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Ocular Avirulence of a Herpes Simplex Virus Type 1 Strain Is Associated with Heightened Sensitivity to Alpha/Beta Interferon

YING-HSIU SU, JOHN E. OAKES, AND ROBERT N. LAUSCH*

Department of Microbiology and Immunology, University of South Alabama College of Medicine, Mobile, Alabama 36688

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BALB/c mice infected on the scarified cornea with herpes simplex virus type 1 strain 35 [HSV-1(35)] rarely developed ocular disease even at challenge doses as high as 10^7 PFU per eye. In contrast, HSV-1(RE) consistently induced stromal keratitis at an inoculum of 2×10^4 PFU. The goal of this study was to determine the reason for the difference in virulence between the two HSV strains. Both HSV-1 strains replicated to similar titers in excised corneal "buttons." However, after in vivo infection of the cornea, the growth of strain 35 was evident only during the first 24 h postinfection, whereas the replication of strain RE persisted for at least 4 days. In vitro tests revealed that HSV-1(35) was >10 times more sensitive to alpha/beta interferon (IFN-α/β) than HSV-1(RE). Both strains induced comparable serum levels of IFN after intraperitoneal inoculation. The kinetics of HSV-1(35) clearance from the eye was markedly altered by treatment with rabbit anti-IFN-α/β. Virus titers exceeding 10^4 PFU per eye could be demonstrated 4 to 5 days postinfection in mice given a single inoculation of antiserum 1 h after infection. Furthermore, anti-IFN treatment in 3-week-old mice infected with HSV-1(35) led to the development of clinically apparent corneal disease which subsequently progressed to stromal keratitis in the majority of recipients. These results indicate that the striking difference in the capacity of HSV-1(35) and HSV-1(RE) to induce corneal disease was related to the inherently greater sensitivity of strain 35 to IFN-α/β produced by the host in response to infection.

One of the intriguing and as yet not fully understood observations made with herpesvirus isolates is their considerable variation in virulence. The studies of Dix et al. (8) provide a good illustration of this phenomenon. They tested 23 strains of herpes simplex virus type 1 (HSV-1) for their pathogenic potential in BALB/c mice. On the basis of comparative 50% lethal dose values, the strains segregated into three groups: those which were highly virulent by both the peripheral and intracerebral routes of inoculation, those which were virulent only by the intracerebral route, and those that were highly attenuated by both routes of infection.

The reasons for variation in virus virulence are only beginning to be explored. When the infected hosts are genetically identical, the differences in virulence cannot be attributed to variation in the immune repertoire of the host. Rather, the results must reflect some important differences in the genetic makeup of the virus strains themselves. Genetic diversity in the virus genome could influence virus virulence by several mechanisms. Various studies have shown that mutations in the genes for key enzymes such as thymidine kinase (11, 30), DNA polymerase (6, 10), and ribonucleotide reductase (2) can critically affect the capacity of an isolate to induce disease. Presumedly, this is because small changes in the amino acid sequence of these enzymes markedly influence how efficiently the virus will replicate in different types of host cells. Variations in virulence among HSV strains might also result as a consequence of differences in immunogenicity. Finally, isolates may vary in their capacity to induce disease because they differ in susceptibility to nonspecific host defense mechanisms such as those mediated by macrophages (16, 17), natural killer cells (14, 19), and interferon (IFN) (1, 31–33).

In our laboratory we have identified two HSV-1 strains which differed dramatically in their capacity to induce stromal keratitis. Specifically, ocular infection of BALB/c mice with HSV-1 strain RE results in a high incidence of persistent corneal disease, whereas infection with HSV-1 strain 35 rarely induces any clinical disease in the inoculated eye. We have carried out studies to identify the reason for the strikingly different results. Our findings are presented in this report.

