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# Orofacial Herpes Simplex Virus Infection in Hairless Mice: Latent Virus in Trigeminal Ganglia After Topical Antiviral Treatment†

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Inoculation of herpes simplex virus on the forehead and/or snout of hairless mice resulted in a significantly lower mortality rate than inoculation of the skin in the lumbosacral area. Latent herpes simplex virus infections were detected in all forehead-inoculated and in 90% of snout-inoculated mice. Phosphonoacetic acid was highly effective in preventing the development of skin lesions, and no latent infections were detected when phosphonoacetic acid ointment was applied 3 h after infection. Neither adenine arabinoside nor adenine arabinoside monophosphate prevented the establishment of latent infections in the trigeminal ganglia, although they protected the mice from the fatal outcome of the infection. The antibody response after adenine arabinoside or adenine arabinoside monophosphate treatment was similar to that observed in untreated animals, and it was six to eight times higher than in mice treated with phosphonoacetic acid. Mice without evidence of latent infection had, in general, lower serum antibody titers than those with latent infections in the ganglia. An analysis of the pathogenesis of herpes simplex virus infection in mice treated with adenine arabinoside showed that virus penetration into the nerve endings was delayed and that the amount of free virus in ganglionic homogenates was 10 to 100 times less than that for untreated mice.

We showed previously that topical treatment of herpes simplex virus (HSV) skin infection of the lumbosacral area of hairless mice with adenine arabinoside (ara-A) and ara-A monophosphate (ara-AMP) ointments prevents death by the infection. However, these antiviral compounds have only a limited effect on the evolution of skin lesions and the subsequent establishment of latent infections in the spinal ganglia of the mice (4). On the other hand, a 2% phosphonoacetic acid (PAA) ointment is highly effective in preventing the development of skin lesions, the fatal outcome of the infection, and the establishment of latent infections in the spinal ganglia (5, 14). We have shown that consistent and reproducible results can be obtained with PAA when the treatment is initiated 3 h after virus inoculation and applied four times daily over a period of 5 days (5).

One disadvantage of experimental HSV skin infection in the lumbosacral area is that latent infections in the spinal ganglia are detected in only about 60 to 70% of surviving untreated animals (4, 5). This relatively low rate of detection makes it difficult to assess the real frequency of latent infections after antiviral treatment. We have developed, therefore, an experimental model of HSV infection involving the orofacial area of hairless mice in which the frequency of detectable latent infections in the trigeminal ganglia approaches 100% (R. J. Klein, A. E. Friedman-Kien, and E. Brady, Arch. Virol., in press). The experimental orofacial HSV infection has the additional advantage of a significantly lower mortality rate among untreated survivors than that observed after lumbosacral infection. As a consequence, adequate numbers of untreated control mice are available for evaluating the efficacy of antiviral compounds in preventing the establishment of latent infections in the trigeminal ganglia.

In the present study, we evaluated the effect of ara-A, ara-AMP, and PAA ointments on the evolution of orofacial skin lesions, the mortality rate, and the establishment of latent infections in the trigeminal ganglia of hairless mice. We likewise monitored the humoral immune response of treated mice and investigated the

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pathogenesis of the orofacial infection during antiviral treatment.

## MATERIALS AND METHODS

**Virus.** The type 1 HSV strain was identical to that utilized in earlier studies (5).

**Infection of mice.** Two kinds of infections were employed during these experiments: a severe infection obtained by rubbing the virus suspension over the scarified skin of the forehead and snout, and a less severe one involving only the snout area.

The techniques for neutralizing antibody assay and identification of virus isolates have been outlined in a previous report (5). Trigeminal ganglia were collected after removing the skull and brain and sectioning the posterior and anterior roots of the ganglion. Latent HSV was detected by co-cultivation of ganglia fragments in the presence of virus-sensitive human fibroblasts (5). The amount of free virus present in ganglionic homogenates (one ganglion per 1 ml of tissue culture medium) was determined by a plaque assay in Vero cell monolayers.

