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Immune Response and Latent Infection After Topical Treatment of Herpes Simplex Virus Infection in Hairless Mice[1]

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Treatment of herpes simplex virus (HSV)-infected hairless mice with a 2% phosphonoacetic acid (PAA) ointment prevented the appearance of virus-induced skin lesions and subsequent central nervous system (CNS) involvement. Treatment started 24 h after infection significantly reduced the intensity of the skin lesions and also prevented CNS involvement. After four to six applications of PAA ointment, a moderate skin erythemia developed, followed by scaling and complete healing 7 days after cessation of the treatment. Mice treated early after HSV infection had low or undetectable levels of virus-specific antibodies but were completely resistant to reinfection. Early treatment prevented the development of a latent ganglionic infection, but treatment initiated 24 h after infection could not prevent the establishment of the latent infection. PAA-treated and HSV-infected mice with nondetectable levels of antibodies did not develop, with a single exception, a latent ganglionic infection upon reinfection. The cell-mediated immune response determined by levels of [^{14}C]thymidine incorporation in Ficoll-Hypaque-purified spleen lymphocytes cultures was low in PAA-treated mice; it increased slightly after challenge infection but was strong in mice that proved to harbor a latent HSV infection in the ganglia.

Phosphonoacetic acid (PAA) is a potent in vitro inhibitor of human (7, 8, 21, 22, 28), simian (8), murine (8), and avian (14) herpesviruses. It was shown that PAA is a specific inhibitor of the herpesvirus-induced (1, 7, 15, 17, 18) and vaccinia virus-induced (1) deoxyribonucleic acid polymerase. PAA-resistant herpes simplex virus (HSV) mutants can be readily isolated (9, 11), and it has been shown that these mutants possess a deoxyribonucleic acid polymerase activity that is less sensitive to PAA action (6). As opposed to many antiviral compounds, active only in vitro or with considerable toxicity in vivo, PAA possesses a definite therapeutic effectiveness in experimental animal models. PAA prevents the fatal outcome of HSV-induced skin infections in mice when given orally (27), topically (27, 31), or systemically (11). The compound applied topically or systemically is likewise active in HSV-induced keratoconjunctivitis in rabbits (4, 19, 27), but it is less effective in genital HSV infection in mice (E. R. Kern, J. C. Overall, and L. A. Glasgow, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, A26, p. 5). When applied topically, PAA also proved to be able to suppress the formation of pustular lesions induced by vaccinia virus or the benign tumors caused by Shope fibroma virus in rabbits (3). Systemic administration of PAA was ineffective both in the genital HSV infection in mice (E. R. Kern, J. C. Overall, and L. A. Glasgow, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, A26, p. 5) and in the prevention of poxvirus-induced lesions in the rabbit skin (3). Recently, it has been shown (29) that PAA inhibits human B lymphocyte transformation in vitro, suggesting that deoxyribonucleic acid synthesis is required for lymphocyte proliferation.

Although PAA is not toxic at the doses employed in the systemic treatment of viral infections, the topical application has a strong irritating effect on the skin when applied as 5% ointment: an erythema, followed by ulcerations with a slow tendency toward healing, is ob-

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served after 10 to 12 applications of the 5% ointment (R. J. Klein and A. E. Friedman-Kien, unpublished observations).

In the present study, we evaluated the efficacy of a less irritating 2% PAA ointment (Abbott Laboratories, North Chicago, Ill.) on the HSV-induced skin infection in hairless mice. At the same time, we monitored the immune response of the treated animals by measuring humoral antibody titers, the cell-mediated immune response, and resistance to reinfection. The efficacy of the treatment was also evaluated with respect to the ability of the compound to prevent the initiation of a latent HSV infection in the spinal ganglia of the surviving hairless mice (31). The in vitro sensitivity to PAA of the virus isolated form the ganglia of PAA-treated and nontreated mice was tested in parallel.

MATERIALS AND METHODS

Chemicals. PAA disodium salt, as a 2% ointment in petrolatum base, as well as a similar placebo ointment, was kindly supplied by Abbott Laboratories, North Chicago, Ill.

