Pathogenesis of Experimental Skin Infections Induced by Drug-Resistant Herpes Simplex Virus Mutants†

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The comparative analysis of the pathogenicity of a parental herpes simplex virus type 1 strain and its phosphonoacetic acid (PAA)-resistant and acyclovir (ACV)-resistant mutants showed marked differences among them. After orofacial skin inoculation of hairless mice the parental and PAA-resistant viruses were detected during the first 4 days after infection at high and increasing titers in the trigeminal ganglia; the ACV-resistant mutant was present at low and decreasing titers in the ganglia. Severe and slow-healing skin lesions were produced by the parental and PAA-resistant viruses; mild and rapidly healing lesions were produced by the ACV-resistant mutant. Viral titers in ganglia and the intensity of skin lesions were related to the virus dose used in the primary infection. Latent infections became established in trigeminal ganglia of mice inoculated with $10^{8.6}$ plaque-forming units of the parental or PAA-resistant virus; no latent infections were detected in ganglia of mice inoculated with $10^{7.0}$ plaque-forming units of the ACV-resistant mutant. Serum antibody titers attained similar values 4 weeks after primary infection with both mutants and the parental virus. Mice infected with the ACV-resistant mutant were reinfected with the parental and PAA-resistant viruses; the degree of protection against development of skin lesions, mortality, and latency was related to the dose of ACV-resistant virus used in the primary infection. Mortality was prevented by a dose of $10^{6.0}$ plaque-forming units, skin lesions were prevented by a dose of $10^{4.2}$ plaque-forming units, and latency was prevented by a dose of $10^{5.0}$ plaque-forming units of the ACV-resistant mutant. Protection against reinfecion with the PAA-resistant mutant was achieved with lower doses than protection against the parental virus. Serum antibody titers showed a 4- to 15-fold increase after reinfecion. The results suggest that the ACV-resistant, latency-negative mutant has many attributes of a live attenuated herpes simplex virus vaccine.

With the increase in the use of antiviral compounds active against herpes simplex virus (HSV), the emergence of drug-resistant virus can be expected during the treatment of viral infection. Thus far, however, 5-iodo-2'-deoxyuridine (IUdR)-resistant HSV mutants from treated patients have been isolated infrequently (10, 11, 30) and the emergence of arabinosyladenine-resistant mutants has not yet been reported in drug-treated patients. Likewise, with the exception of IUdR-resistant mutants (29), drug-resistant mutants have not been isolated from experimentally infected animals treated with a variety of compounds active against HSV (5). On the other hand, the isolation of drug-resistant HSV mutants has been achieved with relative ease in vitro by using in the selection procedure a variety of antiviral compounds: IUdR (22), phosphonoacetic acid (PAA) (15), arabinosylthymine (Ara-T) (27), and 9-(2-hydroxyethoxymethyl)guanine (acyclovir; ACV) (5, 6). Recently, it was reported that arabinosyladenine-resistant mutants were obtained in the presence of high (100-µg/ml) drug concentrations (28), although earlier attempts to obtain them have failed (13).

Previous studies have shown that Ara-T-resistant (25, 26) and ACV-resistant mutants (5, 14) have a decreased pathogenicity in experimental animal infections, whereas PAA-resistant mutants (15) have a pathogenicity similar to that of the parental HSV strain. Intermediate degrees of pathogenicity were observed in experimental infections induced by IUdR-resistant mutants (21, 22, 28). Only Ara-T- and ACV-resistant mutants were evaluated for their abil-

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ity to induce latent infections, whereas PAA- and IUDR-resistant mutants were tested only for their potential to induce skin or ocular lesions and a fatal outcome of the infection. The ability of these mutants to invade sensory ganglia during the acute phase of skin infection has not yet been fully investigated.

In this report we present data on the ability of ACV- and PAA-resistant mutants derived from an HSV-1 strain to invade the trigeminal ganglia and to induce skin lesions during the acute phase of orofacial infection of hairless mice. The frequency of latent ganglionic infections in mice surviving the primary infections with drug-resistant and parental viruses was also determined. In addition, mice inoculated with a latency-negative ACV-resistant mutant (11) were reinfected with the PAA-resistant and parental viruses and observed for the development of skin lesions and the establishment of ganglionic latency. We have also determined the level of neutralizing serum antibodies after primary infection and after reinfecion of the mice.