MATERIALS AND METHODS

Animals. Female BALB/c mice 3 to 4 weeks old were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

Viruses. HSV-1 strains RE and 35 were originally obtained from Ysolina Centifanto-Fitzgerald (Tulane University, New Orleans, La.) and Fred Rapp (Hershey Medical Center, Hershey, Pa.), respectively. Virus stocks were grown in Vero cells after being plaque purified. Infectious titers of virus preparations were determined by plaque assay on Vero cells as previously described (18). Vero cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum, 0.075% sodium bicarbonate, and antibiotics.

Vesicular stomatitis virus was grown and titrated on L-929 fibroblasts.

Corneal infection. For corneal infection, the mice were anesthetized with 0.2 ml of a 1:10 dilution of sodium pentobarbital (50 ml of stock solution) and one eye was scarified by three twists of a 2-mm corneal trephine. A 2-μl volume containing the desired concentration of HSV-1 was dropped onto the corneal surface and gently massaged into the eye with the eyelids. Eyes were examined 2 to 3 times a week for the first 2 weeks postinfection and then weekly thereafter by using a Kowa slit-lamp biomicroscope. Stromal keratitis was graded on a scale of 0 to +5 as previously described (21). Scoring was done by personnel who did not know what treatment was given.

To assay for infectious virus content in ocular tissue, eye

* Corresponding author.

globes were excised and placed in 1.0 ml of Dulbecco modified Eagle medium with 5 to 10% calf serum and antibiotics. Preparations were frozen at −70°C and then thawed and homogenized in Potter-Elvehjem (Wheaton Scientific, Millville, N.J.) tissue grinders fitted with Teflon-coated stainless steel grinders. The homogenates were sonicated for 10 to 20 s by using a Sonic 300 dismembrator (Arteck Systems Inc., Farmingdale, N.Y.). The supernatants were then titrated for infectious virus on Vero monolayers in a 48-h plaque assay.

In vitro growth of HSV-1 in mouse corneal "buttons." Corneas were excised from BALB/c mice and trimmed with a 2-mm trephine to obtain buttons of uniform size. The buttons were pooled in 0.5 ml of medium and infected with the desired concentration of virus. After 1 h of adsorption at room temperature, the infected buttons were washed five times with medium and dispersed into test tubes, two buttons per tube. The tissues were inoculated in 0.5 ml of Dulbecco modified Eagle medium containing 10% calf serum and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at 37°C in 5% CO_2 for the desired time period. Preparations were then frozen at −70°C. Subsequently the buttons were thawed, sonicated, and assayed for infectious virus content on Vero cell monolayers.

Assay for interferon sensitivity of HSV-1 strains. Excised corneal buttons prepared as described above were placed in test tubes (two per tube) and incubated with the desired concentration of mouse alpha/beta interferon (IFN-α/β) (Lee Biomolecular Research, Inc., San Diego, Calif.). The IFN-treated tissues and untreated (control) corneal buttons were infected 18 to 24 h later with HSV-1 as described above. Following an additional 24-h incubation period, the preparations were frozen at −70°C and subsequently assayed for infectious virus. The percent inhibition of virus growth was calculated by using the formula percent inhibition = [(virus titer of untreated buttons − virus titer of IFN-treated buttons)/virus titer of untreated buttons] × 100.

IFN induction in vivo. Mice were inoculated intraperitoneally with 1×10^7 PFU of HSV-1(35) or HSV-1(RE). Serum samples prepared from blood collected 2, 6, or 24 h postinfection were frozen at −20°C until assayed for IFN.

IFN assay. To measure IFN concentrations in the serum, a modification of the method of Havell and Vilcek (15) was used. Briefly, the assay involved protection of L-929 cells against infection with vesicular stomatitis virus as assessed by inhibition of cytopathic effect. The test samples were diluted in a series of twofold dilutions in 96-well plates. Each well was then seeded with 4×10^4 L-929 cells. After 18 to 24 hours of incubation, the monolayers were washed and infected with 10^3 PFU of vesicular stomatitis virus. The cultures were then incubated until the virus controls showed complete destruction of the L-929 cell monolayers (usually 48 h). The cultures were then stained with 2% crystal violet, and the titrations were scored microscopically. The highest dilution of the titrated sample producing at least 50% protection of the cells was taken as the endpoint. An internal laboratory mouse IFN standard was included with each titration and served as the reference for calculating the IFN units in each test sample.

