Biological Basis for Virulence of Three Strains of Herpes Simplex Virus Type 1

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SUMMARY
Herpes simplex virus type 1 (HSV-1) strains F, HF and HFEM were studied with respect to pathogenicity in mice and growth characteristics in vivo and in vitro compared to the neurovirulent HSV-1 strains 17 syn + and KOS. All three viruses demonstrated reduced virulence in mouse brains and were completely avirulent after footpad inoculation. They were shown to express high levels of thymidine kinase activity. Investigations concerning the virulence phenotype indicated that the defect(s) in strains F, HF and HFEM related to general replication deficiency in mouse cells. It was also shown that although the replication restriction observed for strains F and HF was specific for murine cells, strain HFEM did not replicate well in any cell type tested. Additional studies indicated that the avirulence phenotype which followed peripheral inoculation was related to different genotypes, since strain F complemented HF and HFEM and, as expected, the latter two agents did not complement each other. All three agents were found to complement the non-neuroinvasive strain KOS. Finally, the data also show that two herpesviruses which are highly restricted in murine cells (e.g. strains F and HF) can still interact in the animal and produce a lethal infection.

INTRODUCTION
Herpes simplex virus (HSV) infections commonly involve the skin, mucous membranes, cornea and central nervous system. Previous work with animals has shown that most HSV strains, when injected intracranially at an appropriate dose, have the capacity to cause encephalitis and ultimately death. However, some isolates are atypical in that they fail to kill the animal when inoculated with similar, or even higher doses. Such isolates could serve as valuable research tools to study various aspects of HSV pathogenesis.

We chose to study the three HSV-1 strains F, HF and HFEM, and to compare them to the virulent strain 17 syn +. Strains F and HFEM have been reported to be apathogenic in animal models tested (Centifanto-Fitzgerald et al., 1982; Dix et al., 1983; Rosen et al., 1985; Becker et al., 1986) and strain HF (Flexner & Amoss, 1925) is the agent from which the strain HFEM was originally derived (Burnet & Lush, 1939; Watson et al., 1966). We also included the HSV-1 strain KOS (Dix et al., 1983; Thompson et al., 1986) which, although fully virulent when inoculated intracranially, is completely avirulent in mice when inoculated by a peripheral route. Our goal was first to characterize the virulence phenotype of these viruses in mice. If they were in fact apathogenic, we were interested in determining whether the lesion(s) related to specific defects in virulence functions or were due to general replicative defects in murine cells. Finally, the number of genetic lesions responsible would then be established.

In this report we present data indicating that the three test viruses demonstrated reduced virulence in the mouse brain and were completely avirulent when inoculated on mouse footpads. The results of experiments in which the replicative capacity of these viruses was investigated show that the avirulence phenotype of the three test viruses was due to general replication defects in murine cells. The results of our co-inoculation experiments show that the genetic
lesion responsible for the observed phenotype of strain F is different from that in strains HF and HFEM, and the lesions in the latter two agents could not be separated. The genetic lesion in strain KOS differs from that of strains F, HF and HFEM. Finally, our results also indicate that two growth-restricted herpesviruses (e.g. F and HF) could still interact in the animal to bring about a lethal infection.

METHODS

**Cells and viruses.** Rabbit skin cells (Watson et al., 1980) were grown in Eagle's minimal essential medium (EMEM) supplemented with 5% bovine serum (BS) and antibiotics (250 µg/ml penicillin, 250 µg/ml streptomycin, 0-25 µg/ml fungizone). Primary mouse embryo fibroblast cultures (MEF) were prepared from 16 to 18 day Swiss-Webster mice embryos (Merchant et al., 1969) and grown in EMEM supplemented with 10% foetal calf serum (FCS) and antibiotics. Vero cells (CCL-81) were obtained from the American Type Culture Collection (ATCC) and grown in EMEM supplemented with 5% FCS and antibiotics. LM(TK-) cells (Kit et al., 1963) were kindly provided by Dr S. Kit (Baylor College of Medicine, Houston, Tx., U.S.A.) and grown as described for rabbit skin cells.