MATERIALS AND METHODS

Parental virus and drug-resistant mutants. The parental HSV type 1 (HSV-1) strain 5 was described in our previous publications (13–15). The selection and cloning in microtiter plates of the ACV-resistant mutant was described in detail in our recent publication (14). The mutant stock had a titer of 10^6.8 50% tissue culture infective doses in the absence of ACV and 10^6.5 50% tissue culture infective doses in the presence of ACV (5 µg/ml). The PAA-resistant mutant was selected likewise by the passage of the parental strain in the presence of increasing drug concentrations (25, 50, and 100 µg of PAA per ml), and by cloning in microtiter plates (13). The mutant stock had the same titer (10^6.5 tissue culture infective doses) in the presence (100 µg/ml) and absence of PAA. There was no cross-resistance between the ACV- and PAA-resistant mutants: the titer of the PAA-resistant mutant in the presence of ACV and that of the ACV-resistant mutant in the presence of PAA were about 4 log_{10} units lower, similar to that shown by the parental virus. Titers of the stock virus preparations of the parental and mutant viruses were determined by a plaque assay in Vero cells, and the values obtained were used as a reference throughout the described experiments.

Inoculation of mice. Female hairless mice, strain HRS/J, were obtained at the age of 4 to 6 weeks from the breeding facilities of New York University Medical Center. The mice were inoculated percutaneously in the orofacial area by rubbing into the scarified skin various doses of the parental or mutant virus preparations. The intensity of the developing skin lesions was recorded and graded from a scale from 0 to 4 (19). Groups of mice infected with the ACV-resistant mutant were reinfected after 4 weeks with the PAA-resistant or parental virus at the same site, and the evolution of the resulting skin lesions was recorded.

Monitoring of acute infection in trigeminal ganglia. Groups of mice were exsanguinated by heart puncture under pentobarbital sodium anesthesia during the first 4 days after infection. The trigeminal ganglia were removed and immediately homogenized by sonication (Branson Sonifier Cell Disruptor 200). The suspension was clarified by centrifugation, and its virus content was determined by a plaque assay in Vero cells.

Isolation of latent virus from trigeminal ganglia. Mice were exsanguinated by heart puncture under anesthesia 3 to 4 weeks after the primary infection and at the same interval after reinfecion. The trigeminal ganglia were explanted in a 24-well tissue culture plate (Linbro, Camden, Conn.). After the ganglia became attached to the bottom of the well, 2 ml of Eagle minimum essential medium with 2% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) was added to each well. After 4 days in culture the ganglia were homogenized by sonication, and after centrifugation the supernatant was tested for the presence of reactivating virus in human foreskin fibroblasts (FS-7 cells).

Antiviral compounds. Disodium PAA was obtained from Abbott Laboratories, North Chicago, Ill. The compound was dissolved in phosphate-buffered saline adjusted to pH 7.4 and diluted in minimum essential medium to desired concentrations for selection of PAA-resistant mutants. ACV (Wellcome Research Laboratories, Research Triangle Park, N.C.) was dissolved in dimethyl sulfoxide and diluted in minimum essential medium to desired concentrations for selection of ACV-resistant mutants.

RESULTS

Initiation of acute infection in trigeminal ganglia. (i) Parental HSV strain. As shown in Fig. 1, free virus was detectable on the second day postinfection, and the average titer was related to the dose of virus used for infection. The titers increased over the next 2 days and tended to equalize (about 1 x 10^6 to 5 x 10^4 plaque-forming units [PFU]) by day 4 postinfection irrespective of the virus dose. Consistent acute infections could be initiated with a dose of 10^5.5 PFU, and only one mouse of four did not become acutely infected by day 4 postinfection when infected with 10^6.0 PFU.