Virus. HSV type 1, strain S (courtesy of Paul Came, Schering Corp., Bloomfield, N.J.) was used as our fourth laboratory passage on primary rabbit kidney cell cultures. The virus had a titer of 2.5×10^6 plaque-forming units (PFU) when assayed on a Vero cell monolayer covered with methylcellulose. Throughout the experiments, samples of the same batch of virus were stored at −70°C until used. Consistently, reproducible infection of mouse skin was obtained by using a virus dilution containing 2.5×10^5 PFU.

Mice. Female hairless mice, randomly bred at the New York University School of Medicine, Laboratory Animal Facility, were originally derived from the HRS/y strain from Jackson Laboratories, Bar Harbor, Me. Mice weighing 20 ± 2 g were kept separated in groups of five.

Infection of mice. The infection of mice, evolution of the lesions, and scoring procedures were similar to those described in earlier publications (11, 12). Three to 10 weeks after the primary infection, selected groups of surviving mice were challenged percutaneously with 2.5×10^5 PFU of virus.

Neutralizing antibody assay. Mice were bled by heart puncture, and the serum was separated and inactivated for 30 min at 56°C. Twofold dilutions of individual sera were mixed with equal volumes of 200 PFU of virus (HSV type 1, strain S) and incubated for 30 min at 37°C in a water bath. All serum and virus dilutions were performed in Eagle minimal essential medium (MEM; Grand Island Biological Co.) supplemented with 2% fetal calf serum. Human foreskin fibroblasts (cell line FS-7, kindly provided by J. Vilček, New York University School of Medicine, New York, N.Y.), were grown in Microtest II plastic trays (Falcon Plastics, Oxnard, Calif.) to confluency in MEM supplemented with 7% fetal calf serum. The growth medium was removed, and quadruplicate wells were inoculated with 0.2 ml of serum-virus mixture. The highest serum dilutions that protected 50% of the wells from the virus-induced cytopathic effect were taken as the serum antibody titer and expressed in $\log_{10}$ units. Pooled sera of the various experimental animals groups were tested for antibody titers by a plaque reduction assay in Vero cells. Titers thus obtained paralleled very closely those obtained in microplates.

Cell-mediated immune response. (i) Virus and antigens. The HSV antigen consisted of the same type 1 strain used in the infection of mice and was propagated on the human FS-7 foreskin cell line. The viral antigen and control antigen of uninfected cells were prepared as described by Rasmussen et al. (24). The antigen was diluted to contain 10^8 PFU/ml and inactivated by exposure to ultraviolet light for 20 min at a distance of 15 cm from a 15-W lamp. After irradiation, the antigen was free of infectious virus.

(ii) Spleen lymphocyte cultures. Spleens were removed aseptically from mice, minced with scissors, and filtered through cotton gauze to remove large particulate material. The suspension was layered on a Ficoll-Hypaque gradient (2) and centrifuged for 40 min at 1,400 × *g*. The lymphocytes collected from the interface were washed three times in MEM. The cell suspension in MEM containing 15% fetal calf serum was dispensed in loosely capped plastic tubes (12 by 75 mm) containing 5×10^5 cells in a volume of 1 ml. The cultures were incubated at 37°C in an atmosphere containing 5% CO_2.

(iii) Lymphocyte stimulation. Phytohemagglutinin (PHA; purified PHA, Wellcome Research Laboratories, Beckenham, England) stimulation was performed by adding 2 μg of PHA in 0.1 ml of MEM to each culture tube. After a 48-h incubation, 0.05 ml of MEM containing 0.1 μCi of [^{14}C]thymidine per ml was added, and the cultures were harvested after another 18 h of incubation. The HSV and control antigen response was elicited by adding 0.1 ml of HSV antigen (10^7 PFU before inactivation) or control antigen to triplicate culture tubes. The cultures were harvested after an incubation of 5 days. [^{14}C]thymidine (0.1 μCi per ml) in 0.05 ml of MEM was added for a pulse of 6 h prior to the harvest. The lymphocyte cultures were harvested using a multiple cell culture harvester (Skatron AS and Flow Laboratories, Inc.) as described (30). The [^{14}C]thymidine incorporation was determined in a Packard Tri-Carb scintillation counter. The degree of stimulation was expressed by the ratio between the mean number of counts of triplicate PHA-, HSV-, or control antigen-stimulated cultures versus the nonstimulated cultures.