Rabbit anti-mouse IFN-α/β treatment. Mice were given 17,000 to 21,000 National Institutes of Health neutralizing units of rabbit anti-mouse IFN-α/β (Lee Biomolecular Research, Inc., San Diego, Calif.). A single 0.2-ml inoculum was given intraperitoneally either 24 h before or within 1 h after HSV-1 corneal infection. Controls received 0.2 ml of normal rabbit serum (Cappel, West Chester, Pa.).

TABLE 1. Variation in capacity of HSV-1 strains to induce corneal opacity

Virus strain	Corneal challenge dose (PFU/eye)	Ocular opacity[a]
35	1×10^7	0/10
	3×10^6	3/16
	3×10^5	0/10
RE	2×10^5	20/21
	2×10^4	8/8

[a] Animals were monitored for development of ocular opacity for 21 days postinfection.

RESULTS

HSV-1 strains vary in ocular virulence. HSV-1(35) when dropped onto the trephined corneas of BALB/c mice only rarely induced stromal keratitis. This was the case even when challenge doses as high as 10^7 PFU per eye were given (Table 1). In contrast, HSV-1(RE) consistently induced >90% incidence of stromal disease when applied at a 500-fold lower dose. Thus, strain 35 is avirulent, whereas strain RE is virulent after corneal infection.

HSV-1(35) growth in corneal tissue in vitro. It was possible that strain 35 avirulence simply reflected an inability to replicate in BALB/c corneal cells. To test this premise, mouse corneal buttons, prepared as described in Materials and Methods, were infected in vitro with strain 35 or strain RE. The growth of each virus was then followed over a 3-day period (Fig. 1). Avirulent 35 strain grew readily in mouse corneal cells. In fact, its growth was quite analogous to that observed with the virulent RE strain. Thus, HSV-1(35) avirulence was not due to an inability to grow in murine corneal cells.

HSV-1(35) growth in the cornea in vivo. We next investigated to what extent strain 35 grew in vivo after corneal infection. The results are shown in Fig. 2. After an eclipse phase, HSV-1(35) grew to high titer, exceeding 10^5 PFU at 24 h postinfection. However, this was followed by a precipitous drop in titer to nondetectable levels by 48 h. This transient growth of strain 35 in the eye was observed in three independent experiments. In contrast, virulent strain RE was present at high titer over the 4-day observation period. These results suggested that some early host defense mech-

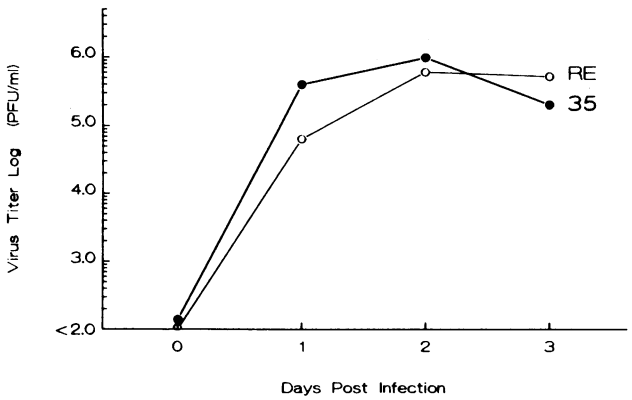

FIG. 1. Growth of HSV-1 strains 35 and RE in excised BALB/c corneas. Corneal buttons were infected in vitro with 10^5 PFU of virus. At daily intervals, samples were collected and the amount of infectious virus was determined.