HSV-1 strain HF (Flexner & Amoss, 1925) used in this study was obtained from the ATCC, and strains F (Ejercito et al., 1968) and HFEM (Burnet & Lush, 1939; Watson et al., 1966) were kindly provided by Dr B. Roizman (University of Chicago, Chicago, Ill., U.S.A.) and Dr I. Halliburton (University of Leeds, Leeds, U.K.), respectively. The HSV-1 strain KOS (Dix et al., 1983) was kindly provided by Dr M. Levine (Ann Arbor, Mich., U.S.A.). The procedure for virus propagation, titration and plaque purification were essentially as described elsewhere (Cook & Stevens, 1973; Watson et al., 1980). All incubations were at 37 or 38.5 °C, as indicated in the text, in 5% CO2 atmosphere. All viruses were plaque-purified three times on rabbit skin cells at 37 °C before use in this laboratory.

**Mouse inoculation procedures.** Outbred, 4- to 5-week-old male Swiss-Webster mice were used throughout this project. Intracerebral inoculations were accomplished by injecting 30 µl of appropriate tenfold dilutions of virus into the left cerebral hemisphere. The animals were observed daily for 3 weeks, deaths scored, and plaque-forming unit/50% lethal dose (p.f.u./LD50) values were determined for each virus by the method of Reed & Muench (1938).

Footpad inoculation was performed by inoculating serial tenfold dilutions of each virus on both rear footpads of mice (lightly abraded with few strokes of an emery board) according to a procedure described (Cook & Stevens, 1973). Mice were observed for a period of 21 days, deaths were scored, and p.f.u./LD50 values were determined as described above.

**Viral replicative kinetics in mouse brains.** Thirty µl inocula containing 10⁸ p.f.u. of each virus were injected intracranially into groups of mice, and at 0, 10, 24, 34 and 48 h post-infection (p.i.), brains were removed from three mice and stored at −70 °C. No samples were obtained for strains KOS and HFEM at 48 h p.i. since animals inoculated with these viruses did not survive beyond 34 h p.i. The samples were then thawed, 20% (w/v) suspensions prepared in EMEM with 5% BS, clarified and the virus content was determined on rabbit skin cells as described previously (Watson et al., 1980).

**Viral replication kinetics in various tissue culture cells.** The procedures to study viral multistep replication kinetics in MEF, rabbit skin cells and Vero cells were essentially as described (Thompson & Stevens, 1983). Briefly, monolayers were infected with an appropriate virus at an m.o.i. of about 0-001 p.f.u./cell and after 1 h adsorption at 37 °C the inoculum was replaced with fresh medium and incubation continued at 38.5 °C. At 0, 10, 24, 34, 72 and 96 h p.i., cells were harvested from duplicate wells and stored at −70 °C. At the end of each experiment, samples were thawed, and viral titres were determined on rabbit skin cells.

**Thymidine kinase (TK) assay.** The TK activity of each virus stock was determined essentially according to the procedure of Jamieson & Subak-Sharpe (1974), except that the infected cells were incubated at 38.5 °C instead of 37 °C. LM(TK-) cells were infected with each virus stock at an m.o.i. of 3 p.f.u./cell, and at 5 h p.i. cells were collected and assayed for their ability to phosphorylate [³H]thymidine. The radioactive phosphorylated thymidine generated was then adsorbed to DE-81 chromatography paper and the radioactivity determined in a scintillation counter. Enzyme activity was expressed as total c.p.m. (phosphorylated thymidine) bound to DE-81 paper per µg protein of each sample.

