Endogenously produced interferon α protects mice from herpes simplex virus type 1 corneal disease

Robert L. Hendricks,[1,2]* Peter C. Weber,[3]† Jerry L. Taylor,[4] Alexandra Koumbis,[1] Terrence M. Tumpey[1] and Joseph C. Glorioso[3]‡

Departments of [1]Ophthalmology, and [2]Microbiology and Immunology, University of Illinois at Chicago College of Medicine, Chicago, Illinois 60612, [3]Department of Microbiology and Immunology, Unit of Laboratory Animal Medicine, University of Michigan, Ann Arbor, Michigan 48109 and [4]Departments of Microbiology and Ophthalmology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, U.S.A.

Intravenous (i.v.) injection of u.v. light-inactivated herpes simplex virus type 1 (UV HSV-1) at the time of HSV-1 corneal infection reduced the cytotoxic T lymphocyte (CTL) response to HSV-1, and significantly reduced the incidence of HSV-1-induced corneal stromal disease in A/J mice. The spread of HSV-1 through the eye after corneal infection, detected using engineered HSV-1 (US3::Tn5-*lacZ*) with the *lacZ* gene under the transcriptional control of the viral late gene promoter for glycoprotein C, was also markedly reduced by i.v. UV HSV-1 injection. The restriction of HSV-1 corneal invasiveness in i.v. UV HSV-1-injected mice preceded the onset of a detectable specific cell-mediated or humoral immune response to HSV-1, and was accompanied by an elevated serum titre of interferon (IFN-α), reversed by anti-IFN-α/β antibody, and mimicked by systemic IFN-α treatment. IFN-α-treated mice developed a normal CTL response to HSV-1 after corneal infection, but the corneal invasiveness of the virus was markedly reduced and none of the treated mice developed corneal stromal disease. Together with our previous findings that HSV-1-specific CTLs participate in the pathogenesis of corneal stromal disease, these results indicate that i.v. injection of UV HSV-1 at the time of corneal infection may prevent stromal disease by the combined effects of IFN-mediated reduction of the spread of virus in the cornea and inhibition of the activity of the HSV-specific T lymphocytes that induce tissue destruction in the corneal stroma.

Introduction

Herpes simplex virus type 1 (HSV-1) is the leading infectious cause of blindness in the United States. HSV-1 corneal disease can involve the epithelial cell and/or the corneal stroma; the latter form of the disease is most frequently associated with permanent visual impairment. The factors influencing susceptibility to HSV-1 corneal stromal disease apparently involve an interaction between certain characteristics of the infecting virus and the immune and natural defence functions of the host (Centifanto-Fitzgerald *et al.*, 1982; Newell *et al.*, 1989; Stulting *et al.*, 1985; Metcalf *et al.*, 1979; Russell *et al.*, 1984; Ksander & Hendricks, 1987; Hendricks *et al.*, 1989*a*; Metcalf & Kaufman, 1976; Hendricks & Tumpey, 1990).

In our murine model of HSV-1 corneal stromal disease, infection of the corneal epithelium with HSV-1 results in the development of dendritic epithelial lesions 24 h after infection. These lesions are limited to the corneal epithelium and heal within 96 h. The cornea then appears normal until 7 to 9 days after infection when stromal disease begins to develop (Hendricks *et al.*, 1989*b*). The epithelial lesions appear to be due to virus replication in the epithelial cells, whereas stromal disease appears to be due to an immunopathological response to viral antigens in the corneal stroma. The source of the viral antigens that induce stromal disease in this model is not clear. Some possibilities include slowly replicating HSV-1 in stromal fibroblasts, reactivation of HSV-1 in latently infected sensory neurons and subsequent shedding of virus at the cornea (Hill *et al.*, 1987) or, as has

† Present address: Department of Microbiology and Immunology, Pennsylvania State College of Medicine, Milton Hershey Medical Center, University Park, Pennsylvania 16802, U.S.A.

‡ Present address: Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15260, U.S.A.

recently been suggested (Abghari & Stulting, 1988; O'Brien & Taylor, 1989; Sabbaga *et al.*, 1988; Cook *et al.*, 1987), latently infected cells in the cornea.

Partial or complete compromise of the T lymphocyte response to HSV-1 can result in a marked reduction in the incidence and severity of HSV-1 corneal stromal disease. HSV-1 corneal infection of nude mice that are congenitally athymic and incapable of mounting a T lymphocyte response is characterized by a virtual absence of corneal stromal disease (Metcalf *et al.*, 1979). Nude mice do develop superficial epithelial corneal lesions which are thought to result from virus replication in, and destruction of, epithelial cells. Reconstitution of nude mice with T lymphocytes, especially from HSV-1-infected donors, renders them as susceptible to HSV-1 corneal stromal disease as their euthymic litter mates (Russell *et al.*, 1984). Specific inhibition of T lymphocyte responses to HSV-1 antigens also reduces the susceptibility of otherwise immunologically competent mice to corneal stromal disease (Ksander & Hendricks, 1987). Moreover, specific cytotoxic T lymphocyte (CTL) unresponsiveness to HSV-1 antigens in the presence of a normal antibody and delayed-type hypersensitivity response was associated with significantly reduced susceptibility to corneal stromal disease in A/J mice infected with the KOS strain of HSV-1 (Hendricks *et al.*, 1989*a*). However, the particular T lymphocyte subpopulation that mediates corneal stromal disease appears to depend on the strain of HSV-1 used to infect the cornea (Newell *et al.*, 1989; Hendricks & Tumpey, 1990).

Previous studies had demonstrated that intravenous (i.v.) injection of HSV-1 inhibited some components of the cell-mediated immune response to HSV-1 (Nash & Ashford, 1982). In our murine model of HSV-1 corneal infection we found that i.v. injection of u.v. light-inactivated HSV-1 (UV HSV-1) at the time of ocular infection with the KOS strain of HSV-1 prevented the development of corneal stromal disease. To determine the mechanism(s) responsible for this inhibition we examined the CTL response to HSV-1 as well as the spread of virus within the eye and to the trigeminal ganglion following simultaneous corneal HSV-1 infection and i.v. injection of UV HSV-1. A sensitive method for identifying productively infected cells in tissue sections was employed, which utilizes a recombinant HSV-1 (US3::Tn5-*lacZ*) with the *lacZ* gene under the transcriptional control of a viral late gene promotor.