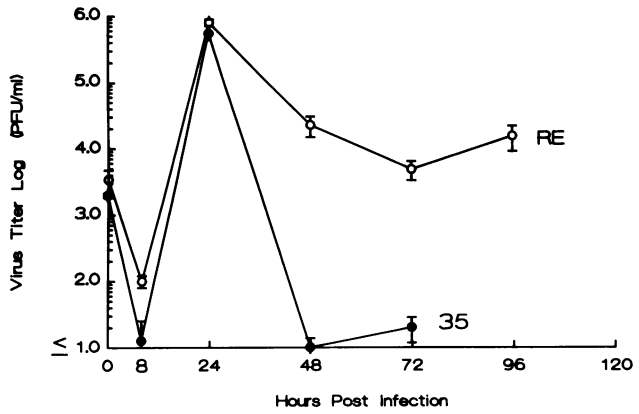

FIG. 2. Ocular growth of HSV-1 strains in BALB/c mice. Scarified mouse corneas were infected topically with 10^7 PFU of strain 35 or strain RE. At selected intervals postinfection, two or three eyes per time point were removed and the virus titers were determined. Vertical bars indicate standard errors of the mean.

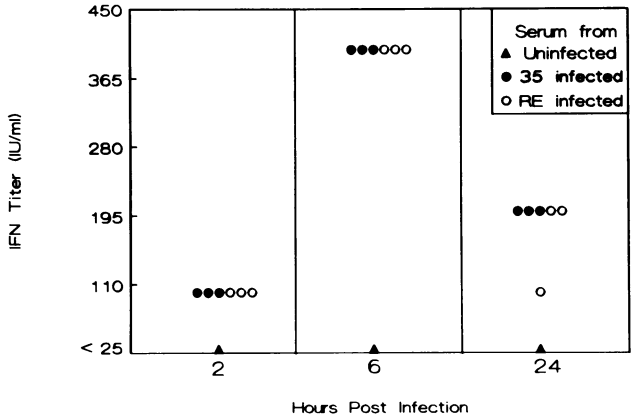

FIG. 3. Interferon serum titers following HSV-1 intraperitoneal infection. BALB/c mice were given 10^7 PFU of strain 35 or strain RE intraperitoneally. At the indicated times postinfection, serum specimens were collected and assayed for interferon content. Serum specimens from uninfected mice served as the control. Each datum point represents an individual serum assay.

anism may be inhibiting the growth of HSV-1(35) but not HSV-1(RE).

Sensitivity of HSV-1 strains 35 and RE to mouse IFN-α/β. IFN is a well-known early antiviral defense mechanism (7, 33). We therefore investigated whether production of this cytokine may have contributed to the abrogation of strain 35 replication in the eye. Excised corneal buttons were pretreated with various concentrations of IFN-α/β or left untreated. The next day all samples were infected with 8×10^3 PFU of strain 35 or RE, and virus titers were determined 24 h later. Table 2 shows that avirulent strain 35 replication was consistently inhibited by IFN at concentrations as low as 1 IU/0.2 ml. In contrast, strain RE replication in corneal buttons was not inhibited at even a 10-fold higher concentration of IFN. These results indicated that avirulent strain 35 was significantly more sensitive than virulent strain RE to mouse IFN inhibition in vitro.

Kinetics of IFN induction in vivo. The capacity of strains 35 and RE to stimulate IFN production in vivo was compared. Mice were inoculated intraperitoneally with virus, and serum samples were subsequently collected at selected intervals for IFN assay. It was found that mice infected with HSV-1(35) uniformly produced IFN detectable at all three time points (Fig. 3). Peak serum titers were observed at 6 h postinfection. The IFN response of RE-infected mice was virtually identical to that of strain 35. No IFN was detected in sera from uninfected controls. Thus, the two HSV-1 viruses did not differ significantly in their capacity to induce IFN.

It was pertinent to test whether IFN appeared in the cornea after ocular infection with strain 35. Corneal tissues from 20 eyes infected 24 h earlier with 10^7 PFU per eye were pooled in 0.5 ml of Dulbecco modified Eagle medium and incubated for 18 additional hours at 37°C in 5% CO_2. The preparation was then frozen and thawed. Following clarification by centrifugation, the supernatant was treated with anti-HSV-1 serum to neutralize any infectious HSV-1(35) before being assayed for IFN. Supernatant from a pool of 20 mock-infected corneas served as the control.

