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Effective Chemotherapy of Equine Herpesvirus 1 by Phosphonylmethoxyalkyl Derivatives of Adenine Demonstrated in a Novel Murine Model for the Disease

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Equine herpesvirus 1 was established in adult mice by means of intranasal inoculation. A disease developed that showed several features closely resembling the infection in the natural host. These included the restriction of virus replication to the respiratory tract and blood, the replication of virus in ciliated mucosa, and development of viremia for several days during the acute phase of the infection. Infected mice were treated with the antiviral agent (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine. A marked effect was observed on virus replication in the respiratory tract when chemotherapy was commenced 1 day before virus inoculation; it also cleared the viremia and reversed the progression of clinical signs. When chemotherapy was commenced 1 day after virus inoculation, a moderate, though less marked effect was observed. The efficacy of this drug in the murine infection correlated with activity of the drug against equine herpesvirus 1 in cell cultures. The prospects for chemotherapy in the natural host are discussed in the light of these findings.

Equine herpesvirus 1 (EHV-1) is a common infection among horses (17), producing clinical signs which include fever, respiratory distress, and abortion in pregnant mares (1). Neurological signs, including hind limb paresis, are also associated with some outbreaks (9, 16). The infection appears to establish a state of latency as evidenced by the recurrence of virus shedding following a variety of natural (4, 10) or experimental (9) stimuli. The site of latency or state of the virus between episodes of virus shedding has yet to be elucidated, but unlike several other alpha herpesviruses of humans and domestic animals, with EHV-1 there is no evidence of latency within neurons of the peripheral nerves.

Natural immunity in the horse appears to be short-lived, and the efficacy of available vaccines is questionable (1, 19). The infection is regarded as very important to the thoroughbred industry, and a safe and efficacious therapeutic agent could have a valuable role in prophylaxis or therapy or in preventing virus shedding during stress.

The drug (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine (HPMPA) is a phosphonylmethoxyalkyl derivative of adenine that has been shown to be active against a variety of herpesviruses including EHV-1 (6–8), and this paper describes its marked effects on the pathogenesis of this virus in a murine model.

MATERIALS AND METHODS

Antiviral agents. The drugs HPMPA and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) were a gift from E. De Clercq, Rega Institute, Leuven, Belgium. The compounds were originally described by Holy and Rosenberg (13), and structural formulas are depicted in Fig. 1. The compounds were received as dry powders, which were dissolved in water and stored frozen at a concentration of 1 mg/ml. The drugs were administered to mice by subcutaneous injection of a solution also containing 0.5% Tween 80 and 0.5% carboxymethyl cellulose in a volume of 0.25 ml to give a total daily dose of 50 μg/g in two divided doses. Control groups of infected and mock-infected mice were given twice-daily injections of the same solution without drug.

Virus strains and tissue cultures. The EHV-1 strain AB4 was a gift from N. Edington, Royal Veterinary College, London, United Kingdom. This strain was originally isolated from a case of equine herpes with neurological complications (paresis). The rabbit kidney fibroblast cell line (RK-13) was used for virus plaque titrations and growth of virus for working stocks. Cells were cultured in Eagle minimal essential medium (EMEM) supplemented with 10% newborn calf serum. For virus titration, EMEM was supplemented with 2% fetal calf serum and 1% carboxymethyl cellulose. For preparation of working stocks, cells were infected at a multiplicity of infection of 0.1 PFU per cell and the cells were harvested after 3 to 4 days, when the cytopathic effect was approximately 90% complete. Working stocks of virus were stored at −70°C in small aliquots.

Intranasal (i.n.) inoculation of mice. BALB/c mice were obtained from Bantin & Kingman Ltd., Hull, United Kingdom, and used at 3 to 4 weeks of age. Mice were lightly anesthetized with ether, and 40 μl, or in some cases 50 μl, of EMEM containing a suspension of virus was placed on the nares until all inoculum was inspired, usually within a few seconds. On completion of the inoculation procedure, surplus virus suspension was retitrated in RK-13 cells to confirm the precise dose.

Assessment of clinical signs and disease. Mice were examined daily, and the weight of each mouse was recorded. Obvious signs of abnormality such as ruffled fur, hunched crouching, dragging movements of the limbs, and abdominal breathing were noted subjectively.

Virus isolation from murine tissues. Previous study showed that the important target organs for virus replication in the present model are nasal mucosa and lungs (A. R. Awan, Y.-C. Chong, and H. J. Field, J. Gen. Virol., in press), with

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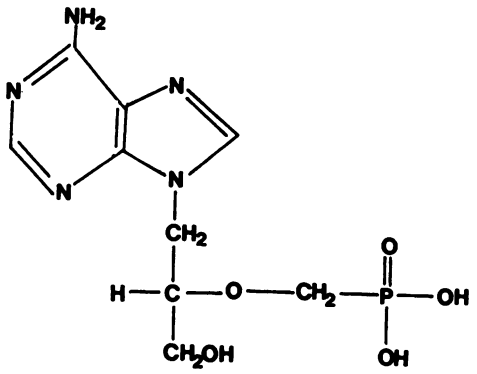
A.