Our studies have identified two independent factors that influence the development of corneal stromal disease in our mouse model. Stromal disease develops when (i) the initial infection of the corneal epithelium results in dissemination of the virus to the corneal stroma as well as to other ocular tissues and (ii) HSV-1-specific T lymphocyte responses are induced. The current studies show that endogenously produced interferon α (IFN-α) can reduce the corneal invasiveness but not the neuroinvasiveness of HSV-1. The reduced corneal invasiveness was associated with reduced susceptibility of mice to corneal stromal disease.

Methods

Mice. Female A/J mice (Jackson Laboratories), 8 to 12 weeks old, were acclimatized for 1 week before the experiments. Mice were anaesthetized with 2 mg of ketamine hydrochloride (Vetalar, Parke-Davis) and 0·04 mg of acepromazine maleate (Aveco Co.) in 0·1 ml of RPMI 1640 medium injected intramuscularly in the left hind leg.

Viruses. The recombinant HSV-1 (US3::Tn5-*lacZ*) containing the *Escherichia coli* β-galactosidase gene under the transcriptional control of the viral late gene promoter for glycoprotein C (gC) was prepared and characterized as follows.

pFH100::Tn5 (no. 16), the cloned *Bam*HI N fragment of HSV-1 containing a Tn5 insertion within the US3 open reading frame, was generated in previous work (Weber *et al.*, 1987). p0N1 contains the *E. coli lacZ* gene, a simian virus 40 (SV40) polyadenylation signal and an

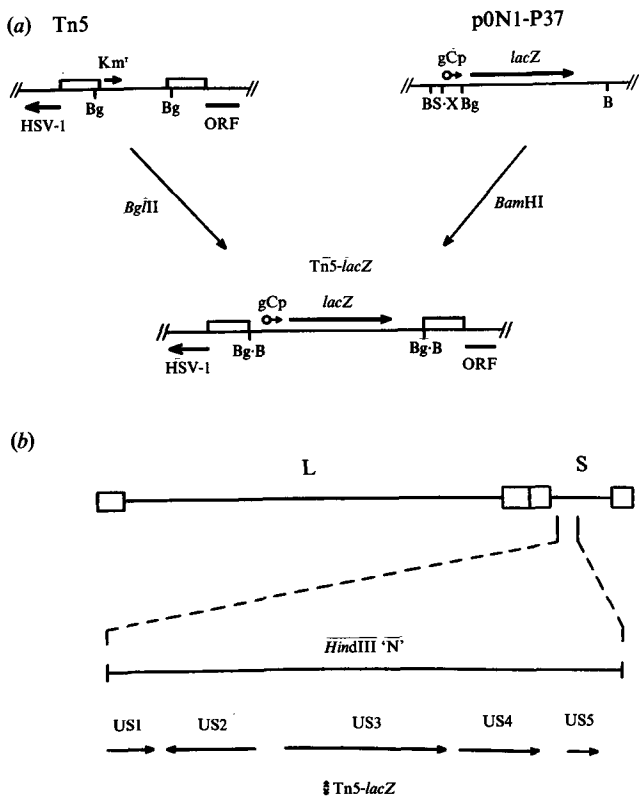

Fig. 1. (*a*) Construction of Tn5-*lacZ* insertions within HSV-1 genes. The generation of the plasmid shown is described in Methods. (B, *Bam*HI; Bg, *Bgl*II; S, *Sal*I; X, *Xho*I; gCp, glycoprotein C gene promoter; Km^r, kanamycin phosphotransferase gene of Tn5; HSV-1 ORF, any HSV-1 open reading frame). (*b*) HSV-1 genome showing location of the US3 open reading frame and its Tn5-*lacZ* insertion within the *Hin*dIII N fragment. L and S are the long and short components of the viral genome, respectively.

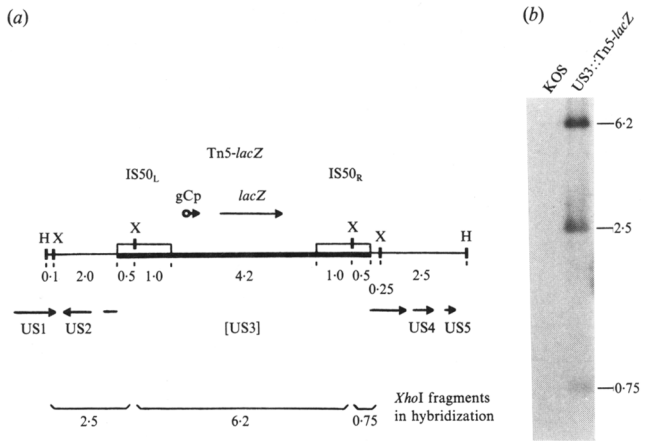

Fig. 2. Mapping of the US3::Tn5-*lacZ* insertion within the HSV-1 genome. (*a*) Location of the US3::Tn5-*lacZ* insertion within the *Hin*dIII N fragment of the HSV-1 genome (see Fig. 1*b*). An *Xho*I (X) and *Hin*dIII (H) map of the region is shown, along with the location of novel *Xho*I fragments which hybridize in the blot in (*b*). (*b*) Hybridization of novel *Xho*I fragments of HSV-1 (US3::Tn5-*lacZ*) to radiolabelled pUC19::Tn5 DNA. HSV-1 (KOS) DNA was used as a control. (gCp, glycoprotein C gene promoter; $IS50_L$ and $IS50_R$, inverted insertion sequences present within Tn5.)

upstream polylinker for insertion of eukaryotic promoter elements (Spaete & Mocarski, 1985). p0N1-P37 was constructed by Delia J. Dorney (unpublished results) by insertion of the 268 bp *Sal*I/*Bgl*II fragment of pgC (Homa *et al.*, 1986) into the *Xho*I and *Bgl*II sites of the p0N1 polylinker. This places the −144 to +124 sequences of the gC promoter upstream of the *lacZ* gene, and enables the entire gC promoter–*lacZ*–SV40 poly(A) cassette to be removed as a single 4·2 kb *Bam*HI fragment. The construction of the US3::Tn5-*lacZ* plasmid by replacement of the *Bgl*II fragment of pFH100::Tn5 (16) with the *lacZ*-containing *Bam*HI fragment of p0N1-p37 is illustrated schematically in Fig. 1. The location of the US3::Tn5-*lacZ* insertion within the *Hin*dIII N fragment of the HSV-1 genome is illustrated in Fig. 2. Generation of recombinant virus by cotransfection of viral and plasmid DNAs, and Southern blot analysis of viral DNA were performed as described previously (Homa *et al.*, 1986).