A total of 24 IU/0.6 ml of IFN was detected in the supernatant from the strain 35-infected corneal tissue pool, whereas <3 IU/0.6 ml of IFN was detected in the control supernatant. Similar results were obtained in a second independent experiment. The antiviral activity was neutralized by rabbit anti-mouse IFN-α/β. These results indicated that resident corneal cells, passenger cells, or both types of cells in the cornea could be stimulated by strain 35 to make IFN.

Effect of rabbit anti-mouse IFN-α/β treatment. Would administration of antibody to IFN-α/β facilitate HSV-1(35) growth in the eye? To investigate this question, mice were given rabbit anti-mouse IFN-α/β serum 1 h after strain 35 infection. Controls received normal rabbit serum. Virus titers in the eye were then monitored over the next 5 days (Fig. 4). In the control animals, strain 35 growth was transient, as expected, with little or no virus detected in the eyes 2 days postinfection and thereafter. The results were strikingly different in the anti-IFN-treated mice. Mean virus titers exceeding 10^4 PFU per eye were observed on days 4 and 5 postinfection. Thus, antibody treatment was accompanied by markedly enhanced virus growth in the eye.

However, enhanced strain 35 growth was not accompanied by overt clinical disease. We reasoned that the anti-IFN treatment protocol was inefficient at neutralizing endogenous IFN in the corneas of the 4-week-old mice used in the experiment. For example, the inhibitory effect of residual IFN could account for the low virus titer seen at day 3 (Fig. 4). Accordingly, to more efficiently suppress IFN activity in vivo, 10-g mice rather than 14-g mice were used, and anti-IFN was given 24 h before virus infection instead of after virus infection. In addition, the eyes were trephined the day before as well as the day of infection in an effort to facilitate corneal vascularization. Under these conditions, strain 35 infection resulted in the development of overt

TABLE 2. Sensitivity of HSV-1 strains 35 and RE to mouse IFN-α/β as assayed in excised mouse corneal buttons

IFN concentration (IU/0.2 ml)	Range of percent inhibition for[a]:	
	35	RE
1	84.8–88.4	0
10	94.3–99.8	0
100	>99.9	96.9–99.9

[a] Data obtained from three independent experiments.

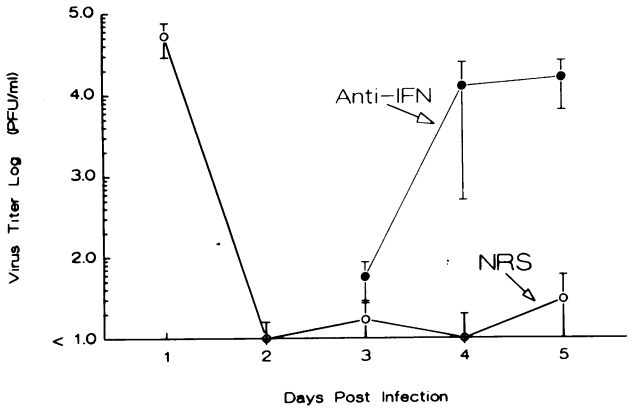

FIG. 4. Effect of anti-IFN-α/β treatment on strain 35 growth in BALB/c eye. The scarified right corneas were infected with 10^7 PFU of strain 35. One hour later, mice were given 21,000 neutralizing units of rabbit anti-mouse IFN-α/β antiserum intraperitoneally. The controls received normal rabbit serum (NRS). At selected times postinfection, eyes were removed and individually assayed for infectious virus content. Each datum point represents the mean infectious titer of three eyes. Vertical bars indicate the standard error of the mean.