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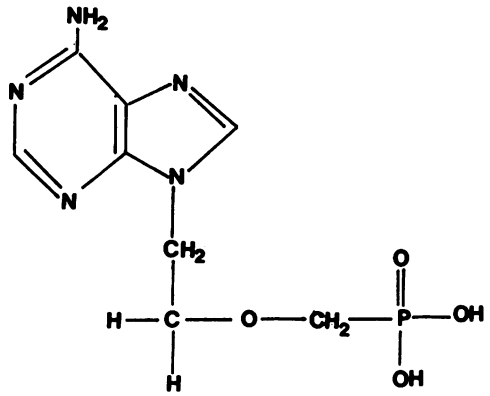
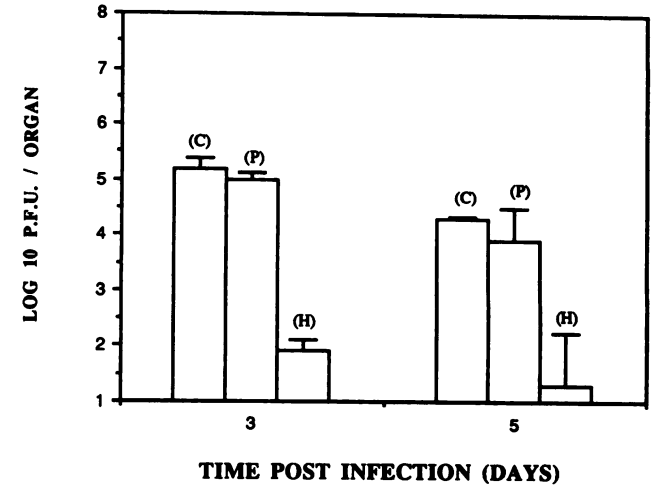


FIG. 1. Structural formulas for HPMPA (A) and PMEA (B).



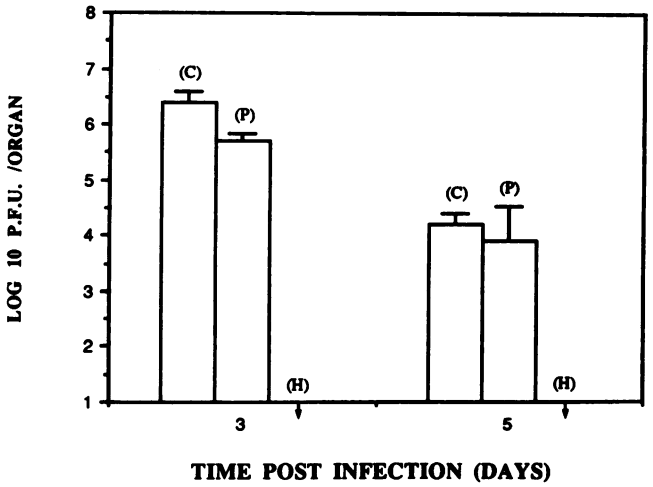


FIG. 2. Reduction of virus titers in the respiratory tissues of mice undergoing chemotherapy. Comparison of virus titers in the turbinates (top) and lungs (bottom) of mice infected i.n. with 10^7 PFU of EHV-1 and treated with HPMPA (H) or PMEA (P) or untreated (C). Therapy was 50 μg/g per day given in 12-h subcutaneous doses commencing 1 day before virus inoculation. Each bar is the geometric mean titer with standard deviation, obtained from four mice processed individually.

peak titers approximately 3 to 5 days after infection. Viremia was also detected during the acute stage of infection.

Lungs and turbinate bones were therefore sampled on days 1, 3, 5, and 8 after inoculation. Tissues were minced with scissors and homogenized in an electric blender in a small quantity of EMEM. The suspension was sonicated for 1 min and centrifuged at 500 × *g* for 10 min to remove cellular debris. Dilutions of the supernatant were made in EMEM, and samples were inoculated onto confluent RK-13 monolayers. After 45 min of adsorption, EMEM containing 2% fetal calf serum and 1% carboxymethyl cellulose was added and the cultures were incubated at 37°C. Cultures were examined after 2 or 3 days, and plaques were stained with crystal violet for enumeration.

Infectious-center assays. To assess viremia, heparinized blood (2 mg/ml of EDTA) was collected and the leukocytes were counted. The blood was centrifuged in microdilution tubes, and the buffy coat was mixed in distilled water for 1 min to lyse the erythrocytes. The osmotic balance was restored with 10×-strength phosphate-buffered saline. The cells were counted in a hemacytometer, and a given number of cells was added to confluent monolayers of RK-13 cells; the development of plaques was determined as above.