(ii) ACV-resistant mutant. Similar to the parental virus, the ACV-resistant mutant was detected in trigeminal ganglia by day 2 postinfection (Fig. 2). However, the titer observed after inoculating 10^6.0 PFU of the mutant was 10 times lower than that observed after inoculating 10^6.0 PFU of the parental virus. The free virus titers in the ganglia were related to the dose of virus used for inoculation. As opposed to the continuous increase of free virus in trigeminal ganglia of mice inoculated with the parental virus (Fig. 1), the titer of free mutant virus showed a continuous decrease over the 4-day observation period (Fig. 2). Consistent acute infection was obtained only with a dose of 10^6.5 PFU of the mutant, whereas a dose of 10^5.5 PFU induced
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Fig. 1. Penetration of the parental HSV-1 strain in the trigeminal ganglia during the acute phase of infection. Mice inoculated in the orofacial area with various doses of virus were killed at the indicated days. The ganglia were removed and homogenized by sonication. The titer of the homogenate was determined by a plaque assay in Vero cells. Each point represents the titer of free virus in the homogenate from a single mouse. Height of bar indicates mean titer per group.

Fig. 2. Penetration of the ACV-resistant mutant in trigeminal ganglia during the acute phase of infection. For details see legend to Fig. 1.

only erratic responses. A dose of $10^{5.0}$ PFU was unable to initiate an acute ganglionic infection.

(iii) PAA-resistant mutant. The PAA-resistant mutant behaved essentially in the same way as the parental virus. Although the titers of free mutant virus increased somewhat slower than those of the parental strain, they eventually attained the same level by day 4 postinfection.
This difference was more visible using a low (Fig. 3A) rather than a high dose of virus (Fig. 3B). Consistent acute infections were obtained by using a dose of 10^6.0 PFU of the PAA-resistant mutant.

**Evolution of skin lesions during the primary infection.** The average lesion score observed in the orofacial area was dose dependent after primary infections with the parental virus and its PAA- and ACV-resistant mutants (Fig. 4). The evolution of skin lesions induced by the parental and PAA-resistant viruses was similar, and severe skin lesions (lesion score, >2) developed in mice inoculated with 10^6.0 PFU of either the parental or the PAA-resistant virus (Fig. 4A and B). Only minor skin lesions developed in mice infected with the ACV-resistant mutant even when inoculated with a dose of 10^7.0 PFU (Fig. 4C). In addition, the lesions healed rapidly, as opposed to the protracted healing of lesions induced by the parental and PAA-resistant viruses.

**Mortality and latent infections after primary infections.** Mortality induced by the parental HSV-1 strain is known to be low with a dose of up to 10^6.0 PFU (17). However, when a high dose (10^7.0 PFU) was used, the mortality approached 100%. With lower doses the mortality was reduced to a statistically significant extent (P < 0.05, Fisher's exact test). The PAA-resistant mutant inoculated at a dose of 10^7.0 PFU induced a lower mortality than that observed with the parental strain, but the difference is statistically not significant. No death
occurred among mice inoculated with any dose of the ACV-resistant mutant (Table 1).

Latent infections were established in almost all mice (92%) inoculated with the parental or PAA-resistant virus with doses of at least 10^6.0 PFU. Even with a dose of 10^5.5 PFU, 50% of the mice became latently infected. No latent infection was established in mice inoculated with any dose of the ACV-resistant mutant (Table 1).

Skin lesions in mice infected with the ACV-resistant mutant and reinfected with the parental or PAA-resistant virus. Mice infected in the orofacial area with various doses of the ACV-resistant mutant (from 10^4.0 to 10^7.0 PFU) were reinfected at the same site with 10^6.0 or 10^7.0 PFU of the parental or PAA-resistant virus. No lesions developed in mice infected with 10^6.0 or 10^7.0 PFU of the ACV-resistant mutant and reinfected with either dose of the parental or PAA-resistant virus. Reinfecion with 10^6.0 PFU of the parental or PAA-resistant virus caused only minimal lesions (score <1) only in mice which were infected with less than 10^6.0 PFU of the ACV-resistant mutant (data not shown). Reinfecion with 10^5.0 PFU of the parental or PAA-resistant virus induced severe lesions in mice infected with 10^5.0 PFU of the ACV-resistant mutant, moderate lesions in mice infected with 10^5.5 PFU, and only mild lesions in mice infected with 10^6.0 PFU (Fig. 5).

