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Comparative Effectiveness of Six Antiviral Agents in Herpes Simplex Type I Infection of Mice (39392)

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It has previously been shown that several antiviral compounds, interferon, and inducers of interferon are effective in preventing Herpes simplex virus, type 1, (HVH) infections (1-7). The comparative efficacy of these agents has been difficult to evaluate, partly because they generally have been studied under different experimental conditions. In addition combination therapy for HVH infection has not been thoroughly explored. The present study therefore was undertaken to compare under similar experimental conditions the therapeutic effectiveness of six antiviral agents during systemic HVH infection in mice. Combinations of some of the drugs also were evaluated. The drugs tested were adenine arabinoside (ARA-A) cytosine arabinoside (ARA-C), idoxuridine (IDU), phosphonoacetic acid (PAA), and the interferon inducer polyinosinic.polycytidylic acid (In.Cn). Particular emphasis was placed on examining the toxicity of the various compounds and effectiveness against HVH-induced mortality.

Materials and methods. Mice and virus. HVH, type 1 strain VR_3, was originally obtained from Dr. A. J. Nahmias and was passaged in primary rabbit kidney cells. The virus infectivity was determined by the microplaque assay in BHK-21 cells. NIH General Purpose Swiss male mice of 22-27 g were used throughout the study. They were inoculated ip with 2 LD_{50} ($10^{5.0}$ $TCID_{50}$) of HVH in 0.2 ml of Eagle's MEM medium with 2% fetal bovine serum. Most deaths in these mice occurred between the seventh and eleventh days after infection. On Day 7 after infection histologic examination of the livers of infected mice revealed multiple areas of focal necrosis with polymorphonuclear cell infiltration: less pathologically severe lesions were usually found in the brains and lungs of HVH-infected mice. HVH was consistently isolated from the brains (10^3-10^6 PFU/g) but rarely from the livers of such mice.

Drugs. All drugs were administered ip. Drugs injected on the same day as virus were given 2 hr after the virus. ARA-A was obtained in powder form from the Parke Davis Co., and suspended in distilled water to a final concentration of 40 mg/ml. The ARA-A was not soluble in water, and therefore the fine particles of ARA-A were well suspended before the injection. IDU was obtained from Calbiochem and diluted with phosphate buffered saline at pH 7.2, to a final concentration of 40 mg/ml. ARA-C was obtained from the Upjohn Co. and diluted with sterile water to a concentration of 5 mg/ml. Double-stranded In.Cn was purchased from P. L. Biochemicals, and prepared as a 1.0 mg/ml solution in phosphate buffered saline, pH 7.6.

Interferon was prepared in mouse C-243 cells using Newcastle disease virus as described previously (8). The final concentration was 10^4 reference units per ml. Phosphonoacetic acid (PAA) was obtained from Abbott Laboratories and diluted with distilled water to a final concentration of 20 mg/ml.

Statistics. Significance of differences in mortality was determined using the chi-square test. Significance of differences in survival time was determined using the Mann-Whitney *U* test. Percent protection was calculated by the formula, mortality rate in virus controls minus rate in treated group divided by mortality rate in virus controls multiplied by 100.

Results. Comparison of ARA-A, ARA-C,

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TABLE I. IDU, ARA-C, OR ARA-A TOXICITY AND PROTECTION AGAINST HSV IN MICE[a]