Plaque reduction assay. Approximately 100 PFU of virus was inoculated onto preformed monolayers of RK-13 cells in multiwell dishes. After 1 h of adsorption, medium containing various concentrations of HPMPA or PMEA was added and incubation was continued at 37°C for 3 days. The monolayers were then fixed and stained, and virus plaques were enumerated. The percent reduction in plaques in the presence of drug was plotted against the $\log_{10}$ drug concentration, and the 50% effective doses were determined directly from the curve.

Isolation of drug-resistant mutant. RK-13 cells were inoculated with EHV-1 (AB4) at a multiplicity of infection of 0.1 PFU per cell in the presence of 0.1 μg of HPMPA per ml. After 3 days, infected cells from single plaques were aspirated from the dish and inoculated into RK-13 cells in the absence of drug. This virus preparation was then used to inoculate additional cultures in the presence of successively higher concentrations of HPMPA as follows: 2, 4, 6, 10, and 15 μg/ml; single plaques were picked at each stage. Working

TABLE 1. Comparison of infectious-center-forming cells in blood cells of mice treated with HPMPA or PMEA[a]

Individual mouse no.	No. of infectious-center-forming cells/10^6 leukocytes at given time postinfection	
	3 days	5 days
1	316	347
2	500	19
3	471	ND[b]
4	ND	ND
5	400	0
6	22	0
7	100	0
8	23	ND
9	0	0
10	0	0
11	0	0
12	0	0

[a] Mice were inoculated i.n. with 10^7 PFU of EHV-1, and therapy (50 mg/kg per day subcutaneously) was commenced starting 1 day before virus inoculation. Animals were given medium only (mice 1 to 4), PMEA (mice 5 to 8), or HPMPA (mice 9 to 12).

[b] ND, Not determined.

stock was prepared from the final virus yield termed EHV-1 AB4 (R1) and was found to have acquired an approximately 100-fold decrease in susceptibility to HPMPA compared with the parental strain AB4.

Histology. Mice were killed by lethal injection of pentabarbitone sodium. Small pieces of tissue were carefully excised, immediately fixed in 10% formal saline, and embedded in paraffin wax. Sections were stained by means of hematoxylin and eosin, using standard methods.

RESULTS

Comparison of HPMPA and PMEA in tissue culture. HPMPA was found to inhibit the replication of EHV-1 in RK-13 cells with a 50% effective dose concentration of 0.1 ± 0.05 μg/ml. PMEA was less active (50% effective dose, 5.0 ± 4.0 μg/ml). Mutant AB4 (R1), which was selected for resistance to HPMPA, showed no reduction in plaque number in the presence of 15 μg of HPMPA per ml.

Comparison of HPMPA and PMEA in mice. Mice were inoculated i.n. with the AB4 strain of EHV-1. Therapy was begun 24 h before virus inoculation and continued by means of twice-daily injections (50 μg/g per day) throughout the period of observation. The infected mice receiving HPMPA treatment showed reduced clinical signs with no obvious respiratory distress and less ruffled fur compared with the infected controls receiving medium only. PMEA-treated mice were observed to have more clinical signs than those receiving HPMPA but less than the untreated controls. These subjective observations were supported by measurement of virus titers in the lungs and nasal turbinate bones.

At days 3 and 5 after inoculation, the groups treated with HPMPA showed a significant reduction in virus titer (of the order of 3 $\log_{10}$ virus per organ) in both sites tested (Fig. 2). In mice receiving PMEA, small reductions in virus titer were observed, but these were not significant.

The clearance of virus-infected cells from the blood as evidenced by the infectious-center assay was even more striking (Table 1). In mice receiving HPMPA, no infectious centers were detectable on day 3 or 5 after inoculation. In mice receiving PMEA, infectious centers were observed on day 3 but not on day 5. Another experiment was then carried out with HPMPA to confirm the effectiveness of this form of therapy.

Effect of delayed treatment with HPMPA. Eighty-two mice were inoculated as above with EHV-1 (AB4). The infected