The KOS 321 and US3::Tn5-*lacZ* strains of HSV-1 were grown in Vero or Hep-2 cells, and intact virions were purified on Percoll (Pharmacia) gradients as previously described (Hendricks & Sugar, 1984). To ensure the HSV specificity of immunological assays, the virus used to infect mice was prepared from HSV-1-infected Hep-2 cells grown in RPMI 1640 plus 10% normal rabbit serum, whereas the virus used for *in vitro* restimulation and i.v. injection was prepared from HSV-1-infected Vero cells grown in RPMI 1640 plus 10% foetal calf serum. The latter virus suspension (10^8 p.f.u./ml) was inactivated by exposure to a germicidal (254 nm) lamp for 10 min at a distance of 5 cm (720·6 mJ/cm^2), reducing the infectious titre to less than 100 p.f.u./ml (UV HSV).

Virus infection. Topical corneal infection of anaesthetized mice was achieved by applying a 3 µl inoculum of virus suspension (5×10^4 p.f.u.) topically to a scarified cornea and rubbing it in with the eyelid. Within 1 h of corneal infection, mice received an i.v. injection of UV HSV-1 (5×10^4 erstwhile p.f.u.) alone or in combination with either 350 neutralizing units of rabbit anti-IFN-α/β antibody or preimmune rabbit serum (Lee Biomolecular). Controls received a mock i.v. injection of Hanks' balanced salt solution.

In some experiments, topical corneal HSV-1 infections were followed by systemic treatment with mouse IFN-α (catalogue no. 20071, Lee Biomolecular). Eight hours after infection an i.v. injection of 1000 international units (IU) of IFN-α in saline was administered, followed by intraperitoneal injections of 10000 IU of IFN-α at 24 and 48 h after infection. Controls received corneal infections followed by similar injections of saline.

Clinical observation of HSV-1-infected corneas. Daily for the first week, and then on alternate days after infection, mice were observed for the severity of corneal disease by slit-lamp examination. All clinical observations were made by the same observer who was unaware of the treatment group to which the animal belonged. Visualization of epithelial lesions was aided by instillation of fluorescein drops in the eye and by examination under cobalt blue light. The degree of stromal opacity was scored on a scale of 0 to 3+, where 0 indicated no opacity, 1+ was slight haze, 2+ was a moderate opacity and 3+was severe opacity obliterating the view of the iris.

Cytotoxicity assays. The regional (preauricular) lymph nodes (RLN) were excised on day 8 after HSV-1 infection, and single-cell suspensions were prepared. Each RLN cell suspension was tested for cytotoxic activity in both bulk culture and limiting dilution (LD) assays.

In bulk culture assays, 5×10^6 RLN cells were suspended in 2 ml of assay medium (RPMI 1640 plus 5% foetal calf serum, 10 mM-HEPES buffer, and antibiotics) and stimulated for 72 h with UV HSV-1 in the 16 mm diameter wells of a 24-well cluster plate as previously described (Hendricks *et al.*, 1989*a*). Cytotoxic activity was measured against HSV-1-infected L929 (L929-HSV) cells (clone CCL 1, American Type Culture Collection). The L929 cells (compatible with A/J mice at the H-2k locus) were infected with HSV-1 at a multiplicity of 5·0 for 2 h and labelled with ^{51}Cr (200 µCi/2×10^6 cells) for 1 h. At least 80% of the L929-HSV targets expressed HSV antigens as assessed by lysis with anti-HSV antibody and complement. A standard 4 h ^{51}Cr release assay was performed as previously described (Ksander & Hendricks, 1987).

The CTL precursor (CTLp) frequency in RLN cells was determined in LD cultures that were prepared as previously described (Hendricks *et al.*, 1989*a*). To summarize, stimulator cells for LD cultures were spleen cells from normal A/J mice which had been incubated for 2 h with UV HSV-1 (5 p.f.u. per cell), treated for 30 min with mitomycin C (50 µg/ml, Sigma), washed, counted, and adjusted to 2×10^6/ml in LD medium (RPMI 1640 supplemented with 10% foetal calf serum, 15% supernatant from concanavalin A-stimulated Lewis rat spleen cells, 5×10^{-5} M-2-mercaptoethanol, and antibiotics buffered to pH 7·2 with HEPES buffer). LD cultures consisted of graded numbers of RLN responder cells that were plated in the wells of 96-well round-bottom microtitre plates in 0·1 ml of LD medium. Twenty-four control cultures did not receive responder cells. All cultures received 0·1 ml of stimulator cells (2×10^5 cells) and were incubated for 7 days at 37 °C in a humid 5% CO_2–95% air atmosphere.

Each LD culture was split and tested for cytotoxic activity against $1{\cdot}0 \times 10^3$ HSV-1-infected and uninfected L929 targets in a 4 h ^{51}Cr release assay. The data from LD assays were analysed with a computer program (provided by Dr Peter Krammer, Institute for Immunology and Genetics, German Cancer Research Center, Heidelberg, Germany). The threshold for positive cultures was the mean plus 3 S.D. of the ^{51}Cr release in control cultures. A few cultures that were positive for both infected and uninfected targets were detected at very high RLN cell inputs at which all cultures were positive for lysis of infected targets. Excluding these cultures from the analyses did not influence the estimate of the CTLp frequency. The natural log of the fraction of negative cultures at each RLN cell input was fitted to a straight line using both the maximum likelihood and the minimum chi-square methods. The CTLp frequency and the 95% confidence limits were

estimated from the regression line. All LD data reported herein conformed to the single-hit Poisson distribution ($P < 0.05$). The significance of differences in CTLp frequencies was assessed by the global test for homogeneity of independent slopes.

Histological examination of infected corneas. Infected eyes were enucleated and immediately fixed in 10% neutral buffered formalin, paraffin-embedded and 5 μm sections were prepared. The sections were stained with haematoxylin–eosin, mounted with Permount and coverslipped for microscopical examination (Hendricks *et al.*, 1989*b*).