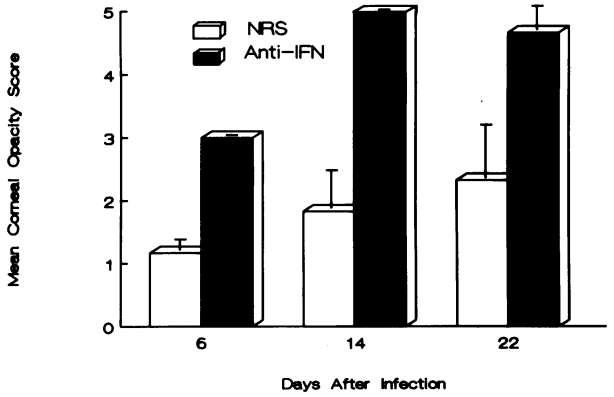

FIG. 6. Effect of anti-IFN-α/β treatment on induction of corneal opacity after HSV-1(RE) ocular infection. BALB/c mice were infected topically and then given 20,000 U of rabbit anti-mouse IFN-α/β antibody or normal rabbit serum (NRS). Ocular opacity was monitored as described previously (21). There were 5 to 6 mice in each group. Corneal opacity was significantly greater in the anti-IFN group at 6 ($P < 0.05$) and 14 ($P < 0.01$) but not 22 ($P < 0.1$) days postinfection as assessed by the Mann-Whitney U test.

disease (Fig. 5). Mild corneal opacity was evident in four of five anti-IFN-treated hosts by day 6, and by day 14, three animals had developed blinding stromal keratitis. Blepharitis was also clearly evident during the first 10 days of infection in 80% of the mice. In contrast, only one of the six control animals developed very mild blepharitis, and none displayed any evidence of corneal disease.

As noted above, strain RE infection of the mouse cornea invariably resulted in stromal disease. Might endogenous IFN production also influence the pathogenicity of this virulent HSV strain? To investigate this question, 6-week-old BALB/c mice were given anti-IFN-α/β 30 min after infection with 8×10^4 PFU of strain RE and then were monitored for the development of ocular disease. Both treated and control animals developed corneal pathology, as expected (Fig. 6). However, the development of severe stromal opacity was significantly accelerated in the anti-IFN group. Blepharitis was also more severe ($P < 0.05$) in the antiserum-treated hosts (data not shown). These results suggest that IFN is also induced in ocular tissue after strain RE infection. However, the amount synthesized by the host is apparently capable only of slowing the development of stromal keratitis induced by this more IFN-resistant HSV strain.

FIG. 5. Effect of anti-IFN-α/β treatment on induction of corneal opacity after HSV-1(35) ocular infection. BALB/c mouse corneas were scarified, and 17,000 U of rabbit anti-mouse IFN-α/β antibody or normal rabbit serum (NRS) were given 1 day before viral infection. At day 0, the corneas of both groups were scarified again and infected topically with 10^7 PFU of strain 35. Ocular opacity was monitored as described previously (21). There were 5 to 6 mice in each group.

DISCUSSION

Our laboratory has been studying the response of the mouse to infection of the scarified cornea with HSV-1. Ocular infection mimics one of the natural routes by which this virus can induce serious disease in humans, the natural hosts. Infection of BALB/c mice with HSV-1(RE) consistently resulted in the development of stromal keratitis. In the course of examining other HSV-1 strains, HSV-1(35) was found to be quite avirulent, rarely inducing disease even when a very high challenge dose (10^7 PFU) was given.

The goal of the present study was to investigate why strain 35 was less virulent than strain RE after ocular infection. A number of lines of evidence were generated which suggest that the sensitivity of strain 35 to IFN-α/β contributed to its inability to cause corneal disease. First, after the initial 24 h of infection, avirulent strain 35 growth in vivo abruptly ceased. It is known that IFN synthesis can be induced shortly after HSV-1 infection in the mouse (33) and that this early antiviral defense mechanism has the potential to limit virus replication and so prevent clinical disease (12, 13). Second, avirulent strain 35 proved to be unusually sensitive to IFN. In contrast, virulent strain RE exhibited prolonged growth in the eye and was >10-fold less sensitive than strain 35 to inhibition by IFN-α/β in vitro. Third, we found that passive transfer of rabbit anti-IFN-α/β had a major enhancing effect on the growth of strain 35 in the eye. Fourth, and most significantly, antibody treatment that efficiently neutralized endogenously synthesized IFN-α/β enabled HSV-1(35) to induce persistent corneal disease. Taken collectively, these results indicate that the difference in the capacity of the two isolates to cause corneal disease is due to

differences in their sensitivity to IFN. Even though HSV-1(RE) was 10-fold more resistant to IFN than was strain 35, it was found that this strain induced necrotizing stromal keratitis more rapidly in anti-IFN-treated hosts. This result suggests that the quantities of IFN produced by the host could not inhibit the spread of HSV-1(RE) within infected corneas, but the cytokine was capable of slowing the development of corneal pathology.