Mortality and latent infections in mice infected with the ACV-resistant mutant and reinfected with the parental or PAA-resistant virus. No mortality was recorded among mice infected with 10^6.0 or 10^7.0 PFU of the ACV-resistant mutant and reinfected with 10^7.0 PFU of the parental virus (Table 2). In mice infected with lesser amounts of the ACV-resistant mutant, the mortality rate induced by 10^7.0 PFU of the parental virus increased with the decrease of the virus dose used in primary infection. With two exceptions, no mortality was recorded in mice reinfected with 10^6.0 or 10^6.0 PFU of the PAA-resistant mutant or 10^6.0 PFU of the parental virus.

### TABLE 1. Mortality and latency in hairless mice inoculated with the parental HSV-1 strain and its PAA- and ACV-resistant mutants

<table>
<thead>
<tr>
<th>Virus inoculum (PFU)</th>
<th>Mortality and latency in mice inoculated with HSV-1 strain:</th>
<th>PAA resistant</th>
<th>ACV resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parental mortality latency</td>
<td>PAA resistant mortality latency</td>
<td>ACV resistant mortality latency</td>
</tr>
<tr>
<td>10^7.0</td>
<td>5/6 1/1 2/6 4/4 0/6 0/6</td>
<td>1/1 2/2 4/4 0/6 0/6</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>10^6.5</td>
<td>1/6 4/5 0/6 6/6 0/6 0/6</td>
<td>0/6 0/6 0/6 0/6 0/6</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>10^6.0</td>
<td>1/6 5/5 1/6 4/5 0/6 0/6</td>
<td>0/6 0/6 0/6 0/6 0/6</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>10^5.5</td>
<td>0/6 2/6 0/6 4/6 0/6 0/6</td>
<td>0/6 0/6 0/6 0/6 0/6</td>
<td>0/6 0/6</td>
</tr>
</tbody>
</table>

* Percutaneous inoculation in the orofacial area. Data are expressed as number of animals with mortality or latency per total number of animals tested.

### TABLE 2. Mortality and latency in hairless mice inoculated with the ACV-resistant mutant and reinfected with the parental strain or the PAA-resistant mutant

<table>
<thead>
<tr>
<th>Primary infection with ACV-resistant mutant (PFU)</th>
<th>Mortality and latency in animals reinfected with the following virus:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parental (10^7 PFU)</td>
</tr>
<tr>
<td></td>
<td>Mortality Latency</td>
</tr>
<tr>
<td>10^7.0</td>
<td>0/6 1/6</td>
</tr>
<tr>
<td>10^6.5</td>
<td>0/6 2/2</td>
</tr>
<tr>
<td>10^6.0</td>
<td>1/6 1/5</td>
</tr>
<tr>
<td>10^5.5</td>
<td>2/6 2/4</td>
</tr>
<tr>
<td>10^4.5</td>
<td>4/6 2/2</td>
</tr>
</tbody>
</table>

* Percutaneous infection and reinfecion in the orofacial area. Data are expressed as in Table 1.

* Statistically significant (P < 0.01 and P < 0.04); different mortality compared with that observed in mice inoculated with 10^5 PFU in the primary infection.

**FIG. 5.** Evolution of skin lesions induced in mice inoculated with (●) 10^5.0 PFU, (●) 10^5.5 PFU, or (●) 10^6.0 PFU of the ACV-resistant mutant and reinfected with 10^7.0 PFU of (A) the parental HSV-1 strain or (B) its PAA-resistant mutant.
Latent infections after reinfection with the parental virus were completely prevented only in mice infected with \(10^{6.0}\) PFU of the ACV-resistant mutant and with a challenge dose not higher than \(10^{6.0}\) PFU. Latent infections after reinfection with the PAA-resistant mutant were completely prevented in mice infected with only \(10^{6.0}\) PFU of the ACV-resistant mutant and challenged with \(10^{7.0}\) PFU. In mice infected with \(10^{6.0}\) PFU or less of the ACV-resistant mutant, the frequency of latent infections induced after reinfection with either dose of the parental or PAA-resistant virus increased generally with the decrease of the virus dose used in the primary infection (Table 2).

Serum antibody titers after primary infection and reinfection. Neutralizing serum antibody titers measured 4 weeks after primary infection did not show a dose-dependent effect. The relative high titer of 3.20 log\(_{10}\) units was observed in the single mouse which survived a primary infection with \(10^{15}\) PFU of the parental virus. The antibody titers in mice inoculated with the ACV-resistant mutant were somewhat lower than those observed in mice infected with the parental or PAA-resistant virus, although the difference was statistically not significant (Table 3).