Isolation of latent virus. Starting 4 weeks after the primary infection or 4 weeks after the challenge infection, groups of mice were bled by heart puncture. The bony structure of the vertebral column was exposed and opened by lateral incision of the vertebrae. The sacral and dorsal ganglia were removed with the spinal cord left in situ. The pieces were washed in saline, and the 20 to 30 fragments were seeded in a small plastic flask of 20 cm² (Falcon

Plastics) in which a monolayer of FS-7 cells had been grown. MEM with 2% fetal calf serum was added, and the flasks were kept under observation for 30 days. During this interval the medium was changed once.

Serological identification of viral isolates. Serological identification of viral isolates from co-cultivated ganglia was performed by a plaque reduction assay on Vero cells as described by Knotts et al. (13). Under these conditions, both the original virus used for inoculating the mice and 12 ganglionic isolates from PAA-treated and nontreated mice were neutralized to the same extent with HSV type 1 rabbit serum.

PAA sensitivity of HSV isolated from ganglia. Cell cultures with virus-specific cytopathic effect were frozen and thawed three times, and the clarified supernatant obtained after centrifugation was tested without any additional passage for PAA sensitivity of the virus. Vero cell monolayers grown in 6-cm diameter plastic dishes (Falcon Plastics) were inoculated in duplicate with suitable dilutions of virus in a volume of 0.5 ml. After a 1-h adsorption at 37°C, the inoculum was removed, and the cell layer was washed three times with Hanks balanced salt solution and covered with 5 ml of an overlay of 2% methylcellulose containing 100 μg of PAA per ml in MEM supplemented with 2% fetal calf serum. After 3 days of incubation at 37°C, the cells were stained with neutral red, and the plaques were counted the next day. Titers were calculated according to the method of Lorenz (16).

RESULTS

Effect of PAA ointment on HSV skin infection in hairless mice. In a first series of experiments, 2% ointment was applied twice daily, starting the treatment either 3 h after virus inoculation or 24 and 48 h later (Table 1). Except in experiment no. 4, where the early treatment yielded a highly satisfactory result, the ointment in all other experiments and treatment schedules had no significant effect on the intensity of the developing skin lesions and the mortality rate but provided in several instances, a prolongation of the mean survival time.

In a second series of experiments, the 2% PAA ointment was applied four times daily at 4-h intervals over a period of 12 h. As may be seen from Table 2, early treatment provided complete protection against the development of the skin lesions, preventing involvement of the central nervous system and death. A slight irritating action was evidenced by the appearance of erythema and consecutive scaling (Fig. 1) at the site of the treatment, but healing occurred completely in 3 to 6 days after the cessation of topical application of the ointment.

When the treatment was initiated 24 h after virus inoculation, the development of skin lesions could not be completely prevented (Fig. 2), but all mice survived the infection. Furthermore, only half the mice in this group showed skin lesions, the other half being completely protected. It is worth noting that prolonged treatment (experiments 1 and 3, Table 2) did not confer better protection than that provided by only six applications of the ointment (experiment 2, Table 2).