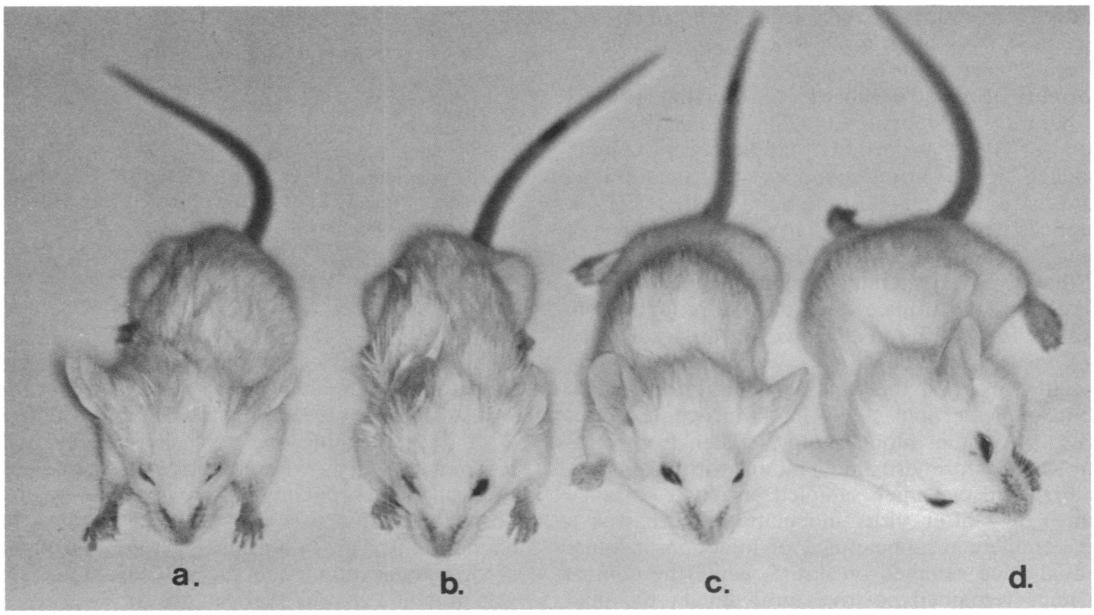


FIG. 3. Photograph showing gross appearance of infected mice with and without treatment with HPMPA. One mouse was selected at random from each treatment group 4 days after inoculation. (a) Infected mouse receiving medium only; (b) infected mouse treated with HPMPA from 1 day after virus inoculation; (c) infected mouse treated with HPMPA from 1 day before virus inoculation; (d) HPMPA-treated, mock-infected (inoculated with RK-13 cells) mouse.

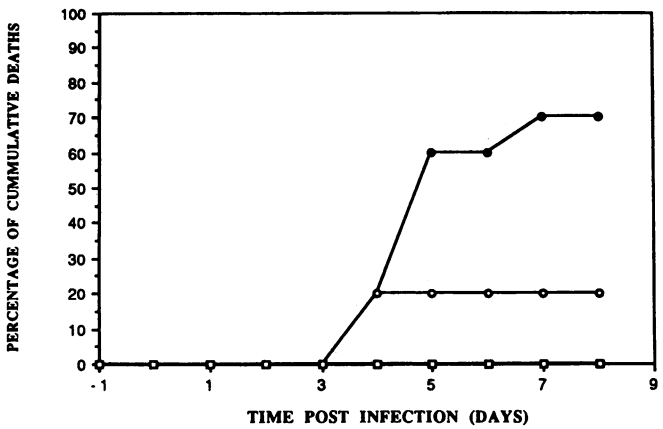


FIG. 4. Cumulative frequency of death in mice with or without chemotherapy. Groups of 10 mice were inoculated i.n. with $10^{6.8}$ PFU of EHV-1. Therapy (HPMPA, 50 μg/g per day given subcutaneously in two doses) was commenced from the day before (□) or the day after (○) virus inoculation; ●, infected, untreated mice.

mice were divided into four treatment groups as follows: (i) HPMPA by means of twice-daily injection commencing 1 day before virus inoculation; (ii) 1 day after virus inoculation; and (iii) "treated" with medium only; (iv) a small control group which were mock infected and treated with HPMPA from 1 day before virus inoculation. Groups (i), (ii), and (iii) were further subdivided into groups of 10 mice which were kept separately to observe clinical signs and mortality was compared with that in group (iv). The remaining treatment groups of mice were sampled at different times after virus inoculation.

There were subjective differences in the clinical signs observed in each group, with untreated mice showing the most severe signs and mice receiving drug from the day before infection being the least affected. The changes observed were very uniform and the photograph (Fig. 3) shows the comparative appearance of mice from each group, randomly selected, 4 days after virus inoculation. Of the 10 untreated mice, 7 died from day 4 after inoculation; however, no mortality occurred in those mice receiving HPMPA from 1 day before virus inoculation (Fig. 4). Furthermore, administration of HPMPA starting 1 day before virus inoculation produced significant reduction in virus titers in lung and nasal turbinate tissues at all time points tested (Fig. 5). These differences were also reflected in a rapid clearance of infectious-center-forming cells from the blood (Table 2). In those mice that received therapy commencing 1 day after virus inoculation, 2 of 10 mice died and the reductions in virus titer were also less marked. However, an accelerated clearance of virus from the tissues was observed upon commencing therapy, and this was particularly noticeable for the blood samples (Table 2). No infectious-center-forming cells were present in blood samples taken from mice treated from 1 day before virus inoculation, with the exception of low levels in two mice sampled on day 1. In mice treated from 1 day after virus inoculation, there was a sudden and complete disappearance of infectious centers from individual mice sampled on day 5, while the control (untreated) mice remained positive until day 8, the final sampling time.