Mice infected with various doses of the ACV-resistant mutant had an average neutralizing serum antibody titer of about 2.20 log\(_{10}\) units (Table 3). After reinfection with \(10^{6.0}\) or \(10^{7.0}\) PFU of the parental or PAA-resistant virus the titers increased about 5- to 15-fold (Table 4). The increase was more pronounced with a challenge dose of \(10^{6}\) PFU than with \(10^{7}\) PFU and stronger with the parental virus than with the PAA-resistant mutant. There was no apparent relation between the extent of the titer increase after reinfection and the dose of the ACV-resistant virus used in the primary inoculation. Reinfection with the parental virus induced on the average a 15-fold (\(10^7\) PFU) to 4-fold (\(10^6\) PFU) increase, whereas in reinfections with the PAA-resistant mutant the increases ranged from more than 6-fold (\(10^7\) PFU) to almost 4-fold (\(10^6\) PFU).

**DISCUSSION**

The comparative analysis of the pathogenicity of the parental HSV-1 strain and its drug-resistant mutants showed marked differences among them. The parental strain was highly pathogenic as indicated by its ability to invade and attain high titers in the trigeminal ganglia during the acute phase of infection, by inducing severe skin lesions, by causing an increased mortality when inoculated at high dose (\(10^7\) PFU), and by its ability to induce latent ganglionic infections in all mice, even when inoculated at a relative low dose (\(10^6\) PFU). Furthermore, only mice which were inoculated with at least \(10^{6.5}\) PFU of the ACV-resistant mutant showed a satisfactory degree of protection when reinfeeted with a high dose of the parental strain (Table 2).

The PAA-resistant mutant behaved much in the same way as the parental virus. However, the results suggest that this mutant is somewhat less pathogenic: the mortality rate after primary infections was slightly lower, and the invasion of the trigeminal ganglia during the acute phase of the infection proceeded at a slower rate. Mice inoculated with the ACV-resistant mutant showed a better degree of protection against reinfection with the PAA-resistant mutant, and antibody titers elicited after reinfection with a high dose of the mutant (\(10^7\) PFU) were slightly lower than those elicited by the parental strain (Table 4). None of these criteria was significantly different when examined separately; however, considered together they suggest that the PAA-resistant mutant is less invasive than the parental strain.

Resistance to PAA is caused by a mutation of the deoxyribonucleic acid polymerase gene in the HSV genome, which results in the synthesis

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**Table 3. Neutralizing HSV-specific serum antibody titers of mice after primary infection with the parental virus and its drug-resistant mutants**

<table>
<thead>
<tr>
<th>Virus dose (PFU)</th>
<th>Parental virus</th>
<th>PAA-resistant mutant</th>
<th>ACV-resistant mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10^7)</td>
<td>3.20</td>
<td>2.76 ± 0.91</td>
<td>2.48 ± 0.24</td>
</tr>
<tr>
<td>(10^6)</td>
<td>2.61 ± 0.51</td>
<td>2.73 ± 0.21</td>
<td>2.18 ± 0.32</td>
</tr>
<tr>
<td>(10^5)</td>
<td>2.57 ± 0.29</td>
<td>2.58 ± 0.15</td>
<td>2.05 ± 0.40</td>
</tr>
<tr>
<td>(10^4)</td>
<td>2.20 ± 0.54</td>
<td>2.65 ± 0.27</td>
<td>2.28 ± 0.53</td>
</tr>
<tr>
<td>(10^3)</td>
<td>Not done</td>
<td>Not done</td>
<td>1.98 ± 0.32</td>
</tr>
</tbody>
</table>

* Data are expressed as log\(_{10}\) units ± standard deviations.

* Determined in the single surviving mouse (see Table 1).
of an enzyme insensitive to the inhibitory action of the drug (9). Whether the observed decreased invasiveness of the PAA-resistant mutant is due solely to the altered polymerase cannot be determined on the basis of the present experiments.

