1 (*A*) or 2×10^4 pfu of HSV-2 (*B*). For demonstration of apoptotic cells we used the ApopTag kit (immunoperoxidase detection of digoxigenin-labeled DNA fragments) in fresh-frozen brain tissue, counterstained with methyl green. Cells were counted in similarly located sagittal sections, including the optic tract and the dorsal and ventral parts of the geniculate nuclei. Serial sections of six mice per group were studied, and cells were counted under ×20 light-microscopic magnification with a transparent 1-mm grid as reference. Rho, rhoγ transgenic mice; nontr, nontransgenic control mice; nontr IFN, nontransgenic mice treated with 10^4 international units of IFN-γ injected intravitreally together with the virus and repeated once at day 4 after infection. Error bars represent the SD of each group ($\sigma\, n - 1$).

Up-Regulation of *Bcl2* in the Brains of Transgenic Mice. The reduction of apoptosis in the transgenic brains may reflect the activation of endogenous protective mechanisms. The protein encoded by the *Bcl2* gene has been correlated with protection from programmed cell death, and we therefore studied Bcl2 protein expression by immunochemistry in infected tissues. We found that in the brains of noninfected controls there was a low amount of constitutive expression in the Purkinje cells of the cerebellum and isolated neurons in the cortex. Noninfected transgenic mice, nontransgenic mice infected with HSV-2 (Fig. 1*G*), and all mice infected with HSV-1 had similarly sparse patterns of *Bcl2* expression. However, transgenic mice infected with HSV-2 had considerably more *Bcl2* expression in the cortex of their brains (Fig. 1*H*). *Bcl2*–expressing cells in transgenic mice were mostly neurons (Fig. 1*I*) and were present in areas displaying viral antigen.

DISCUSSION

Transgenic rhoγ mice, which express IFN-γ in the retina, survived infection with a highly neuroinvasive encephalitogenic HSV-2 strain, whereas their BALB/c mice nontransgenic littermates died from encephalitis when infected under

identical conditions. The transgenic mice had more inflammation in their eyes than the BALB/c controls. The virus was minimally sensitive to the replication effects of IFN-γ, and increased inflammation and decrease of the viral load were restricted to the eyes of rhoγ mice. In the brain, transgenic and nontransgenic mice showed equal amounts of inflammation and infectious virus. Most significantly, rhoγ transgenic mice underwent far less neuronal apoptosis in the brain than nontransgenic control mice, probably accounting for the selective survival of these transgenic mice. In contrast to control mice, we found an up-regulation of the protooncogene *Bcl2* in the brains of infected transgenic mice, suggesting a mechanism of protection from apoptosis in these animals.

HSV-2 reached equal levels of infectious virus in the brains of transgenic and nontransgenic mice. Nevertheless, transgenic mice survived the infection with only minor neurological disorders, whereas the control mice suffered from encephalitis and died. The factor correlating with survival of the rhoγ mice was the decreased extent of apoptosis in their brains. The fact that a large number of the cells undergoing apoptosis were negative for HSV antigen makes it unlikely that the differences between transgenic and nontransgenic mice were caused predominantly by the action of cytotoxic T cells, which are known to induce this reaction in HSV-infected cells (19), especially because transgenic and nontransgenic mice have equal numbers of T cells in their brains and IFN-γ concentrations did not appear to increase.

Consistent with earlier reports investigating the spreading characteristics of HSV by immunostaining (18), we found viral antigen in the second eye by day 6 or 7 and found the antigen slightly earlier in the brain. However, the more sensitive method of viral recovery assay yielded infectious virus in the second eye and in the brain 1 day after infection. Therefore, viral spread must be much faster than previously thought. A possible explanation could rest with the assumption of higher numbers of infected macrophages than published for other viruses (20). Alternatively, viral spread to the brain could occur through the optic nerve sheaths via the cerebrospinal fluid, accounting for the unusual locations of the virus we found in the brain. However, precise determination of propagation mechanisms in the brain will need further study.

The observation of increased *Bcl2* expression in the CNS reflects a complex interaction between the cytokine, virus, and cellular factors elicited in neurons. Because *Bcl2* up-regulation was not seen in uninfected transgenic mice, it cannot be accounted for by a direct effect of IFN-γ. Indeed our results are counterintuitive because IFN-γ has been associated with inducing apoptosis *in vitro* in multiple cell lines (3–5). Likewise, the induction of the *Bcl2* gene is not the direct effect of HSV because in infected nontransgenic mice *Bcl2* is not hyperexpressed. Thus, our observations can only be accounted for by the cumulative interaction of virus and cytokine. One possibility is that exposure of the virus to the cytokine in the eye could impose either genetic selection or alteration of viral protein expression, which, in turn, can affect protooncogene expression by brain neurons. Alternatively, *Bcl2* up-regulation could result from a fundamental difference in the cytokine-influenced inflammatory response. A parallel interaction where cytokines favor the expression of viral proteins that prevent apoptosis of infected neurons is known for Tat expression in human immunodeficiency virus infection (21) or p35 of baculoviruses (22). Similarly, the latent membrane protein 1 of the Epstein–Barr virus (23) has been shown to up-regulate *Bcl2* and block apoptosis in infected B-cell lines. Here we demonstrate the significance of such regulation *in vivo* within the CNS, favoring neuronal survival over death. It is interesting to note that HSV-1 virions lacking the γ1 34.5 gene cause a response similar to programmed cell death in neurons, suggesting that the γ1 34.5 gene product might be critical for fostering such neuronal survival (24). Interestingly, in the case of the lethal HSV-2, the interference with programmed cell death observed here also requires exposure to IFN-γ. Whereas the antiviral activity of IFN-γ has previously been attributed to effects on viral replication or enhancement of the antigen-specific immune response, here we document that IFN-γ promotes survival of virus-infected neuronal cells by a distinct mechanism.

The mechanisms by which IFN-γ influences *Bcl2* expression and programmed cell death in the brain need further experimental investigation. These findings warrant additional perspectives concerning infections by other viruses. Mechanisms superficially beneficial in acute viral infection of the CNS may ultimately allow for the survival of cells infected with multiple pathogenic viruses. Such viruses could affect the genetic programming of terminally differentiated cells and be deleterious to their function in a manner such as seen in postencephalitic neuronal disorders and AIDS dementia. Additionally these viral genomes may not remain quiescent forever, allowing reactivation and localized inflammatory reactions to endure beyond where the death and elimination of infected cells would allow.

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