RESULTS

Initially, the virulence phenotype of the viruses was established, and the data obtained (Table 1) show that strains F, HF and HFEM demonstrated markedly reduced virulence in mouse brains when compared to the neurovirulent strains 17 syn+ and KOS. While the neurovirulent agents killed mice at 25 p.f.u. or less, 350 p.f.u. or more was required to kill mice with the test
**HSV-1 virulence**

Table 1. *P.f.u./LD*$_{50}$* ratios in mice after brain and footpad inoculation of HSV-1 strains 17 syn*+, KOS, F, HF and HFEM

<table>
<thead>
<tr>
<th>Virus</th>
<th>Brain P.f.u./LD*$_{50}$</th>
<th>Footpad P.f.u./LD*$_{50}$</th>
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<tbody>
<tr>
<td>17 syn*+</td>
<td>4-6</td>
<td>10*4</td>
</tr>
<tr>
<td>KOS</td>
<td>25</td>
<td>&gt;10*8</td>
</tr>
<tr>
<td>F</td>
<td>6-2 × 10*5</td>
<td>&gt;10*8</td>
</tr>
<tr>
<td>HF</td>
<td>6-2 × 10*2</td>
<td>&gt;10*7</td>
</tr>
<tr>
<td>HFEM</td>
<td>3-5 × 10*2</td>
<td>&gt;10*7</td>
</tr>
</tbody>
</table>

* Values are mean of two experiments. Mice (five/dilution) were inoculated with tenfold dilutions of the viruses noted, deaths scored, and p.f.u./LD*$_{50}$* ratios calculated using the formula of Reed & Muench (1938). Details concerning methods are presented in the text.

Table 2. *Thymidine kinase enzyme activity of HSV-1 strains 17 syn*+, KOS, F, HF, HFEM and TK$^{-}$

<table>
<thead>
<tr>
<th>Virus</th>
<th>C.p.m./µg protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 syn*+</td>
<td>1.9 × 10*3</td>
</tr>
<tr>
<td>KOS</td>
<td>2.1 × 10*3</td>
</tr>
<tr>
<td>F</td>
<td>3.1 × 10*3</td>
</tr>
<tr>
<td>HF</td>
<td>1.6 × 10*3</td>
</tr>
<tr>
<td>HFEM</td>
<td>2.1 × 10*3</td>
</tr>
<tr>
<td>TK$^{-}$</td>
<td>1.5 × 10*3</td>
</tr>
</tbody>
</table>

* LM(TK$^{-}$) cells were infected with each stock virus at an m.o.i. of 3 p.f.u./cell, and at 5 h p.i. cells were harvested and assayed for TK activity as described in the Methods section. Values were adjusted by subtracting that obtained for the uninfected cells (20 c.p.m./µg protein). The TK activity in each case is expressed as total radioactivity of [*H]thymidine phosphorylated and bound to DE-81 chromatography paper.

agents. As Table 1 also indicates, neither the test viruses nor KOS killed mice when inoculated on footpads at the highest doses obtainable. They possessed p.f.u./LD*$_{50}$* ratios of >10*7* (HF and HFEM) or >10*8* (F and KOS) while the virulent strain 17 syn*+* demonstrated a p.f.u./LD*$_{50}$* of 10*4*.

It is widely appreciated that expression of the virus-encoded TK enzyme activity is necessary for maximal demonstration of the virulence phenotype of herpes simplex viruses. To determine whether the observed reduced neurovirulence of the viruses tested here was due to lack of such activity, a TK enzyme assay was performed on LM(TK$^{-}$) cells infected with each of the above viruses. A TK-negative variant of HSV-1 strain 17 syn*+* (TK$^{-}$) was also included in this assay as a negative control. The results presented in Table 2 show that all of the test viruses expressed levels of TK activity comparable to that of the virulent strain 17 syn*+. From these results, it was concluded that the reduced neurovirulence of the viruses tested was not due to lack of expression of TK enzyme activity.