Expt. no.	Drug	Dose (mg/kg)	Mortality			
			Drug only		Virus infected[b]	
			Dead/total	Mortality (%)	Dead/total	Mortality (%)
1	ARA-C	100	5/10	50	15/15	100
	ARA-C	50	2/10	20	12/15	80
	ARA-C	25	0/10	0	13/15	87
	IDU	1000	9/10	90	15/15	100
	IDU	500	2/10	20	11/15	73
	IDU	250	0/10	0	9/13	70
	None				18/30	60
2	ARA-A	1000	0/10	0	2/15	13[c]
	ARA-A	500	2/10	20	0/15	0[c]
	IDU	250	1/10	10	11/15	73
	ARA-C	25	2/10	20	13/15	87
	None				24/30	80
3	ARA-A	1000	2/10	20	1/15	7[d]
	ARA-A	500	0/10	0	0/15	0[c]
	IDU	250	2/10	20	7/15	47
	ARA-C	25	0/10	0	11/14	79
	None				13/30	40
4	ARA-A	250	0/20	0	1/20	5
	ARA-C	15	0/15	0	12/15	80
	ARA-C	10	0/15	0	11/15	73
	ARA-C	5	0/15	0	11/15	73
	IDU	150	0/15	0	10/15	67
	IDU	100	0/15	0	9/15	60
	IDU	50	0/15	0	11/15	73
	None				20/30	67

[a] Treatment started 2 hr after virus and repeated daily for a total of five doses ip.
[b] Virus-infected mice were inoculated ip with 2 LD_{50} ($10^{5.0}$ $TCID_{50}$) of HSV.
[c] $P < 0.01$.
[d] $P < 0.05$.

and IDU for toxicity and protection (Table I). In these experiments treatment was started 2 hr after virus and repeated daily for a total of five doses ip. All three dosage schedules of ARA-A used (1000, 500, and 250 mg/kg/day ip) were highly effective in reducing mortality in HVH-infected mice. ARA-C and IDU both exhibited significant toxicity at the highest doses used as measured by deaths in the uninfected controls. At a dose of IDU without obvious toxicity (150 mg/kg/day or less), no antiviral effect of IDU could be demonstrated. Similarly, a nontoxic dose of ARA-C (15 mg/kg/day or less) was not protective; mice which received the relatively nontoxic dose of 25 mg/kg/day of ARA-C have a significantly decreased survival time when compared to untreated infected mice ($P < 0.01$). Thus, ARA-A was highly effective when begun 2 hr after HVH infection, while ARA-C and IDU over a wide range of doses were not effective under similar experimental conditions.

Toxicity of and protection against HVH in mice by PAA (Table II). It has been reported previously that PAA, at a dose of 200 mg/kg/day, is effective in preventing paralysis and death of mice when given systemically 24 or 72 hr after HVH infection (9). In the present study the same dose of PAA was nontoxic following systemic treatment of mice. However, as shown in Table II, no significant protection was noted. In similar experiments (not shown) there also was no protection even when mice were treated with PAA 24 hr before virus infection.

Postinfection treatment with ARA-A of HVH-infected mice. Experiments were per-

TABLE II. PAA TOXICITY AND PAA PROTECTION AGAINST HSV IN MICE.

Expt. no.	Drug	Dose (mg/kg)	Days of treatment	Mortality: Drug only: Dead/total	Mortality: Drug only: Mortality (%)	Mortality: Virus infected[a]: Dead/total	Mortality: Virus infected[a]: Mortality (%)	Survival time significance
1	PAA	200	3-8	1/10	10	14/15	93	0.7
	None					14/15	93	
2	PAA	200	1-6	ND[b]	ND	8/14	57	0.4
	None					10/15	67	
3	PAA	200	1-3	ND	ND	5/15	33	0.3
	None					9/15	60	

[a] 2 LD_{50} ($10^{5.0}$ $TCID_{50}$) of HSV injected ip on Day 0.
[b] Not done.

formed to determine how late in the course of HVH infection therapy with ARA-A could be initiated and still be effective (Fig. 1). Treatment with ARA-A (1000 mg/kg/day for 5 days) begun 2 or 3 days after virus inoculation was still highly effective in preventing deaths. Thus therapy with ARA-A was effective when begun as late as 3 days after experimental HVH infection.