Detection of the β-galactosidase enzyme in tissues infected with US3::Tn5-*lacZ* HSV-1 was accomplished as previously described (Ho drated with graded alcohol, and cleared in xylene. The sections were imbedded in O.C.T. (Tissue Tek, Miles), snap-frozen in an isopentane dry ice bath, and 6 μm sections were cut at −20 °C. The sections were fixed in 0·5% glutaraldehyde in PBS for 10 min and then exposed to the chromogenic substrate X-gal (2·4 mM-X-gal/12·5 mM-K_3FeCN_6/12·5 mM-$K_4FeCN_6.3H_2O$ in PBS) for 21 h, washed, dehydrated with graded alcohol and cleared in xylene. The sections were then counterstained lightly with nuclear fast red, mounted with Permount and coverslipped.

IFN assay. IFN antiviral activity was determined in a haemagglutination yield-reduction assay in L cells using the picornavirus, GDVII, as the challenge virus (Jameson & Grossberg, 1981). This assay can detect 1 to 2 units of each type of murine IFN (MuIFN). MuIFN-α titres are expressed in IU/ml and calibrated using the WHO standard, Ga02-901-511. IFN subtypes were identified by neutralization of antiviral activity with anti-MuIFN-α and anti-MuIFN-β antibodies (a generous gift from Yamasa Soyu Co.) or anti-MuIFN-γ antibodies (from E. Havell, Saranac Lake, New York State, U.S.A.).

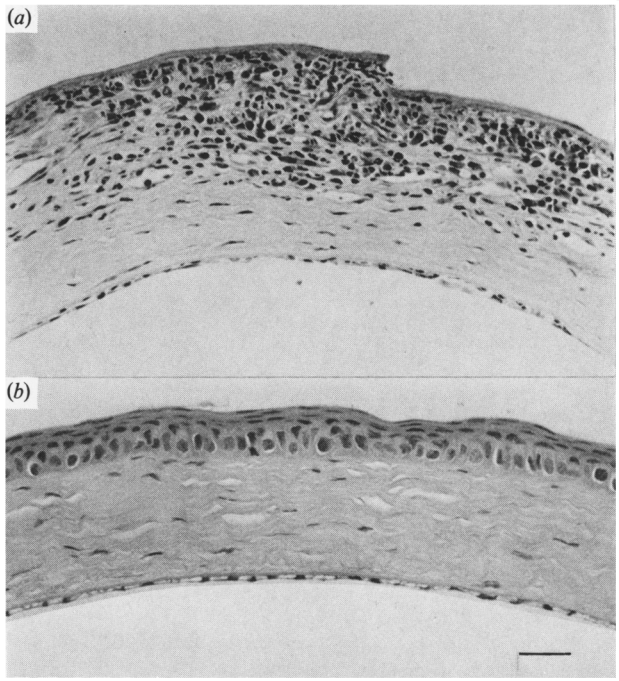

Fig. 3. Micrographs of corneas 21 days after HSV-1 infection. Mice receiving corneal infection alone showed severe stromal opacity and neovascularization (*a*). In mice receiving corneal infection in combination with i.v. injection of UV HSV-1, only one mouse (shown) developd a mild stromal opacity with no corneal neovascularization (*b*). Bar marker represents 100 μm.

Results

Inhibition of the CTL response to HSV-1 through i.v. injection of UV HSV-1

Groups of mice received an i.v. injection of UV HSV-1 or a mock i.v. injection of medium alone at the time of HSV-1 corneal infection. Eight days later, cells from the draining lymph nodes were tested for CTL activity against HSV-1-infected and uninfected H-2k-compatible targets.

As shown in Table 1, i.v. injection of UV HSV-1 at the time of corneal infection resulted in a significant reduction in both the CTL activity and CTLp frequency of RLN cells following restimulation in bulk cultures and LD cultures, respectively. Thus, i.v. injection of UV HSV-1 at the time of corneal infection reduced the HSV-specific CTL response, at least in part, by inhibiting the expansion of CTLp in the RLN. This experiment was repeated two additional times with similar results.

Table 1. *CTL suppression induced by intravenous HSV-1 antigens*

Infection	^{51}Cr Release* (%) E:T Ratio 80:1	40:1	20:1	Inverse of the CTLp frequency (95% CL)
Corneal + i.v. (mock)	34·13	20·82	11·97	2707 (1885–4768)
Corneal + i.v. (UV HSV-1)	10·06	4·68	2.26	46239 (35216–67307)†

* The data are recorded as CTL activity against HSV-1-infected targets at various effector to target (E:T) ratios, and as the inverse of the CTLp frequency and 95% confidence limits (95% CL). Lysis of uninfected targets was less than 5% at an E:T ratio of 80:1 (not shown).

† Significantly ($P = 0.00003$) lower CTLp frequency than the group receiving mock i.v. injection as assessed by the global test for homogeneity of independent slopes.

Effect of i.v. injection of UV HSV-1 on susceptibility to HSV-1 corneal disease

Our previous studies (Hendricks *et al.*, 1989*a*) suggested an immunopathological role for HSV-specific CTL in the stromal disease that begins 9 to 14 days after corneal infection with the KOS strain of HSV-1. In contrast, the epithelial lesions that develop 2 days after infection and resolve by day 4 after infection appear to result strictly from virus replication in and destruction of epithelial cells. Therefore, we hypothesized that the CTL inhibi-