**Antiviral agents.** A 2% PAA ointment in petrolatum base was kindly provided by Abbott Laboratories, North Chicago, Ill. A 10% ara-A gel and water-soluble ara-AMP powder was obtained by the courtesy of Parke, Davis & Co., Ann Arbor, Mich. A 10% ara-AMP ointment in petrolatum base was prepared by the pharmacy of New York University Medical Center. The topical treatment was initiated 3 or 24 h after infection and was applied four times daily over a period of 5 days.

## RESULTS

**Effect of antiviral agents on the evolution of HSV infection in the facial area.** After percutaneous inoculation of HSV on the snout or forehead of untreated hairless mice, severe skin lesions were observed in 60 to 80% of the animals. The mortality rate among mice inoculated on the forehead was 33%, whereas no deaths occurred after inoculation of the snout. The difference is statistically significant ($P < 0.01$). Latent HSV infections in the trigeminal ganglion were detected in 90% of mice inoculated on the snout and in all animals inoculated on the forehead (Table 1).

Topically applied PAA, ara-A, and ara-AMP ointments were able to completely protect mice inoculated on the forehead from the fatal outcome of infection observed in 33% of untreated animals. A statistically significant reduction in the number of mice developing severe skin lesions was observed in most of the treatment groups. Although the difference was not statistically significant, a decreased number of severe lesions was also observed among mice treated with PAA 24 h after forehead inoculation. Ara-A-treated mice 24 h after snout inoculation and ara-AMP-treated mice 3 h after forehead inoculation likewise showed a lower incidence of severe lesions than untreated controls (Table 1).

No latent infections were detected after PAA treatment initiated 3 h after severe forehead inoculation. PAA treatment initiated 24 h after the severe and the mild infections resulted in a significant reduction in the latent infections in the trigeminal ganglia (Table 1). However, the frequency of latent infections after forehead inoculation was significantly higher than that observed after snout inoculation (6 of 11 versus 1 of 10; $P < 0.04$).

TABLE 1. *Effect of topical antiviral treatment on orofacial HSV skin infection in hairless mice*

| Treatment[a] | Inoculation site | Start of treatment[b] | Mortality rate[c] | Severe skin lesions[c] | P[d] | Latent ganglionic infections[c] | P[d] |
|---|---|---|---|---|---|---|---|
| None or placebo | Snout | 3 | 0/20 | 12/20 | | 18/20 | |
| | Forehead | 3 | 6/18 | 15/18 | | 12/12 | |
| PAA, 2% | Forehead | 3 | 0/8 | 0/8 | <0.001 | 0/8 | <0.001 |
| | Snout | 24 | 0/10 | 0/10 | <0.002 | 1/10 | <0.001 |
| | Forehead | 24 | 0/11 | 5/11 | NS | 6/11 | <0.05 |
| Ara-A, 10% | Forehead | 3 | 0/8 | 2/8 | <0.01 | 8/8 | NS |
| | Snout | 24 | 0/10 | 4/10 | NS | 8/10 | NS |
| | Snout | 3 | 0/11 | 2/11 | <0.03 | 6/11 | NS |
| Ara-AMP, 10% | Forehead | 3 | 0/8 | 4/8 | NS | 8/8 | NS |
| | Snout | 24 | 0/10 | 2/10 | <0.04 | 7/10 | NS |
| | Snout | 3 | 0/10 | 1/10 | <0.02 | 6/10 | NS |

[a] Applied four times daily over a period of 5 days.

[b] Hours after virus inoculation.

[c] Numerator: Number of dead mice, mice with severe skin lesions, and mice with detected latent infection in the trigeminal ganglia. Denominator: Number of mice examined.

[d] *P*, Probability that the reduced number of mice with severe skin lesions, or decreased frequency of latent infections is due to chance (Fisher exact test). NS, Statistically not significant.