Combination therapy against HVH infection in mice. Since by themselves ARA-A, interferon, and In.Cn (an inducer of interferon; (10)) have been shown to prevent HVH infection in mice, a series of experiments were performed to determine if combinations of these drugs, could provide even more protection than the drugs alone. In numerous experiments no mortality or obvious morbidity was noted in mice which received just In.Cn or interferon at the dose levels used in the above experiments. Similarly, administration of both In.Cn and interferon was without obvious toxicity in uninfected mice. Treatment with either In.Cn or interferon beginning on Day 3 did not result in significant reduction in mortality (Table III). Treatment with In.Cn was not continued beyond 2 days since hyporesponsiveness of continued interferon production occurs after 2 days of treatment (10). Treatment with interferon (10^4 units daily for 5 days) beginning on Days 3 and 4 gave erratic protection in preliminary experiments. Since many of the deaths in these experiments occurred after termination of interferon treatment, additional experiments

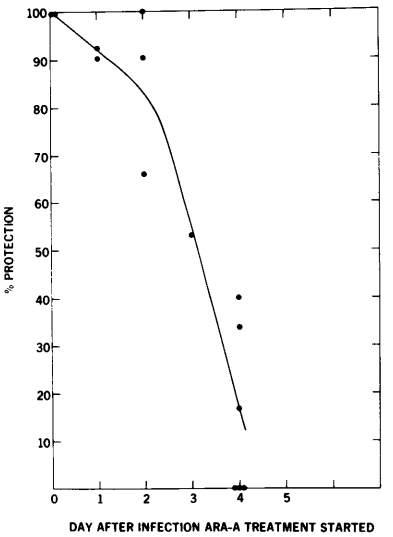

FIG. 1. Postinfection treatment with ARA-A (100 mg/kg/day ip for 5 days) of HVH-infected mice. Each dot represents percentage protection in an individual experiment when therapy was begun on the indicated day.

were performed using 8 days of treatment. Slight but significant protection by interferon as measured by survival time occurred in two of two experiments (Table III) (Experiments 2 and 3), but mortality was not significantly reduced by interferon treatment. Combined In.Cn and interferon therapy resulted in both a decrease in mortality and a prolonged survival time. However, when the combined In.Cn and interferon therapy group was compared directly with the group which received only interferon,

TABLE III. THERAPY WITH INTERFERON AND IN.CN AGAINST HSV INFECTION IN MICE.

Expt. no.	Drug	Dose schedule	Mortality		
			Dead/total	Mortality (%)	Survival time significance
1	In.Cn	100 μg Days 3, 4	6/15	40	0.4
	In.Cn	50 μg Days 4, 5	7/15	47	1.0
	Interferon	10^4 units Days 3–7	11/15	73	0.5
	Interferon	10^4 units Day 4–8	9/15	60	0.9
	In.Cn + Interferon	100 μg Days 3,4 10^4 units Days 5–9	7/15	47	0.18
	None		8/15	53	
2	In.Cn	100 μg Days 3, 4	12/15	80	0.8
	Interferon	10^4 units Days 4–11	9/15	60	0.01
	In.Cn + Interferon	100 μg Days 3, 4 10^4 units Days 4–11	7/15	47	0.03
	None		12/15	80	
3	In.Cn	100 μg Days 3, 4	14/15	93	0.9
	Interferon	10^4 units Days 4–11	12/15	80	0.05
	In.Cn + Interferon	100 μg Days 3, 4 10^4 units Days 4–11	7/15	47[a]	0.03
	None		14/15	93	

[a] $P < 0.05$.

the survival times and the decreased mortality were not significantly different.