Histopathological observations. In untreated mice, pathological changes were clearly visible by day 3 after inoculation. Accumulations of inflammatory cells were present

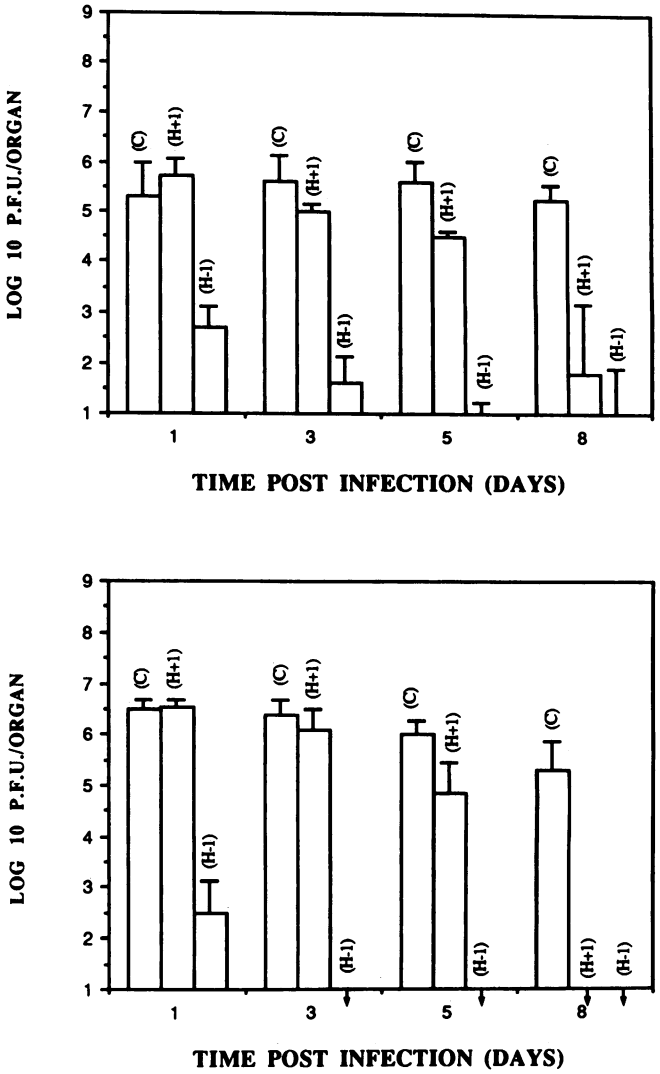


FIG. 5. Isolation of virus from tissues of mice treated with HPMPA commencing before or after virus inoculation. Mice were inoculated i.n. with $10^{6.8}$ PFU, and the organs were tested for the presence of virus on days 1, 3, 5, and 8 after inoculation. (C) Control mice receiving medium only from the day before virus inoculation. (H+1) Infected mice treated with HPMPA from the day after virus inoculation. (H−1) Infected mice treated with HPMPA from 1 day before virus inoculation. Bars represent the geometric mean titers with standard deviation, obtained from four mice processed individually. Top panel, Turbinate bones; bottom panel, lungs.

among the alveolae, and there were signs of damage in the lining of the bronchioles (Fig. 6b). However, virus antigen was localized to the bronchiolar mucosa (data not shown). By day 5, the lung was typically heavily infiltrated with inflammatory cells with complete loss of alveolar architecture and many bronchioles were occluded by the presence of desquamated mucosal cells together with an inflammatory exudate (Fig. 7a). In a previous study, morphological proof of virus replication within these desquamated epithelial cells was obtained (Awan et al., in press).

All histological changes were markedly reduced in the tissue specimens from HPMPA-treated mice. The cellular infiltration was much less (Fig. 6c and 7b), with some preservation of the alveolar architecture. Although some

TABLE 2. Comparison of infectious-center-forming cells in blood of mice treated with HPMPA commencing from 1 day before or 1 day after virus inoculation[a]

Individual mouse no.	No. of infectious-center-forming cells/10^6 leukocytes at given time postinfection			
	1 day	3 days	5 days	8 days
1	387	480	100	0
2	391	300	216	1
3	1,082	183	55	2
4	455	464	31	ND[b]
5	335	425	0	0
6	333	27	0	0
7	86	8	0	0
8	174	260	0	ND
9	0	0	0	0
10	1	0	0	0
11	1	0	0	0
12	0	0	0	0

[a] Mice were inoculated i.n. with $10^{6.8}$ PFU of EHV-1. Animals received medium only (mice 1 to 4), HPMPA from 1 day after virus inoculation (mice 5 to 8), or HPMPA from 1 day before virus inoculation (mice 9 to 12).
[b] ND, Not determined.

changes were visible in the lining to the bronchioles, it was notable that portions of the epithelial surface appeared to remain intact at day 5 (Fig. 7b). When treatment with HPMPA was delayed to 1 day after virus inoculation, the pathological changes were more pronounced, although less severe than in the untreated mice, and the lung tissue sections from all treated mice examined on day 8 after inoculation were found to closely resemble those of the uninfected controls.