The ACV-resistant mutant proved to be devoid of pathogenicity. The skin lesions were minimal and healed rapidly, and no latent infections were detected in the trigeminal ganglia irrespective of the virus dose employed. Nevertheless, the mutant was able to penetrate in the ganglion, but the titers of free virus during the acute phase of infection were about 100 times lower than those observed with the parental or PAA-resistant virus. Similar results were obtained by Tenser et al. (27) after corneal inoculation of guinea pigs with an Ara-T-resistant HSV mutant. In addition, free virus in ganglia decreased rapidly, the maximum titer being observed on the first day (day 2 p.i.) when virus became detectable. However, despite this limited presence of the ACV-resistant mutant in the infected mouse, it was sufficient to elicit an immune response adequate to protect mice against reinfection.

ACV acquires antiviral potential through conversion by the HSV-induced thymidine kinase (TK) into its di- and triphosphate (4, 8). ACV-resistant mutants should, therefore, be TK negative. Field and Wildy (7) have shown that HSV TK-negative mutants have a reduced pathogenicity after intracerebral inoculation of mice and a low propensity to induce latent ganglionic infections. Field and Darby (5) confirmed that TK-negative ACV-resistant mutants were attenuated when injected into mice, as indicated by reduced virus replication and inflammatory response of the ears of the animals; in addition, a dose of $10^{4.0}$ PFU did not result in latent infections in cervical ganglia. The latency-negative character of ACV-resistant mutants was confirmed by our previous (14) and present data which show that percutaneous infection with a dose as high as $10^{4.0}$ PFU does not lead to latent infections in trigeminal ganglia.

Attenuated HSV mutants were isolated also by selection in the presence of Ara-T (27); since this drug is likewise phosphorylated into its active form by the viral TK (1), Ara-T-resistant mutants are TK negative. The Ara-T-resistant mutant has a reduced pathogenicity in guinea pigs as reflected by a decreased frequency of latent infections in the trigeminal ganglia after corneal inoculation (27). It was shown that not all ACV-resistant mutants are TK negative, and that these mutants are not latency negative (5). It appears also that TK-negative and drug-resistant HSV mutants show differences in their ability to induce latent infections; some of the ACV-resistant mutants isolated by Field and Darby (5), as well as the ACV-resistant mutant isolated in our laboratory, are completely latency negative, whereas some TK-negative (7) and Ara-T-resistant (26, 27) mutants show only a reduced frequency of latency after primary infections. Since resistance of HSV to ACV results also from a mutation in the deoxyribonucleic acid polymerase locus (3, 24) it is quite possible that ACV-resistant mutants with an increased ability to induce latent infections have a defect in the polymerase instead of the TK locus.

Our previous (14) and present data, as well as those obtained in other laboratories (2, 26, 27), indicate that TK-negative and drug-resistant mutants penetrate the sensory ganglia during the acute phase of infection. Since latency is, however, not established or present only in a small proportion of the infected animals, some possibilities may be considered. (i) The mutants do not establish latent infections, the virus being eliminated by a process of autosterilization. (ii) The mutant becomes established in a defective, non-reactivable form similar to the latent viruses found in human ganglia, which were rescued only upon reinfection with temperature-sensitive mutants (2). (iii) The mutant may be either totally eliminated or latency established in a defective form, depending on the particular mutants used, the dose of the inoculated virus, the animal species used in the experimental infection, or the route of the primary infection.

The natural outcome of peripheral infection in mice with most wild HSV strains is death from encephalitis (12). The severity of the infection is controlled by the dose of virus, the route of inoculation, and the strain of mice (20). We have shown that mice surviving a primary infection with the neurovirulent HSV-1 strain S are resistant to reinfection with a homo- or heterotypic HSV strain. However, this protection against mortality does not prevent the establishment of latency in ganglia not involved during the primary infection (16). Intraperitoneal inoculation with neurovirulent HSV-1 strains, which does not lead to the establishment of latent infections, produced significant protection against the development of latency after subsequent challenge (23). Likewise, simultaneous intraperitoneal inoculation with pathogenic and apathogenic HSV-1 strains protects mice against the fatal outcome of infection (25). Protection against mortality was obtained also by intracerebral inoculation of avirulent HSV strains (18).

It has been suggested that TK-negative mu-
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LITERATURE CITED


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