Neither ara-A nor ara-AMP ointment showed any statistically significant effect in reducing the frequency of latent infections in the trigeminal ganglia (Table 1). However, our experiments show with regularity a reduction in the number of latent infections relative to the precocity of treatment initiation and especially to the severity of infection. Whereas all forehead-inoculated animals treated 3 h after the infection developed latent infection, only about 60% had detectable latent infection after the less-severe snout inoculation. When taken as a single group, ara-A- and ara-AMP-snout-inoculated mice treated 3 h after infection had a significantly lower frequency of latent infections than did untreated controls (12 of 21 versus 18 of 20, $P < 0.04$).

**Immune response after antiviral treatment.** Untreated control mice with evidence of latent infections in the trigeminal ganglia had a mean HSV-specific serum antibody titer of 3.44 $\pm$ 0.29 $\log_{10}$ units 3 to 4 weeks after virus inoculation. Mice treated with the tested antiviral compounds and with evidence of latent infections in the trigeminal ganglia displayed antibody titers of a similar order of magnitude (Fig. 1). In general, mice without evidence of latent infection had lower antibody titers than mice with latent infections in the ganglia.

**Pathogenesis of orofacial HSV infection.** In forehead-inoculated and untreated mice, free virus was first detected in the trigeminal ganglionic homogenates 2 days after virus inoculation. A peak was observed 4 days after infection; small quantities were still detectable after 7 days, but virus was generally not found after 10 days. Ganglionic homogenates from PAA-treated mice never contained free virus during the 10-day observation period. In ara-A-treated mice, free virus was detected in the homogenates only 4 days after infection, and the ganglions contained 10 to 100 times less virus than those from untreated mice (Fig. 2).

Virus-specific neutralizing antibodies (Fig. 2) were first detected in untreated mice 7 days after infection. Two of four PAA-treated mice possessed antibodies 7 days after infection. After 10 days, antibody titers in untreated and ara-A-treated mice were similar and close to the peak observed 3 to 4 weeks after infection. Only two of four PAA-treated mice had antibodies in their serum after 10 days, and titers appeared to be on the average four to eight times lower than