In initial experiments to investigate whether the observed reduced virulence related to deficiencies in the replication capacity of the viruses in murine cells, replicative kinetics were studied in mouse brains. The data presented in Fig. 1 show that following intracranial injection of 10*5* p.f.u., strain KOS and the virulent strain 17 syn*+* replicated to high titres (approx. 2 × 10*6* p.f.u./g brain tissues) within 34 h p.i. and all mice died by 48 h p.i. In contrast, strains F, HF and HFEM, which replicated to various extents, never reached the levels achieved by the neurovirulent strains. These results indicated either that the agents were specifically restricted in nervous tissues, or that there was a general restriction in murine cells. That the latter explanation was the correct one was established by measuring multistep replicative kinetics in primary MEF at 38.5 °C (murine body temperature). The data presented in Fig. 2 represent the
Fig. 1. HSV-1 replicative kinetics in mouse brains in vivo. Mice were inoculated intracranially with $10^5$ p.f.u. of HSV-1 strains 17 syn+ (■), KOS (■), F (○), HF (□) or HFEM (▲) as described in the Methods section. At 0, 10, 24 and 34 h p.i., animals were sacrificed, brains removed and stored at $-70^\circ$C and later titrated for infectious virus. Values are presented as p.f.u. per g of brain tissue based on titres obtained from three mouse brains.

Fig. 2. Multistep replicative kinetics of HSV-1 strains in primary mouse embryo fibroblasts. Monolayers were infected with 17 syn+ (■), KOS (■), F (○), HF (□) or HFEM (▲) at an m.o.i. of 0.001 p.f.u./cell and cultures were incubated at $38.5^\circ$C. At various times p.i., cells from duplicate wells were collected and stored at $-70^\circ$C and later titrated for infectious virus. The titres obtained for each sample at the indicated times p.i. are presented.

Replicative kinetics in MEF of strains F, HF and HFEM compared to those of strain 17 syn+ and KOS. Clearly, none of the three test viruses replicated efficiently under these conditions. In the case of strain F, delayed replication was observed, the peak titre never reached the level achieved by strains 17 syn+ and KOS, and a precipitous decline in titre was observed by 72 h p.i. Although the replicative kinetics of strains HF and HFEM appeared to be normal when compared to the neurovirulent strains, they failed to produce the viral yields observed for the latter strains.

We also determined whether the inefficient replication of test viruses was specific for murine cells or if they were also restricted in other cell types. Here, multistep replicative kinetics in rabbit skin cells and Vero cells at $38.5^\circ$C were tested. The results indicated that there were marked differences among the viruses with respect to their ability to grow in rabbit skin (Fig. 3a) or Vero cells (Fig. 3b). Although strains 17 syn+ and KOS replicated in both cell systems very efficiently, strains F and HF replicated well only in rabbit skin cells and Vero cells, respectively, while strain HFEM showed reduced replicative capacity in both cell types.
Fig. 3. Multistep replicative kinetics of HSV-1 strains 17 syn*, KOS, F, HF and HFEM in (a) rabbit skin cells and (b) Vero cells. Virus designations, conditions for infection and incubation of monolayers, and titration of the samples at indicated times p.i. were as described for Fig. 2.

Table 3. Mortality of mice infected with HSV-1 strains KOS, F, HF or HFEM alone or in combinations

<table>
<thead>
<tr>
<th>Virus combination*</th>
<th>Mortality (%)</th>
<th>Pathogenic phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With saline†</td>
<td>No saline</td>
</tr>
<tr>
<td>KOS + F</td>
<td>7/7 (100)</td>
<td>9/10 (90)</td>
</tr>
<tr>
<td>KOS + HF</td>
<td>7/7 (100)</td>
<td>3/7 (43)</td>
</tr>
<tr>
<td>KOS + HFEM</td>
<td>3/8 (38)</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td>F + HF</td>
<td>7/7 (100)</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>F + HFEM</td>
<td>7/7 (100)</td>
<td>5/10 (50)</td>
</tr>
<tr>
<td>HF + HFEM</td>
<td>0/7 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>KOS</td>
<td>0/7 (0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>F</td>
<td>0/7 (0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>HF</td>
<td>0/7 (0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>HFEM</td>
<td>0/7 (0)</td>
<td>0/5 (0)</td>
</tr>
</tbody>
</table>

* 10⁶ p.f.u. of each virus alone or mixed with other viruses were inoculated on footpads of mice as described in Methods. Mice were observed for 21 days and deaths scored.
† Mice in this group were injected with 0.1 ml/footpad of a 10% saline solution, 6 to 8 h prior to the viral inoculations (Cook & Stevens, 1973). This procedure increases the effective dose of virus inoculated by some 100-fold (Olitsky & Schlesinger, 1941).