Resistance to reinfection and serum antibody levels. Surviving nontreated mice, as well as mice treated for 1 or 5 days with PAA ointment, were fully resistant to reinfection (Table 3). However, whereas this resistance was correlated with relatively high levels of antibodies in the sera of nontreated mice, mice treated for 5 days with PAA showed resistanct to reinfection in the absence of detectable levels of HSV-specific antibodies. Mice treated for 1 day with PAA had a mean antibody titer seven times

TABLE 1. *Effect of PAA ointment (2%) applied twice daily to HSV-infected hairless mice*

Expt no.	Treatment	Duration	Intensity of skin lesions	Mortality rate (no. dead/no. infected)	Mean survival time (days)
1	None		2.90	10/10	6.2
	Placebo[a]	0–5	2.80	8/10	8.5
	PAA	0–5	3.45	8/10	9.3[b]
2	None		3.60	9/10	9.9
	Placebo	0–5	3.50	8/10	10.0
	PAA[c]	1–6	3.20	6/10	11.7
3	None		3.30	10/10	6.7
	PAA	0–5	2.05	5/10[b]	11.4[b]
4	None		4.0	8/8	6.6
	PAA	0–5	0.5[b]	0/10[b]	>14.0
	PAA[d]	2–7	3.4	7/10	10.5[b]

[a] Treatment started 3 h after infection.
[b] Statistically significant.
[c] Treatment started 24 h after infection.
[d] Treatment started 48 h after infection.

TABLE 2. *Effect of PAA ointment (2%) applied four times daily to HSV-infected hairless mice*

Expt no.	Treatment	Duration	Intensity of skin lesions	Mortality rate (no. dead/no. infected)	Mean survival time (days)
1	None		3.25	9/10	8.2
	Placebo[a]	0–5	3.65	10/10	6.6
	PAA	0–5	E & S[b]	0/10[c]	>14.0[c]
2	None		3.30	6/10	10.7
	PAA	0–1	E & S	0/10[c]	>14.0[c]
	PAA	0–3	E & S	0/10[c]	>14.0[c]
	PAA[d]	1–4	0.95[c]	0/10[c]	>14.0[c]
3	None		3.55	8/10	9.7
	PAA	0–5	E & S	0/10[c]	>14.0[c]

[a] Treatment started 3 h after infection.
[b] E & S, Erythema and scaling.
[c] Statistically significant.
[d] Treatment started 24 h after infection.

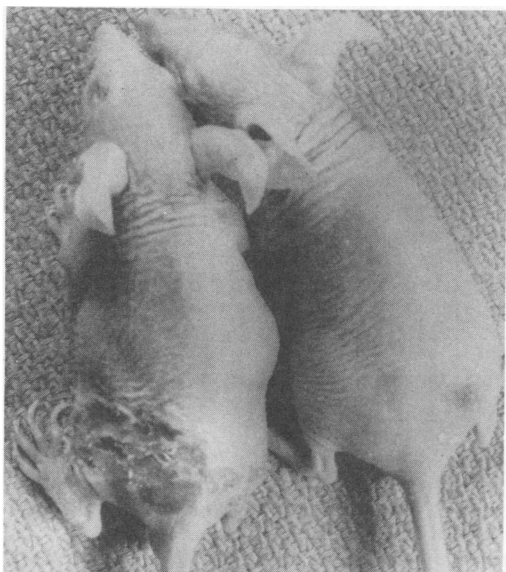

FIG. 1. *Hairless mice 7 days after HSV inoculation. (Left) Nontreated; (right) treated with a 2% PAA ointment, 4 times daily, for 5 days.*

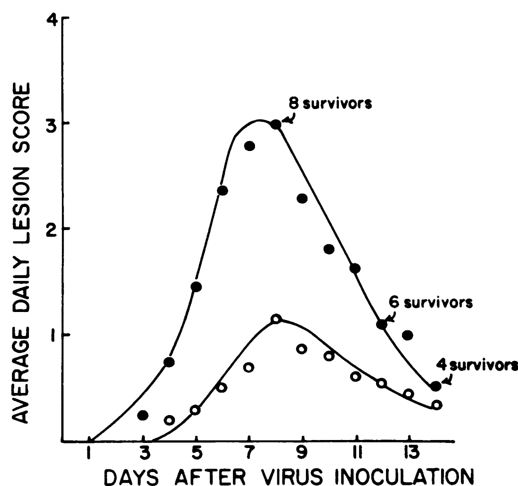

FIG. 2. *Evolution of HSV-induced skin lesions in nontreated mice (●) and mice treated four times daily for 4 days, starting the treatment 24 h after infection* (○).

lower than that observed in nontreated animals. After challenge infection, these mice with nondetectable or low levels of antibodies displayed an increase in titer, similar to that observed in nontreated mice.