It has recently been reported that ARA-A and human interferon are synergistic *in vitro* against HVH (11); the possibility was therefore suggested that combined therapy with interferon and ARA-A might be significantly more effective *in vivo* than therapy with either agent alone. In other experiments, not shown here, ARA-A was combined with either In.Cn (four experiments) or interferon (three experiments) in the therapy of this experimental HVH infection. The dosages of these drugs were: (a) ARA-A, 1000 mg/kg/day for 5 days beginning on Day 3 or 4; (b) In.Cn 50 or 100 μg/day for 2 days beginning on Day 3 or 4; (c) interferon 10^4 units/day for 5 or 8 days beginning on Day 3 or 4. The combinations were no more effective in decreasing mortality or prolonging survival time than the ARA-A alone. In fact the combination of ARA-A and In.Cn proved to be toxic, giving a mortality range of 33 to 90% in the uninfected controls. Even when the dosage of In.Cn was decreased to 12.5 μg daily for 2 days, in combination with ARA-A at 1000 mg/kg daily for 5 days, there was still high mortality in the uninfected controls. Only when the dose of ARA-A was reduced to 500 mg/kg/day for 5 days was there no mortality in the uninfected controls. When HVH-infected mice were treated with this nontoxic combination (500 mg/kg/day ARA-A and 100 μg In.Cn for 2 days and 50 μg In.Cn/day for 3 more days), no reduction in mortality or increase in survival time was observed.

Discussion. The present findings extend the previous demonstrations of efficacy of ARA-A by showing its clear superiority over the five other antiviral agents tested under the same conditions against HVH infection of mice. Interferon at the highest dose used was less protective than was ARA-A while ARA-C, IDU, PAA, and In.Cn had no antiviral effect in the present system. Of the several combinations of antiviral drugs tested, none was significantly more effective than either agent alone. Unexpectedly the protective and nontoxic 1000 mg/kg dose of ARA-A, when combined with nontoxic doses of In.Cn, resulted in a synergistic mortality of 33–90% in uninfected mice.

Similar to the present findings a number of previous studies of ARA-A have shown it to be effective in the treatment of HVH and vaccinia virus infections in mice, even when therapy with ARA-A was initiated as late as

48 hr after infection (1, 4, 5, 7). In three of these previous studies, treatment with ARA-A appeared to be superior to that with IDU when the two drugs were used in the same experiments (1, 4, 5). Particularly encouraging has been the recent report of a double-blind trial which demonstrated the effectiveness of systemic ARA-A in the treatment of herpes simplex keratouveitis in man (12).

It has been somewhat more difficult to demonstrate a significant protective effect of either ARA-C or IDU in experimental viral infections. In mice, ARA-C has been shown to be effective only when HVH and drug were both given by intracerebral inoculation (1, 13, 14). In an experimental HVH infection in rats, which are relatively resistant to the toxic effects of ARA-C, a beneficial effect of ARA-C was observed even when initiation of therapy was delayed for 3 days after virus inoculation (15). Similar to the present findings in mice, ARA-C was noted to have a deleterious effect on disseminated herpes zoster infection of man (16). Reports such as these emphasize the necessity of very careful evaluation of antiviral drugs which have significant side effects, particularly immunosuppressive effects. Consistent with the present findings, no effect of IDU on mortality has been noted in experimental HVH infection of mice or encephalitis and disseminated HVH infection in marmosets (17, 18), although some beneficial effects were noted in guinea pigs (19, 20).

In contrast to our negative findings with PAA, it has been noted to be effective in several experimental HVH infections in mice, even when therapy was delayed 72 hr after initiation of infection (9). Possible reasons for the differences in protection include strain of virus or mice and routes of administration and dissemination of virus.

By using combinations of different antiviral agents which are effective through differing mechanisms of action and which possess differing toxicities, it was hoped that more effective therapy of this experimental infection could be obtained. This was particularly true since ARA-A and interferon have been reported recently to be synergistic *in vitro* against HVH (11). In fact, this sort of added effectiveness was not noted.

The superior therapeutic effectiveness of ARA-A may be related to its relative lack of toxicity. The deleterious effects of ARA-C during HVH infections in mice and man (16) emphasize the need for careful evaluation of potentially toxic drugs. Equal caution must be used in the evaluation of combinations of antiviral drugs, since unexpected toxicities may be noted when different drugs are combined. The observed combined toxicity of nontoxic doses of ARA-A and effective but nontoxic doses of In.Cn (as determined previously (23)) provides such an example.