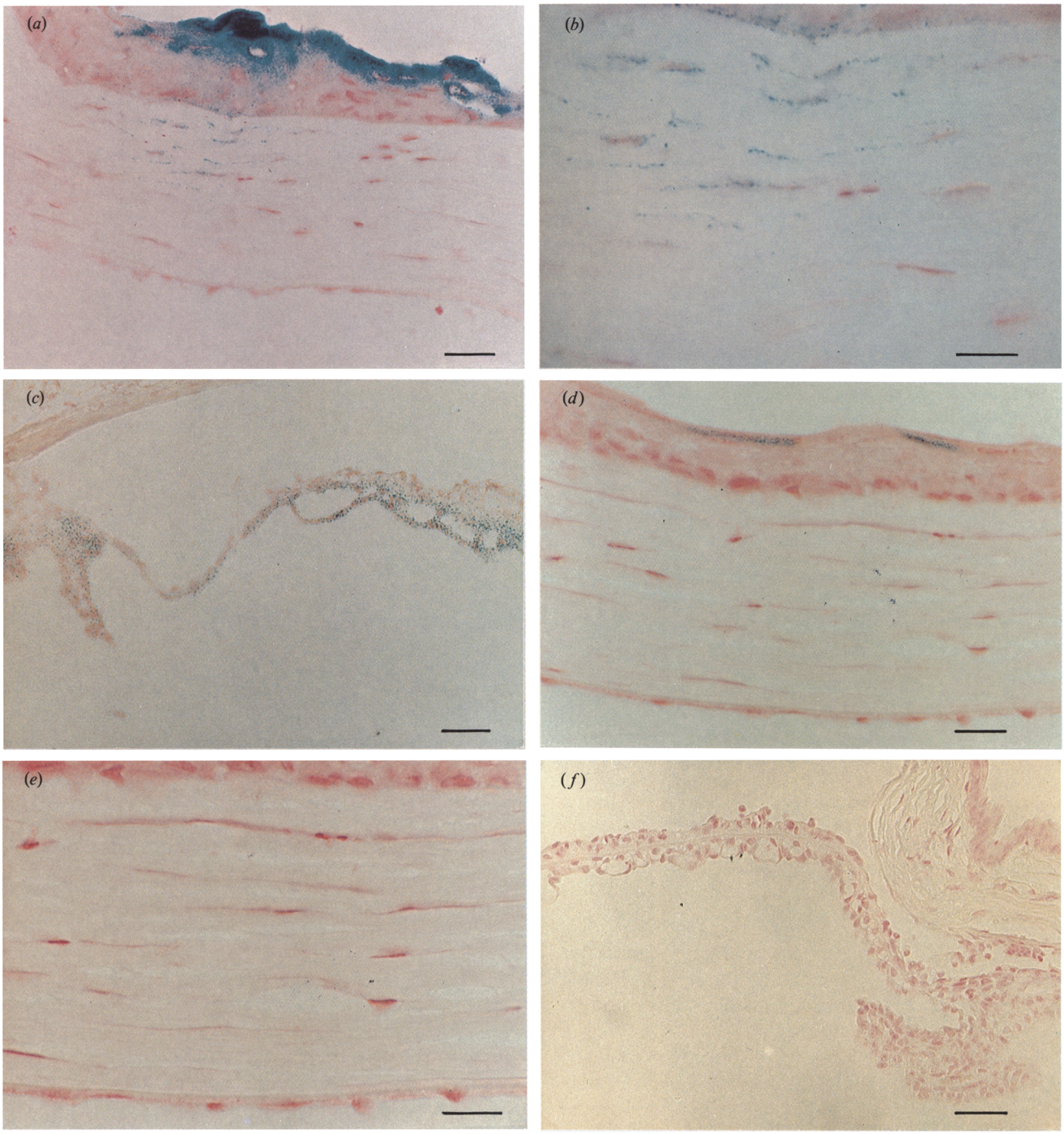

Fig. 4. Micrographs of corneas (*a*, *b*, *d* and *e*), obtained 2 days after infection with US3::Tn5-*lacZ* HSV-1 and stained with X-gal (blue). Corneal infection alone resulted in infection of epithelial cells (*a*) as well as underlying stromal fibroblasts (*b*). Corneal infection in combination with i.v. injection of UV HSV-1 resulted in, at most, a mild infection of the corneal epithelium (*d*) with no spread to the underlying stroma (*e*). Three days after corneal infection alone, the infection had spread to the iris and ciliary body (*c*). The iris and ciliary body remained uninvolved in mice receiving i.v. injections of UV HSV-1 at the time of corneal infection (*f*). These results are representative of three experiments. Bar markers represent: 50 μm, *a*, *d*; 25 μm, *b*, *e*; 100 μm, *c*, *f*).

tion resulting from i.v. injection of UV HSV-1 would prevent the development of stromal disease but have little or no effect on the development of epithelial disease. To test this hypothesis, groups of 10 mice received corneal infections alone or in combination with i.v. injection of UV HSV-1. The infected eyes were then

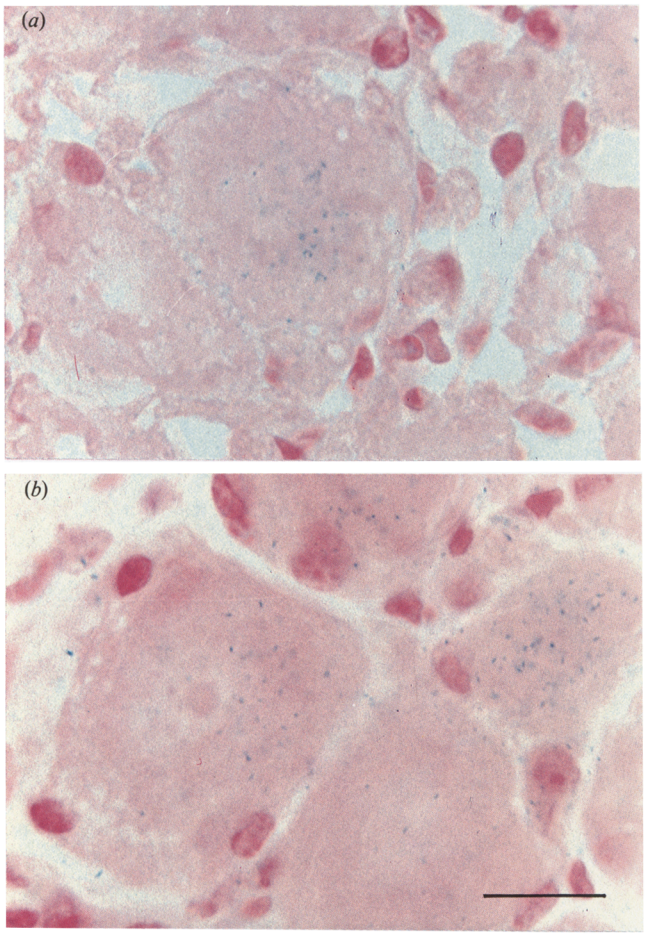

Fig. 5. Micrographs of trigeminal ganglia obtained 2 days after corneal infection with US3::Tn5-*lacZ* alone (*a*), or corneal infection in combination with i.v. injection of UV HSV-1 (*b*). The virus was present in numerous neurons of ganglia obtained from mice of both treatment groups. Bar markers represent 100 μm.