To clarify the basis of this resistance to reinfection in the absence of humoral antibodies, the cell-mediated immune response to HSV was tested in PAA-treated mice.

Cell-mediated immune response. Ficoll-Hypaque-purified spleen lymphocyte cultures from normal mice were unresponsive to HSV antigen stimulation as determined by [^{14}C]-thymidine uptake. After topical PAA treatment, initiated shortly after virus infection, the cell-mediated immune response was slightly, but not significantly, increased. Surviving PAA-treated mice, after a challenge infection, showed on the average a threefold increase of the stimulation ratio as compared to normal mice (Fig. 3). However, the only PAA-treated mouse that proved to be latently infected after challenge infection had a stimulation ratio six times higher than the normal mice. The latter result is similar to that observed in nontreated mice surviving the HSV infection, and which all proved to harbor a latent virus infection in their spinal ganglia (Table 4). The stimulation ratio with the control antigen was always near unity in each group of mice, and the nonspecific responsiveness of the lymphocyte was unimpaired as

TABLE 3. *Resistance to reinfection, serum antibody titers, and presence of latent virus in ganglia of mice surviving HSV infection after topical PAA treatment*

Treatment of experimental group	Mortality after challenge (no. dead/no. infected)	Mean log antibody titer ±SD[a]	Virus isolation from ganglia
None or placebo[b]	0/10	2.35 ± 0.38	6/9
PAA day 0-1	NC[c]	1.51 ± 0.58	0/5
PAA day 0-1 and challenged[d]	0/5	2.61 ± 0.52	0/4
PAA days 0-5	NC	<1.30	0/10
PAA days 0-5 and challenged[e]	0/10	2.54 ± 0.56	1/10
PAA days 1-4 (lesions present)	NC	2.26 ± 0.39	4/5
PAA days 1-4 (no lesions)	NC	2.23 ± 0.53	1/5

[a] SD, Standard deviation.
[b] Placebo-treated or not treated mice surviving HSV infection.
[c] NC, Not challenged.
[d] Challenged 3 weeks after the primary infection.
[e] Challenged 3 weeks (five mice) and 10 weeks (five mice) after the primary infection.

shown by the uniform reaction to PHA stimulation (Fig. 3).

Latent virus infection of the spinal ganglia. Virus was detected in the spinal ganglia in six out of nine nontreated mice, but none of the mice treated 3 h after virus infection presented evidence of a latent ganglionic infection. Mice treated with PAA 24 h after virus inoculation, and developing cutaneous virus-induced lesions, displayed the same frequency of latent ganglionic infection as observed in nontreated mice. However, mice treated with PAA 24 h after infection and not developing cutaneous lesions presented a very low frequency of latent ganglionic infections. Only in one animal did reinfection of mice treated with PAA for 1 or 5 days immediately after virus inoculation result in the establishment of a latent infection, even though antibody levels were low or nondetectable in parallel groups of mice. These results are summarized in Table 3. There was no correlation between the presence of latent ganglionic infection and the magnitude of antibody response. Positive ganglia cultures were detected in mice differing in their antibody titers by a factor of 8. On the other hand, an increased cell-mediated immune response was detected only in the presence of a latent ganglionic infection (Table 4).

Out of the 12 positive co-cultivated ganglia, 75% were detected between the 5th and 8th day of cultivation, 2 were detected on the 12th and 13th day, and only 1 positive culture was observed after 25 days.

PAA sensitivity of ganglionic isolates of HSV. Experiments, performed in parallel with the original challenge virus, with ganglionic isolates from mice treated with PAA 24 h postinfection (two isolates) and from nontreated mice (three isolates) showed the same degree of sensitivity towards PAA, i.e., a difference of about 3 log units between the number of PFU under medium containing no inhibitor and containing 100 μg of PAA per ml. These results are consistent with previously published data (9, 11).