The fields shown in Fig. 6 and 7 were chosen to illustrate these points; however, it should be stressed that they represent typical changes observed in many different sections examined from several mice in each experimental group.

DISCUSSION

There has been a long-standing interest in the possibility of controlling EHV-1 infections by means of chemotherapy. The majority of published work concerns results in the hamster. This model has several differences from the murine model described above. In the hamster, virus is generally a hamster-adapted laboratory strain which is inoculated subcutaneously and the chief target organ is the liver. Acute

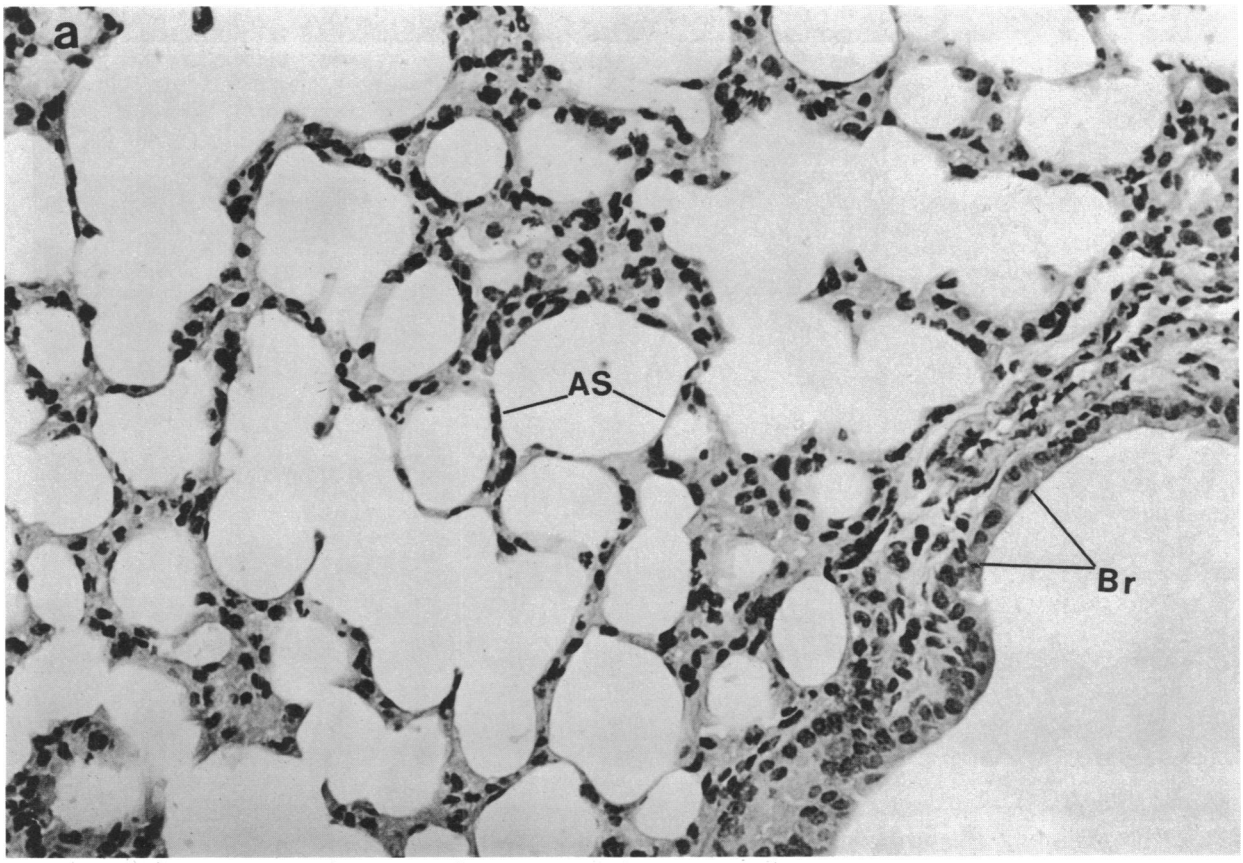
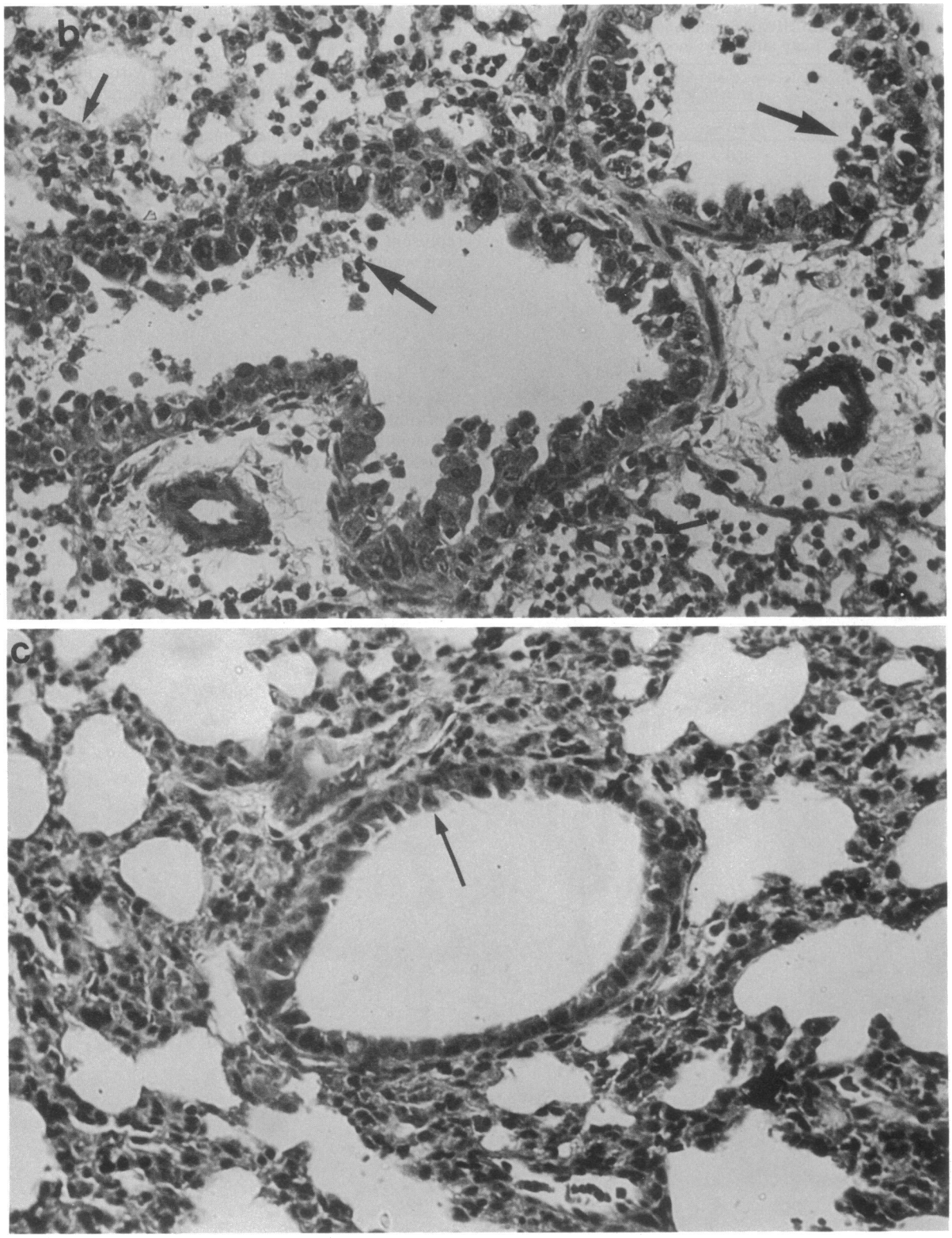