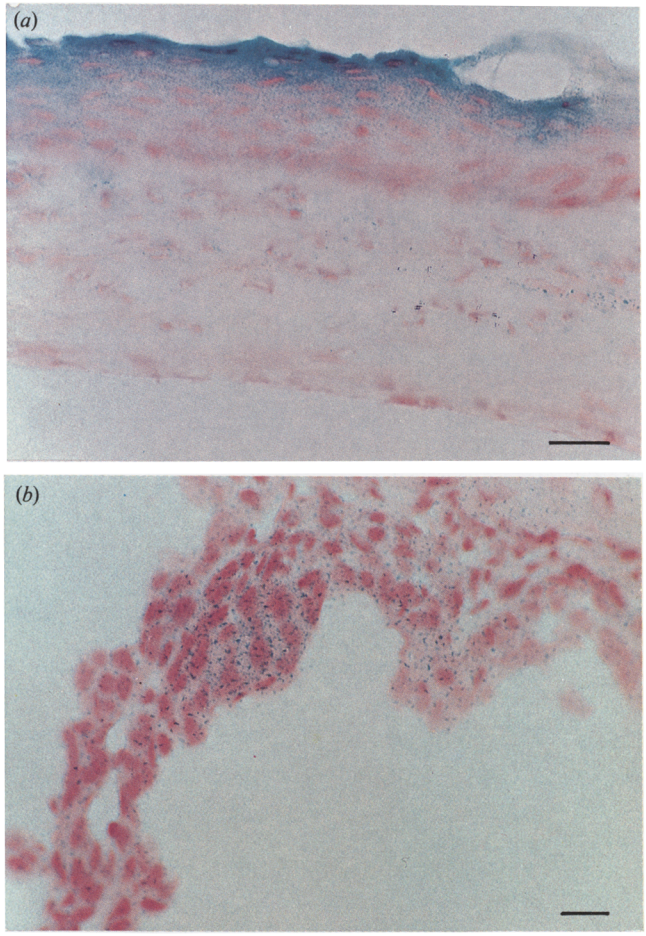

Fig. 6. Micrograph of eyes infected with US3::Tn5-*lacZ* in combination with an i.v. injection of a mixture of UV HSV-1 and 350 neutralizing units of anti-IFN-α/β serum. Note the failure of the i.v. injection of UV HSV-1 to inhibit virus infection of the cornea (*a*) 2 days after infection, or the spread of the virus to the iris and ciliary body (*b*) by day 3 after infection in the presence of IFN-α/β neutralizing antibody. Bar markers represent 50 μm (*a*) and 100 μm (*b*).

examined with a slit-lamp on alternate days after infection. In addition, representative corneas from each treatment group were examined histologically to determine the extent of corneal disease.

The animals receiving corneal infection alone all developed corneal epithelial lesions by day 2 post-infection (p.i.), which healed by day 4. The incidence of corneal stromal disease in mice receiving corneal infection alone reached a peak 21 days p.i. with 10 of 20 mice developing disease. Stromal disease began around day 7 with a mild stromal haze, which progressed to severe stromal opacity with neovascularization (Fig. 3*a*). The degree of stromal opacity in this group reached a plateau 18 days p.i. with a mean score of $1{\cdot}92 \pm 0{\cdot}33$ (S.E.M.). As expected, i.v. injection of UV HSV-1 at the time of corneal infection significantly reduced the incidence of corneal stromal disease ($P < 0{\cdot}05$, Pearson's chi-square test). Only one of 20 animals from this treatment group developed stromal disease, exhibiting only a mild stromal opacity (degree of opacity 1+) and no corneal neovascularization (Fig. 3*b*). A surprising finding was that animals receiving corneal infection in combination with an i.v. injection of UV HSV-1 developed less severe epithelial disease as assessed by slit-lamp examination. This reduced epithelial involvement will be illustrated in experiments described below.

Effect of i.v. injection of UV HSV-1 on the ocular invasiveness of HSV-1

Because epithelial disease is thought to reflect cell destruction due to viral replication, the ocular spread of HSV-1 after corneal infection was followed. Groups of mice received corneal infections with US3::Tn5-*lacZ* HSV-1 in combination with an i.v. injection of UV

HSV-1 or in combination with mock i.v. injection. At various times after infection, the eyes were enucleated and frozen sections were stained with X-gal as described in Methods.

Two days after corneal infection and mock i.v. injection, numerous pockets of staining were observed in the corneal epithelial layer (Fig. 4*a*). Stromal fibroblasts underlying these areas of epithelial infection were also stained (Fig. 4*b*). In contrast, corneas from animals receiving corneal infection in combination with i.v. injection of UV HSV-1 showed either no staining or only small pockets of weak staining in the corneal epithelium (Fig. 4*d*). There was no evidence of infection of stromal fibroblasts in corneas from these mice (Fig. 4*e*).

Three days after corneal infection and mock i.v. injection, the virus had spread from the cornea to the iris and the ciliary body (Fig. 4*c*), and even into the nerve fibre layer of the retina (not shown). Infection of these ocular tissues was not observed in animals receiving corneal infection in combination with i.v. injection of UV HSV-1 (Fig. 4*f*). Although the ocular invasiveness of the virus was clearly reduced by i.v. injection of UV HSV-1, its neuroinvasiveness was not altered. Two days after corneal infection with US3::Tn5-*lacZ* and mock i.v. injection or corneal infection in combination with i.v. injection of UV HSV-1, numerous infected neurons were detected in the trigeminal ganglia (Fig. 5).

Four days after corneal infection, staining was no longer observed in any of the ocular tissues or the trigeminal ganglia from animals of either treatment group (not shown), indicating that the virus replication cycle was unlikely to be active at that time. The corneal epithelium had healed, and there was no apparent loss of corneal stromal fibroblasts or endothelial cells. These findings suggest that after corneal infection alone HSV-1 spreads rapidly from the epithelial layer of the cornea to the posterior segment of the eye, infecting many of the ocular tissues that lie between. Expression of the virus genome in ocular cells appears to be quite transient, and there is no evidence for cellular destruction by the virus except in the corneal epithelium. The corneal invasiveness of the virus is markedly reduced by simultaneous i.v. injection of UV HSV-1.