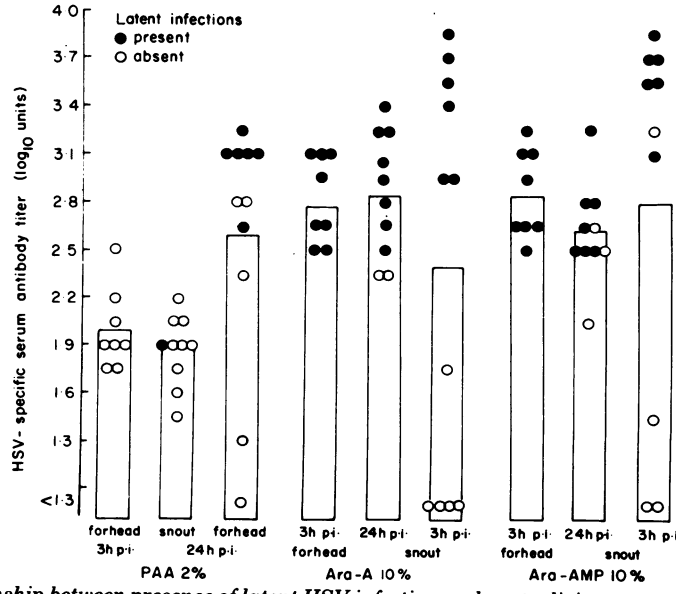


FIG. 1. *Relationship between presence of latent HSV infection and neutralizing serum antibody titers. Mice were sacrificed 3 to 4 weeks postinfection (p.i.) by exsanguination. Latent HSV infections were detected by cocultivating trigeminal ganglia fragments in the presence of virus-sensitive human fibroblasts. Antibody titers were determined by mixing twofold dilutions of mouse serum with equal volumes of 30 to 100 plaque-forming units of HSV type 1 and incubating for 30 min at 37°C in a water bath. Human fibroblasts were grown in Micro Test II plastic trays (Falcon Plastics, Oxnard, Calif.). The growth medium was removed, and duplicate wells were inoculated with 0.2 ml of serum-virus mixture. The highest serum dilution that protected 50% of the wells from virus-induced cytopathic effect was taken as the serum antibody titer and expressed in $\log_{10}$ units. Bars represent the mean antibody titer of indicated treatment group.*

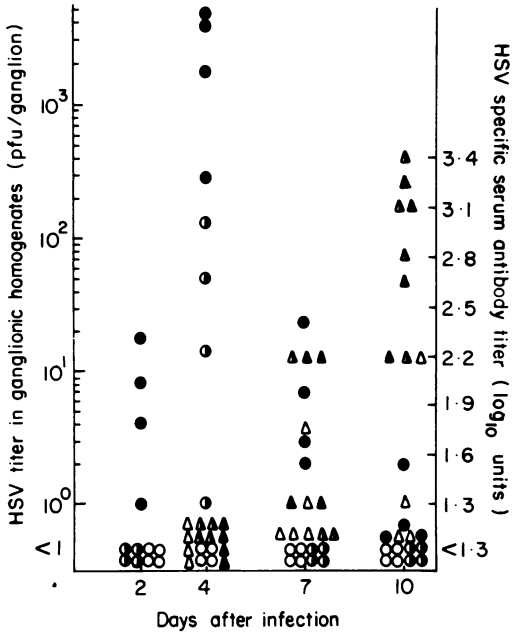


FIG. 2. *Pathogenesis of orofacial HSV infection in untreated and ara-A- and PAA-treated mice. Ara-A and PAA treatments were initiated 3 h after infection and applied four times daily until mice were sacrificed or up to a maximum of 5 days. Groups of four mice were killed by exsanguination at the intervals indicated. Trigeminal ganglia from individual mice were homogenized in Hanks balanced salt solution (2 ml per two ganglia from individual mice), centrifuged, and frozen until assay. Virus titration was performed on monolayers of Vero cells grown in plastic dishes. Virus dilutions (0.5 ml) were inoculated in duplicate dishes, adsorbed for 1 h at 37°C, washed three times, and covered with methylcellulose. After 3 days of incubation, neutral red was added, and plaques were counted after overnight incubation. Titers were calculated by the method of Lorenz (8). Antibody titration was performed as described in the legend of Fig. 1. Plaque-forming units (PFU) in ganglionic homogenates from (●) untreated, (◑) ara-A-treated, and (○) PAA-treated mice. Antibody titers in sera of (▲) untreated, (◭) ara-A-treated, and (△) PAA-treated mice.*

those observed 3 to 4 weeks after infection (Fig. 1).

## DISCUSSION

Our results indicate that HSV infection in the facial area of hairless mice is associated with a relatively low mortality rate and a very high incidence of latent HSV infections in the trigeminal ganglia. The effectiveness of topical antiviral agents against HSV infections cannot be judged by their ability to prevent the development of lesions at the inoculation site or by the death rate of the animals alone. Factors such as the ability of a topically applied compound to prevent the establishment of latent HSV infections in the sensory ganglia of animals surviving the primary infection should also be taken into consideration. The facial route of inoculation has the obvious advantage of providing a large number of survivors with a very high frequency of established latent ganglionic infections in which the effects of various treatment schedules on the establishment of latent infection can be compared.