DISCUSSION

Our data show that PAA applied topically is able to prevent the development of HSV-in-

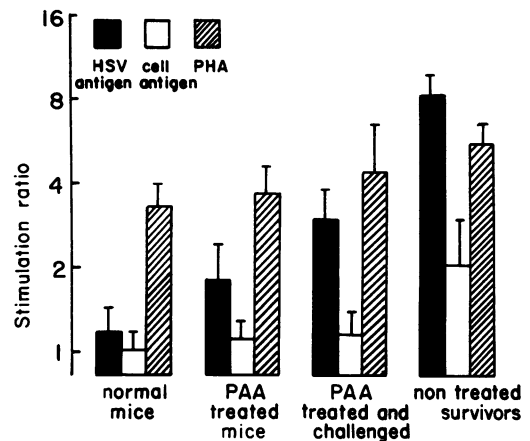

FIG. 3. *Mean stimulation ratios of virus antigen, control antigen, and PHA of normal and HSV-infected mice (PAA-treated, PAA-treated, and challenged and nontreated). Stimulation ratio of PAA-treated and challenged mouse with demonstrated latent infection not included in average. (See Table 4.)*

TABLE 4. *Antibody titers and stimulation ratios in individual mice with demonstrated latent HSV infection in the spinal ganglia*

Treatment	HSV antibody titer ($\log_{10}$)	Stimulation ratio
None	1.90	Not done
None	2.35	8.13
None	2.35	8.90
None	2.50	9.74
None	2.80	6.02
PAA and challenged	2.50	6.54[a]

[a] The mean stimulation ratio of three PAA-treated and challenged mice, without evidence of latent infection, was 2.95 ± 0.69.

duced skin lesions after percutaneous inoculation of the virus, and the subsequent involvement of the central nervous system and death. They confirm earlier reports about the efficacy of PAA ointments in HSV infections of mice (27, 31). Wohlenberg et al. (31), using a 5% ointment applied only twice daily, obtained somewhat irregular results, as exemplified by the variability of latent virus isolation from the spinal ganglia after 5 days of treatment (none out of 14 in one experiment, 4 out of 10 in another). Irregular survival rates were observed in our experiments when the 2% PAA ointment was applied twice daily. These differences can be attributed to random variability of the biological material. Taken together, these results show that PAA has to be applied more frequently in order to obtain consistent results.

The 2% PAA ointment applied four times daily caused a moderately irritating effect on the mouse skin. If similar irritation should occur on the human skin, it would be difficult to justify the use of PAA in order to reduce the relativley minor discomfort caused by the spontaneously healing recurrent herpes eruptions. However, there would be a major benefit from this treatment of recurrent herpes if the "static state" hypothesis of Roizman (25) is true and if a "round-trip" mechanism (10) is operative in the pathogenesis of this condition. This mechanism is based on the supposition that, during recurrences, the entire information for virus synthesis is dislodged from the trigeminal ganglion and channeled towards the skin. Part of the virus is probably destroyed by local defense mechanisms, but part of it will still be able, by centripetal migration, to reestablish the latent infection in the ganglion. As PAA applied locally is able to prevent the establishment of ganglionic infection in mice, early treatment of recurrent herpetic eruptions in man might be able to prevent the reinstatement of the latent infection and result in a complete cure.

Although PAA ointment applied early effectively prevents the development of HSV-induced skin lesions and the establishment of latent ganglionic infections, the compound does not impair the resistance of the mice to reinfection. After a treated primary infection, the animals, whether possessing detectable levels of antibodies or not, were resistant towards challenge infection, as judged by the fact that skin lesions did not appear and spinal ganglia did not become latently infected (with one exception). It was shown (23) that mice immunized intraperitoneally with live HSV type 1 (which in no case developed a latent infection) displayed substantial resistance against reinfection in the presence of antibodies, but their level of cell-mediated immunity was not investigated.