Effect of i.v. injection of UV HSV-1 on systemic levels of IFN

The i.v. injection of UV HSV-1 restricted the spread of virus in infected corneas 2 to 3 days p.i., before the onset of detectable T lymphocyte or antibody responses in this model (Ksander & Hendricks, 1987). Thus the restricted HSV-1 corneal infection in these animals appeared to be due to a natural defence mechanism that was activated by i.v. injection of UV HSV-1. Therefore, we determined whether i.v. injection of UV HSV-1 at the time of corneal infection resulted in elevated systemic levels of IFN. Serum samples were obtained from four mice 24 h and 48 h after HSV-1 corneal infection alone, or corneal infection in conjunction with an i.v. injection of UV HSV-1. Test samples consisted of pooled sera of two mice (i.e. two samples/treatment group). These serum samples, as well as samples of normal mouse serum, were coded and tested for antiviral activity in a masked fashion. There was no detectable IFN activity in the serum of normal mice or of mice receiving only a corneal infection with HSV-1. In contrast, the mice that received corneal infection in conjunction with an i.v. injection of UV HSV-1 exhibited high serum titres of IFN (140 IU/ml and 105 IU/ml, mean = 122 IU/ml) 24 h p.i. By 48 p.i. the IFN titre of these animals had dropped to 28 IU/ml and 27 IU/ml (mean = 27 IU/ml). The IFN was determined to be IFN-α by neutralization with IFN type-specific monoclonal antibodies.

To establish further a role for IFN-α in reducing the ocular invasiveness of HSV-1, groups of mice were given i.v. injections of a mixture of UV HSV-1 and 350 neutralizing units of anti-IFN-α/β antiserum, or UV HSV-1 in preimmune serum. All mice received a simultaneous corneal infection of US3::Tn5-*lacZ*, and the spread of virus in the eye was monitored. As expected, the i.v. injection of UV HSV-1 in preimmune serum restricted the spread of virus to a mild infection of the corneal epithelium (not shown). However, the addition of IFN-α/β neutralizing antibodies to the i.v. injection eliminated this effect, permitting the virus to spread through the cornea 2 days p.i. (Fig. 6*a*) and to the iris and ciliary body 3 days p.i. (Fig. 6*b*).

In similar experiments, groups of two mice received corneal infections with KOS 321 HSV-1 followed within 1 h by i.v. injections of preimmune rabbit serum, UV HSV-1 plus preimmune rabbit serum, or UV HSV-1 plus 350 neutralizing units of rabbit anti-mouse IFN-α/β antiserum. Eight days later RLN cells were restimulated with UV HSV-1 for 72 h, and tested for cytotoxic activity against HSV-1-infected and uninfected targets. As shown in Table 2, anti-IFN-α/β treatment did not abolish the inhibition of the CTL response induced by i.v. injection of UV HSV-1.

To determine whether IFN-α alone could mimic the effects of i.v. injection of UV HSV-1, IFN-α was administered systemically at 8, 24 and 48 h after topical corneal HSV-1 infection as described in Methods. Controls received topical corneal HSV-1 infection followed by similar treatments with saline.

To evaluate the effect of IFN-α on HSV-1 corneal invasiveness, mice received topical corneal infection with US3::Tn5-*lacZ* followed by IFN-α or saline treatment. The eyes were enucleated 48 and 72 h after

Table 2. *Effect of IFN-α on the CTL response to HSV-1*

HSV-1 Infection	Treatment	^{51}Cr Release (%) E:T Ratio* 40:1	20:1	10:1
Corneal†	Saline	39·5	26·4	16·5
Corneal	IFN-α	37·3	22·9	11·6
Corneal‡	None	45·8	28·6	15·8
Corneal + i.v. (UV HSV-1)	Preimmune	4·5	2·5	0·7
Corneal + i.v. (UV HSV-1)	Anti-IFN-α/β	3·7	0·8	0

* Eight days after infection, draining lymph node cells were restimulated with UV HSV-1 for 72 h in bulk cultures, and then tested for cytotoxic activity against HSV-1-infected and uninfected L929 cells in a 4 h ^{51}Cr release assay at various effector to target (E:T) ratios. Lysis of uninfected targets was negligible (not shown).

† Four mice received corneal HSV-1 infections. Eight hours later, IFN-α treatment (see Methods) was begun in two mice, while two control mice were similarly treated with saline.

‡ Groups of two mice received corneal HSV-1 infections followed within 1 h by an i.v. injection of preimmune rabbit serum, UV HSV-1 plus preimmune rabbit serum, or UV HSV-1 plus 350 neutralizing units of rabbit anti-IFN-α/β antiserum.

infection and frozen sections were stained with X-gal. The mice whose eyes were removed at 48 h had received IFN-α injections at 8 h and 24 h only. By 48 h after infection the corneas of control mice had large pockets of epithelial staining as well as staining of the underlying stromal fibroblasts (similar to the staining patterns shown in Fig. 4*a*, *b*). In contrast, IFN-α-treated mice showed no or very slight staining of the corneal epithelium with no evidence of HSV-1 spread to the corneal stroma (similar to the staining patterns in Fig. 4*d*, *e*). Seventy-two hours after infection the virus had spread to the iris and ciliary body of control mice. At this time no staining was seen in any ocular tissues of IFN-α-treated mice.

To determine the effect of IFN-α on the capacity of HSV-1 to induce corneal stromal disease, groups of mice received topical corneal infections with KOS 321 HSV-1 followed by IFN-α or saline treatment. The eyes of all mice were examined with a slit-lamp on alternate days after infection for the presence of corneal stromal disease. None of the 20 IFN-α-treated mice developed stromal disease, whereas 45% of control mice (nine of 20) developed stromal disease.

A third set of experiments tested the effect of IFN-α on the CTL response to HSV-1. Mice received topical corneal infections with KOS 321 HSV-1 followed by systemic treatment with IFN-α or saline. Eight days after infection RLN cells were restimulated with UV HSV-1 in bulk cultures and tested for cytotoxic activity against HSV-1-infected targets. As shown in Table 2, IFN-α treatment did not appreciably affect the level of CTL activity induced by HSV-1 corneal infection.