Summary. The therapeutic effectiveness of six antiviral agents and certain combinations were evaluated under the same experimental conditions during systemic HVH infection of mice. The agents used were adenine arabinoside (ARA-A), cytosine arabinoside (ARA-C), idoxuridine (IDU), polyinosinic.polycytidylic acid (In.Cn), interferon, and phosphonoacetic acid (PAA). Treatment with ARA-A begun as late as 3 days after the virus infection was highly effective in preventing death. Interferon begun on Day 4 prolonged survival. In similar experiments, no antiviral effect of either IDU or PAA could be demonstrated, while therapy with ARA-C potentiated the infection. Of the drug combinations tested, none was more effective than the better agent alone. Unexpectedly a protective and nontoxic dose of ARA-A when combined with a nontoxic dose of In.Cn resulted in a synergistic mortality in uninfected mice.

1. Allen, L. B., and Sidwell, R. W., Antimicrob. Agents Chemother. **2,** 229 (1972).
2. Catalano, L. W., Jr., and Baron, S., Proc. Soc. Exp. Biol. Med. **133,** 684 (1970).
3. Kaufman, H. E., Harefuah **85,** 467 (1973).
4. Klein, R. J., Overall, J. C., Jr., and Glasgow, L. A., J. Infect. Dis. **128,** 290 (1973).
5. Schabel, F. M., Jr., Chemotherapy **13,** 321 (1968).
6. Shipkowitz, N. L., Bower, R. R., Appell, R. N., Nordeer, C. W., Overby, L. R., Roderick, W. R., Schleicher, J. B., and VonEsch, A. M., Applied Microbiol. **26,** 284 (1973).
7. Sloan, B. J., Miller, F. A., and McLean, I. W., Jr., Antimicrob. Agents Chemother. **3,** 74 (1973).
8. Oie, H. K., Gazdar, E. F., Buckler, C. E., and

Baron, S., J. Gen. Virol. **17,** 107 (1972).
9. Friedman-Kien, A. E., and Klein, R. J., Antimicrob. Agents Chemother. **7,** 289 (1975).
10. DuBuy, H. G., Johnson, M. L., Buckler, C. E., and Baron, S., Proc. Soc. Exp. Biol. Med. **135,** 340 (1970).
11. Lerner, A. M., and Bailey, E. J., J. Infect. Dis. **130,** 549 (1974).
12. Kaufman, H. E., and Abel, R., Abstract No. 40, presented at the 14th Interscience Conference on Antimicrobial Agents and Chemotherapy (1974).
13. Prince, H. N., Grunberg, E., Buch, M., and Cleeland, R., Proc. Soc. Exp. Biol. Med. **130,** 1080 (1969).
14. Renis, H. E., Hallowell, C. A., and Underwood, G. E., J. Med. Chem. **10,** 777 (1967).
15. Renis, H. E., Antimicrob. Agents Chemother. **4,** 439 (1973).
16. Stevens, D. A. Jordan, G. W., Wadell, T. F., and Merigan, T. C., New Eng. J. Med. **289,** 873 (1973).
17. Cho, C. T., Liu, C., Voth, D. W., and Teng, K. K., J. Infect. Dis. **128,** 718 (1973).
18. Kern, E. R., Overall, J. C., Jr., and Glasgow, L. A., J. Infect. Dis. **128,** 290 (1973).
19. Tokumaru, T., Arch. Gesamte Virusforsch. **22,** 332 (1967).
20. Tomlinson, A. H., and MacCallum, F. O., Ann. N.Y. Acad. Sci. **173,** 20 (1970).
21. Worthington, M., and Baron, S., J. Infect. Dis. **128,** 308 (1973).
22. Worthington, M., Levy, H., and Rice, J., Proc. Soc. Exp. Biol. Med. **143,** 683 (1973).
23. Worthington, M., and Baron, S., Proc. Soc. Exp. Biol. Med. **136,** 323 (1971).

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