In our experiments, the resistance of PAA-treated mice was not accompanied by a significant increase in [^{14}C]thymidine uptake after the stimulation of purified lymphocytes by HSV antigen. This is not surprising, since it was shown (26) that the stimulation ratio in HSV-immunized rabbits drops after 2 weeks to low values, similar to those observed in our experiments with mice. Data from humans (5) with recurrent herpes showed that Ficoll-Hypaque-purified mononuclear cell cultures obtained 2 to 6 weeks after recurrences display stimulation ratios similar to those observed in our experiments in mice harboring a latent infection. Since it is highly probable that humans with HSV recurrences have likewise a latent ganglionic infection, one may conclude that this condition is correlated with the maintenance of a stable responsiveness of the lymphocytes to HSV antigen stimulation. This would not preclude, however, a certain variability of the cell-mediated immune response connected with the actual recrudescence, although the results are conflicting (20).

Two immediate questions arise in connection with our experiments. (i) Do other antivirals likewise possess the ability to prevent a latent infection? (ii) If specific resistance to HSV after successful PAA treatment is not the function of humoral and cell-mediated immune response, do factors such as interferon or other lymphokines play a major role in this particular model? Both aspects are currently under investigation in our laboratory.

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LITERATURE CITED

1. Bolden, A., J. Aucker, and A. Weissbach. 1975. Synthesis of herpes simplex virus, vaccinia virus, and adenovirus DNA in isolated HeLa cell nuclei. I. Effect of viral specific antisera and phosphonoacetic acid. J. Virol. 16:1584–1592.
2. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21(Suppl. 97):31–89.
3. Friedman-Kien, A. E., A. A. Fondak, and R. J. Klein. 1976. Phosphonoacetic acid treatment of Shope fibroma and vaccinia virus skin infections in rabbits. J. Invest. Dermatol. 66:99–102.
4. Gerstein, D. D., C. R. Dawson, and J. O. Oh. 1975. Phosphonoacetic acid in the treatment of experimental herpes simplex keratitis. Antimicrob. Agents Chemother. 7:285–288.