Discussion

A variety of clinical and experimental observations have suggested that HSV-1 corneal epithelial disease results from HSV-1 replication in and destruction of corneal epithelial cells, whereas HSV-1 corneal stromal disease results from a T cell-mediated inflammation in the corneal stroma (Metcalf & Kaufman, 1976; Metcalf *et al.*, 1979; Russell *et al.*, 1984; Ksander & Hendricks, 1987; Hendricks *et al.*, 1989*a*, *b*). We have previously demonstrated that susceptibility of A/J mice to corneal stromal disease following corneal infection with the KOS strain of HSV-1 can be markedly reduced by inhibiting the HSV-specific and major histocompatibility complex class I-restricted CTL response (Hendricks *et al.*, 1989*a*). CTL unresponsiveness was induced by injection of infective (but not u.v.-inactivated) HSV-1 into the anterior chamber of the eye at the time of HSV-1 corneal infection. Although the corneas of these mice were spared the tissue destruction that normally accompanies a corneal infection, the anterior chamber of the infected eye became severely inflamed, and most of the animals developed cataracts. In studies described in this report, corneal infection was accompanied within 1 h by an i.v. injection of UV HSV-1, which significantly reduced the CTL response to HSV-1 in the regional lymph nodes relative to that in mice receiving corneal infection alone. The decreased CTL activity was due, at least in part, to reduced expansion of HSV-specific CTLp in the lymph nodes. In addition to reducing CTL activity, i.v. injection of UV HSV-1 also rendered mice highly resistant to stromal disease following HSV-1 corneal infection, with no apparent adverse effect on the general health of the animals.

An unexpected finding was the reduced severity of corneal epithelial disease in these mice. The engineered HSV-1, US3::Tn*5*-*lacZ*, was used to trace the spread of virus in HSV-1-infected corneas. We have found that this virus provides a more sensitive means of tracing HSV-1 infection of ocular tissues than immunohistochemical staining for HSV-1 antigens. The virus identifies cells that are productively infected since the transcription of the *lacZ* gene is controlled by an HSV-1 late gene promoter. Our studies with the US3::Tn*5*-*lacZ* virus demonstrated a rapid spread of HSV-1 through the ocular tissues. Two days after corneal infection, several large pockets of infected epithelial cells were observed. In addition, the infection had spread to the stromal fibroblasts underlying these foci of epithelial infection. The rapid virus spread following corneal infection alone was in marked contrast to the limited corneal infection observed in mice that received i.v. injection of UV HSV-1 at the time of corneal infection. In these mice, the infection was restricted to small areas of the corneal epithelium.

The reduced corneal disease was observed 2 to 3 days after infection, prior to the onset of detectable T lymphocyte responses to HSV-1 in this model (Ksander & Hendricks, 1987). This observation suggested that the palliative effect of i.v. injection of UV HSV-1 might not be due entirely to inhibition of the CTL response. Induction of an HSV-specific CTL response may be necessary but not sufficient for the establishment of HSV corneal stromal disease in this model. The spread of the HSV-1 infection from the corneal epithelium to the stromal fibroblasts may also be necessary. The infected fibroblasts may then provide a source of viral antigens to activate and serve as targets for the HSV-specific CTL in the cornea.

The i.v. injection of UV HSV-1 appears to inhibit virus replication in the cornea before the onset of detectable serum anti-HSV antibody or T cell reactivity to HSV-1 antigens in the lymph nodes and spleen (Ksander & Hendricks, 1987). These findings suggested the involvement of a natural defence mechanism(s). This interpretation was strongly supported by our detection of elevated serum titres of IFN-α in animals that received i.v. injection of UV HSV-1. A role for IFN-α in reducing HSV-1 ocular invasiveness was confirmed by our demonstration that the palliative effect of i.v. injection of UV HSV-1 could be mimicked by systemic treatment with IFN-α, and eliminated by IFN-α/β neutralizing antibody. Other investigators have studied the therapeutic efficacy of exogenously applied IFN for treatment of HSV-1 corneal infections (Trousdale & Nesburn, 1984; Reccia *et al.*, 1985). In most cases, topical treatment with IFN alone had minimal effect. Our findings suggest that endogenously produced IFN-α may be much more effective than exogenous (usually recombinant IFN) at reducing HSV-1 replication in the cornea. Very small quantities of IFN have been detected in mouse eyes during corneal infection, but only with doses of topical HSV higher than those given in the studies reported here (Taylor & O'Brien, 1987). Systemically derived IFN-α may increase the IFN levels in the cornea directly or indirectly by priming the corneal cells for greater IFN production upon virus infection. Exposure to low concentrations of IFN has been shown to increase production of IFN by human lymphocytes, fibroblasts and mouse L cells upon treatment with IFN inducers including viruses (Cantell *et al.*, 1981; Raj & Pitha, 1981; Stewart *et al.*, 1971). It is noteworthy, however, that i.v. UV HSV-1 did not reduce the neuroinvasiveness of HSV-1 during corneal infections. Thus, colonization of the trigeminal ganglion may not require extensive HSV-1 replication in the cornea. In fact, we have recently demonstrated that a non-replicating HSV-1 mutant (ICP4 deletion) gains access to the trigeminal ganglion and expresses its latency-associated transcript promoter in ganglionic neurons following corneal infection (unpublished observations). The fact that i.v. injection of UV HSV-1 did block the development of stromal disease demonstrated that colonization of the sensory ganglia is not sufficient for the establishment of stromal disease in this model.

Elevated systemic levels of IFN-α appeared to account for the reduced ocular spread of HSV-1 that resulted from i.v. injection of UV HSV-1. However, the reduced CTL response in these mice was not attributable directly or indirectly to IFN-α, because (i) systemic IFN-α treatment reduced virus replication in the cornea but did not inhibit the CTL response induced by HSV-1 corneal infection and (ii) anti-IFN-α/β antibody treatment did not reverse the CTL inhibition resulting from i.v. injection of UV HSV-1. The mechanism of the CTL inhibition resulting from i.v. injection of UV HSV-1 has not been determined. The inhibition is HSV-specific, but suppressor cells have not yet been identified (our unpublished observation).

IFN-α treatment after HSV-1 corneal infection provided an interesting model in which the CTL response to HSV-1 developed normally, but the infection was restricted to the corneal epithelium. The fact that the IFN-α-treated mice failed to develop corneal stromal disease suggested that the spread of HSV-1 through the eye may be an important prerequisite to the development of stromal disease.

We conclude from these studies that i.v. injection of UV HSV-1 can reduce the susceptibility of A/J mice to HSV-1 corneal stromal disease. The reduced susceptibility to stromal disease may result from the combined effect of inhibiting the T cell responses that mediate tissue destruction in the cornea and preventing the spread of HSV-1 from the corneal epithelium to the underlying stromal fibroblasts. The latter effect may be due directly or indirectly to elevated systemic levels of IFN-α and may serve to reduce or abolish the source of HSV-1 antigens that induce the tissue-destructive T cell responses in the cornea.

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