A central question raised by our study is why was HSV-1(35) so much more sensitive than HSV-1(RE) to the antiviral effects of IFN-α/β. This is a difficult question to answer because different mechanisms are activated during the IFN-induced antiviral state, not all of which have been clearly defined. The stage at which IFN inhibits the replication cycle of HSV-1 has been extensively studied, but the results are controversial and apparently may depend upon the type of cell that is infected. Early studies indicated that IFN acted late in the viral replication cycle, after protein synthesis had been completed (4, 5, 23). More recently, investigators working with HSV-infected murine and human macrophages have shown that IFN can inhibit the synthesis of immediate-early proteins and that this is a consequence of an earlier inhibition at the transcription level (9, 22). It is known that both HSV and host cell proteins contribute to transcription of immediate-early genes (20, 26). Perhaps the proteins of HSV-1(35) make transcription of the immediate-early genes of this strain more sensitive to the inhibitory effects of IFN than those of HSV-1(RE). It has also been observed that HSV can inhibit the (2′-5′)oligoadenylate pathway induced by IFN (3). Thus, a second possibility is that HSV-1(RE) exerts a stronger inhibiting effect on this pathway than does HSV-1(35).

In addition to inducing the antiviral state in potential target cells, IFN-α/β may exert its protective effect in vivo by activating other host defense mechanisms such as natural killer cells (14, 19). Therefore, a third potential explanation for the heightened sensitivity of strain 35 to IFN is that corneal cells infected with strain 35 were more susceptible to lysis by activated effector cells than were corneal cells infected with strain RE. Whatever the explanation, and there may be multiple reasons, the availability of a highly susceptible strain should prove useful for investigating the molecular mechanisms of IFN-α/β action on HSV-1.

It is presently unclear as to what type(s) of cells produced the IFN detected in the eye. Murine corneal cells can be stimulated to produce IFN-β (29) and would appear to be the most likely source. However, this may not be the case. Strain 35 grew as well as strain RE in excised corneal buttons and also replicated to high titer in the eye during the first 24 h of infection. If the corneal cells themselves were producing inhibitory levels of IFN, one might expect to observe less vigorous growth of HSV-1(35). Since the avascular cornea becomes vascularized shortly after HSV introduction (Lausch and Kayes, unpublished observations), leukocytes would have access to the infected tissue. Thus, they may have been the principal source of IFN-α/β synthesized by the host in response to HSV infection.

In this work we have focused on IFN-α/β because this type of IFN is known to be produced early during acute virus infection (7, 33). IFN-γ is not thought to be produced until after T cells have been sensitized to viral antigens and so would not be expected to participate in the first line of defense. The efficacy of IFN-γ in inhibiting herpesvirus growth is reported to vary with the target cells under study (25), and our preliminary in vitro results indicate HSV-1(35) replication is less sensitive to inhibition by IFN-γ than IFN-α/β. Nevertheless, IFN-γ may still contribute to host resistance to strain 35 in vivo (28), and studies are in progress to test this possibility.

In conclusion, the results of this study demonstrate that genetic differences in the sensitivity of HSV-1(35) and HSV-1(RE) to IFN-α/β drastically affected the ability of these two viruses to cause stromal keratitis. To our knowledge, the exquisite sensitivity of certain HSV strains to IFN-α/β has heretofore not been reported. It will be of interest to determine whether sensitivity to IFN can account for differences seen in virulence between other HSV isolates (24, 27).

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