5. **Haahr, S., L. Rasmussen, and T. C. Merigan.** 1976. Lymphocyte transformation and interferon production in human mononuclear cell microcultures for assay of cellular immunity to herpes simplex virus. Infect. Immun. 14:47-54.
6. **Hay, J., and J. L. Subak-Sharpe.** 1976. Mutants of herpes simplex virus types 1 and 2 that are resistant to phosphonoacetic acid induce altered DNA polymerase activities in infected cells. J. Gen. Virol. 31:145-148.
7. **Huang, E.-S.** 1976. Human cytomegalovirus. IV. Specific inhibition of virus induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. J. Virol. 16:1560-1565.
8. **Huang, E.-S., C.-H. Huang, S.-M. Huong, and M. Selgrade.** 1976. Preferential inhibition of herpes-group viruses by phosphonoacetic acid: effect on virus DNA synthesis and virus-induced DNA polymerase activity. Yale J. Biol. Med. 49:93-98.
9. **Klein, R. J.** 1975. Isolation of herpes simplex virus clones and drug-resistant mutants in microcultures. Arch. Virol. 49:73-80.
10. **Klein, R. J.** 1976. Pathogenetic mechanisms of recurrent herpes simplex virus infections. Arch. Virol. 51:1-13.
11. **Klein, R. J., and A. E. Friedman-Kien.** 1975. Phosphonoacetic acid-resistant herpes simplex virus infection in hairless mice. Antimicrob. Agents Chemother. 7:289-293.
12. **Klein, R. J., A. E. Friedman-Kien, and E. Brady.** 1974. Herpes simplex virus skin infection in hairless mice: treatment with antiviral compounds. Antimicrob. Agents Chemother. 5:318-322.
13. **Knotts, F. B., M. L. Cook, and J. G. Stevens.** 1973. Latent herpes simplex virus in the central nervous system of rabbits and mice. J. Exp. Med. 138:740-744.
14. **Lee, L. F., K. Nazerian, S. S. Leinbach, J. M. Reno, and J. A. Boezi.** 1976. Effect of phosphonoacetate on Marek's disease virus replication. J. Natl. Cancer Inst. 56:823-828.
15. **Leinbach, S. S., J. M. Reno, L. F. Lee, A. F. Isbell, and J. A. Boezi.** 1976. Mechanisms of phosphonoacetate inhibition of herpesvirus-induced polymerase. Biochemistry 15:426-429.
16. **Lorenz, R. J.** 1962. Zur Statistik des Plaque-Testes. Arch. Gesamte Virusforsch. 12:108-137.
17. **Mao, J. C.-H., and E. E. Robishaw.** 1975. Mode of inhibition of herpes simplex virus DNA by phosphonoacetate. Biochemistry 14:5475-5479.
18. **Mao, J. C.-H., E. E. Robishaw, and L. R. Overby.** 1975. Inhibition of DNA polymerase from herpes simplex virus-infected WI-38 cells by phosphonoacetic acid. J. Virol. 15:1281-1283.
19. **Meyer, R. F., E. D. Varnell, and H. E. Kaufman.** 1976. Phosphonoacetic acid in the treatment of experimental ocular herpes simplex infections. Antimicrob. Agents Chemother. 9:308-311.
20. **Nahmias, A. J., S. L. Shore, S. Kohl, S. E. Starr, and R. B. Ashman.** 1976. Immunology of herpes simplex virus infection: relevance to herpes simplex virus vaccines and cervical cancer. Cancer Res. 36:836-844.
21. **Nyormoi, O., D. A. Thorley-Dawson, J. Elkington, and J. W. Strominger.** 1976. Differential effect of phosphonoacetic acid on the expression of Epstein-Barr viral antigens and virus production. Proc. Natl. Acad. Sci. U.S.A. 73:1745-1748.
22. **Overby, L. R., E. E. Robishaw, J. B. Schleicher. A. Rueter, N. L. Shipkowitz, and J. C.-H. Mao.** 1974. Inhibition of herpes simplex virus replication by phosphonoacetic acid. Antimicrob. Agents Chemother. 6:360-365.
23. **Price, R. W., M. A. Walz, C. Wohlenberg, and A. B. Notkins.** 1975. Latent infection of sensory ganglia with herpes simplex virus: efficacy of immunization. Science 188:938-940.
24. **Rasmussen, L. E., G. W. Jordan, D. A. Stevens, and T. C. Merigan.** 1974. Lymphocyte interferon production and transformation after herpes simplex infections in humans. J. Immunol. 112:728-736.
25. **Roizman, B.** 1965. An inquiry into the mechanism of recurrent herpes infections of man. Perspect. Virol. 4:283-301.
26. **Rosenberg, G. L., and A. L. Notkins.** 1974. Induction of cellular immunity to herpes simplex virus: relationship to the humoral immune response. J. Immunol. 112:1019-1025.
27. **Shipkowitz, N. L., R. R. Bower, R. N. Appell, C. W. Nordeen, L. R. Overby, W. R. Roderick, J. B. Schleicher, and A. M. von Esch.** 1973. Suppression of herpes simplex virus infection by phosphonoacetic acid. Appl. Microbiol. 26:264-267.
28. **Summers, W. C., and G. Klein.** 1976. Inhibition of Epstein-Barr virus DNA synthesis and late gene expression by phosphonoacetic acid. J. Virol. 18:151-155.
29. **Thorley-Lawson, D., and J. L. Strominger.** 1976. Transformation of human lymphocytes by Epstein-Barr virus is inhibited by phosphonoacetic acid. Nature (London) 263:332-334.
30. **Vesikari, T., and E. Buimovici-Klein.** 1975. Lymphocyte responses to rubella antigen and phytohemmagglutinin after administration of RA27/3 strain of live attenuated rubella vaccine. Infect. Immun. 11:748-753.
31. **Wohlenberg, C. R., M. A. Walz, and A. L. Notkins.** 1976. Efficacy of phosphonoacetic acid on herpes simplex virus infection of sensory ganglia. Infect. Immun. 13:1519-1521.