Although the results presented showed that the observed reduced virulence phenotype was due to replication deficiency in murine cells, it was of interest to determine whether the phenotype related to different genetic lesions. This was accomplished by co-inoculating the agents on the footpads of mice. The validity of this approach was established earlier, when we
showed that under these experimental conditions, the two avirulent viruses ANG and KOS interacted in the animal to produce lethal recombinants (Javier et al., 1986). In the present experiments, 10⁶ p.f.u. of each virus was inoculated on footpads of mice in combinations consisting of KOS + F, KOS + HF, KOS + HFEM, F + HF, F + HFEM and HF + HFEM. Controls consisted of animals inoculated with each virus alone at the same dosage. Any combination that showed a significant increase in mortality compared to a virus inoculated alone was considered to be pathogenic.

The results of these co-inoculation experiments are presented in Table 3, and they show that five of the combinations tested (F + KOS, HF + KOS, HFEM + KOS, F + HF and F + HFEM) killed mice. The KOS + HFEM combination showed the lowest enhancement of mortality (10%). However, as shown in Table 3, when mice were pretreated with a 10% saline solution to increase susceptibility to viruses (Olitsky & Schlesinger, 1941), a 38% mortality was obtained with this combination. Since the procedure did not increase susceptibility to KOS or HFEM inoculated alone, the KOS + HFEM mixture was considered to be pathogenic.

DISCUSSION

We have examined the virulence of the HSV-1 strains F, HF and HFEM in mice, and compared them to the neurovirulent strains 17 syn ^ and KOS. All the test viruses were shown to demonstrate reduced virulence in mouse brains and were completely avirulent when inoculated by a peripheral route. These results were shown not to be related to viral TK activity since all agents expressed high levels of the enzyme.

As to the properties responsible for the phenotypes, analyses of replicative kinetics of the test viruses in mouse brains in vivo and in MEF in vitro (at 38.5 °C) show that the phenotype related to inefficient replicative capacity in murine cells. It is important to note that in previous experiments (Thompson et al., 1986), we showed that KOS also lacks virulence when inoculated by a peripheral route, but does not demonstrate the restriction in murine cells. These results, and the fact that KOS complemented all other agents in the co-inoculation experiments presented here show that the lesion in KOS is different from those in the other viruses. The restriction demonstrated by F and HF seems to be specific for murine cells, since these viruses replicate well in other cell types, but strain HFEM does not replicate well in any cell type tested. The somewhat differing results with strains HF and HFEM were not expected, since these viruses are genetically related (Burnet & Lush, 1939; Watson et al., 1966), and they do not complement each other in the co-inoculation experiments (Table 3). It might also be emphasized that strains F and HF, which expressed highly restricted growth kinetics specific for murine cells, also killed animals when co-inoculated.

Although we have not determined whether mortality in the co-inoculation experiments was a consequence of complementation or recombination, the results do emphasize the multigenic nature of HSV-1 virulence in this system. As for the number of genetic determinants involved, based on our previous studies with ANG and KOS (Javier et al., 1986) and the present results, we would suggest that there are at least four, and these are defined by lesions detected in strains ANG, KOS, F and HF (the lesion in HFEM could not be separated from that of HF in the co-inoculation experiments). However, while the lesions in ANG and KOS are specific for virulence, those in F and HF reflect general replicative defects in murine cells.

Finally, the latter findings clearly emphasize the importance of establishing general replicative properties of viruses in cells from the animal in question when studying specific biological effects.

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