A survey of published observations (Table 2) shows that the overall frequency of latent infections after various routes of inoculation is about equal in the spinal and trigeminal ganglia (66 and 70%, respectively). However, there are marked differences in this frequency depending on the route of inoculation. The footpad inoculation method (6, 10–12) is the most effective in inducing latent infections in the spinal ganglia, whereas the genital route (10, 12) leads to less than a 50% frequency of latent infections. Among inoculation areas innervated by the branches of the trigeminal ganglion, infection of the cornea (7, 9–11) is more effective than that of the lips (10, 12) in inducing latent infections in this ganglion. The highest frequency of latent infections in the trigeminal ganglion was achieved by virus inoculations on the snout and forehead of mice as described in the present study.

The evaluation of antiviral compounds in orofacial HSV infection of mice has confirmed that PAA is highly effective in preventing both the development of skin lesions and the establishment of latent infections in sensory ganglia (5, 14). However, PAA irritates the skin, and a recent report states that it is deposited in bone tissue (B. A. Bopp, C. B. Estep, and D. J. Anderson, Fed. Proc. **36**:939, 1977). Therefore, the use of PAA in humans is unlikely.

The topical use of ara-A and ara-AMP seems quite effective in preventing death after HSV inoculation in the lumbosacral (4) or facial area of mice. However, these drugs have only a limited effect on the development of skin lesions and the establishment of latent infections in the ganglia. We noticed that virus reactivation from co-cultivated spinal root ganglia is delayed in ara-A-treated mice, and we suggested that the drug may limit virus replication in skin and subsequent virus penetration of nerve endings (4). The results of the present experiments tend to support this suggestion. Although in untreated mice free virus is detectable in the trigeminal ganglion as early as 48 h after inoculation and reaches a peak at 96 h, virus was detected in ara-A-treated mice only after the 4th

TABLE 2. *Frequency of latent ganglionic HSV infection in mice after various routes of inoculation*

| Site of latent infection | Route of inoculation | Observed latent infections[a] | Frequency (%) | Reference |
|---|---|---|---|---|
| Spinal root ganglia | Footpad | 16/17 | 94 | 11 |
| | | 36/53 | 70 | 6 |
| | | 40/41 | 98 | 12 |
| | | 32/39 | 82 | 10 |
| | Skin (thigh) | 19/20 | 95 | 10 |
| | Skin (lumbar) | 16/26 | 62 | 5 |
| | Vagina (type 1) | 7/15 | 47 | 12 |
| | | 29/50 | 58 | 10 |
| | Vagina (type 2) | 13/43 | 30 | 12 |
| | | 35/75 | 47 | 10 |
| | Ear | 32/45 | 71 | 2 |
| | Intravenous | 19/23 | 83 | 1 |
| Total | | 294/447 | 66 | |
| Trigeminal ganglion | Cornea | 23/53 | 43 | 7 |
| | | 31/39 | 79 | 11 |
| | | 54/62 | 87 | 10 |
| | | 21/37 | 57 | 9 |
| | Lip | 14/24 | 58 | 12 |
| | | 15/32 | 47 | 10 |
| | Mouth | 29/36 | 81 | 3 |
| | Forehead | 8/8 | 100 | In press[b] |
| | | 12/12 | 100 | Present data |
| | Snout | 18/20 | 90 | Present data |
| Total | | 225/323 | 70 | |

[a] Number infected/number examined.
[b] R. J. Klein, A. E. Friedman-Kien, and E. Brady, Arch. Virol.

day postinfection. Quantitation of the virus showed that ganglia from ara-A-treated mice contained about 100 times less virus than ganglia from untreated mice.

Thus, it appears that, although ara-A and ara-AMP have only a limited effect on HSV infections, they are able to confer a reasonable degree of protection by reducing and delaying virus penetration of the nervous system. It has been shown (13) that the immune response reduces about 10-fold the number of cells latently infected with HSV in the spinal ganglia. Because ara-A- and ara-AMP-treated mice have antibody titers comparable to those of untreated mice, it is possible that the early immune response plays a certain role in the survival of treated mice by restricting the invasion of the nervous system by HSV.

## ACKNOWLEDGMENTS

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