FIG. 6. Histopathological changes in lungs of infected mice with and without treatment. All sections were stained by hematoxylin and eosin; magnification, ×340. (a) Uninfected mice showing the normal architecture of alveoli of the lung. AS, Alveolar septa; Br, bronchiolar epithelium. (b) Mouse 3 days after i.n. inoculation with $10^{6.8}$ PFU of EHV-1 showing the intense inflammatory reaction. There is evidence of damage to bronchiolar epithelium (large arrows) with some destruction of the alveolar architecture and the presence of inflammatory cells (small arrows). (c) Mouse infected as above but treated with HPMPA from the day before virus inoculation. The intensity of inflammation is less marked and there is more preservation of the bronchiolar epithelium (arrow).



hepatitis leads to death, and with moderate inocula, mortality of 100% can be obtained consistently (18).

Early results with chemotherapy in the hamster model were disappointing; Aswell et al. (3) observed 60% survival with arabinofuranosylthymine given intraperitoneally at the relatively high dose of 1 mg/g per day every 4 h for 72 h commencing 4 h preinfection. Phosphonoacetic acid at a dose of 2 mg/g similarly administered gave only 20% survival. Lieberman et al. (14) obtained 80 to 90% survival with adenine arabinoside up to 250 μg/g per day but found

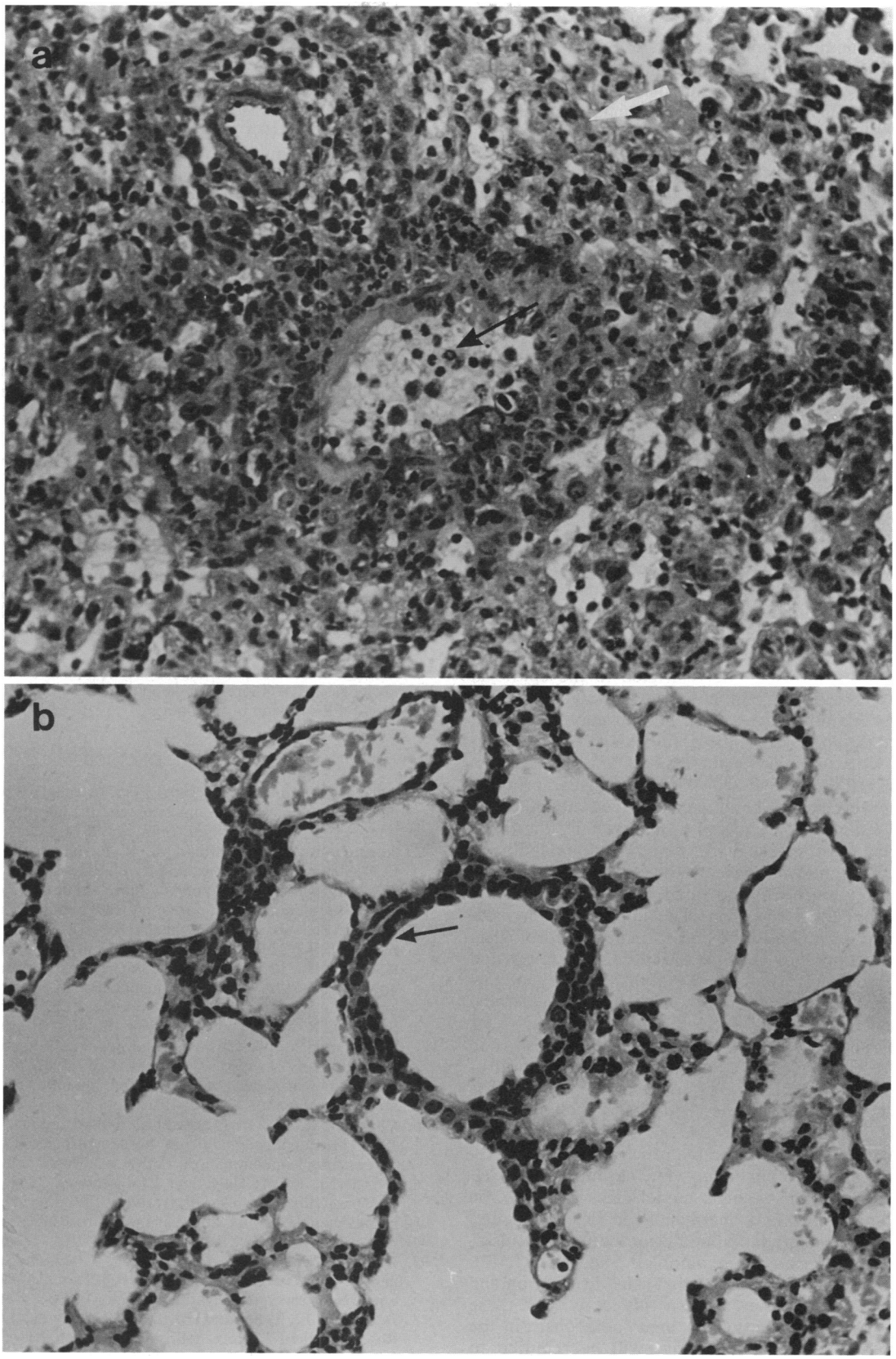


FIG. 7. Reduction in histopathological changes evident in infected mice treated with HPMPA. Typical lung sections obtained from mice from the same experiment as Fig. 6 examined 5 days after inoculation. (a) In the untreated mouse, the inflammation is more intense with almost complete loss of the alveolar architecture by inflammatory cells. The bronchus (arrow) is occluded by desquamated epithelial cells and cellular exudate. (b) In the HPMPA-treated mouse lung, the inflammation is relatively mild, with lung parenchyma relatively normal. The bronchiolar epithelium (arrow) is partially preserved, with relatively few apparently abnormal cells. Magnification, ×330.

iododeoxyuridine, distamycin A, and *N*-ethylisatinthiosemicarbazone all to be ineffective. Allen et al. (2) reported more favorable results with adenine arabinoside and adenine arabinoside monophosphate, which gave a maximum of 80% survival with parenteral therapy of 3.3 and 8.7 μg/g per day, respectively.

Rollinson and White (18) were even more successful with a similar hamster model. These workers found that 100% survival was readily obtained with the nucleoside analog dihydroxypropoxymethyl guanosine (also known as BW759 or BIOLF-62) at doses down to 2 μg/g intraperitoneally starting 5 h before virus infection or 39 μg/g per day in the drinking water. Similar protection was still observed when therapy was delayed until 27 h after infection; in all cases, all infected untreated controls died from the infection. Rollinson and White (18) also made a quantitative assessment of virus growth in the liver, observing reductions in titer of some 3 $\log_{10}$. The histopathology of the acute hepatitis was described, and a reduction in the number of inclusion-bearing cells was reported.

The results we describe were obtained by using a different species as a model, the mouse. Furthermore, a recent clinical isolate (i.e., not mouse adapted) of EHV-1 was inoculated by means of the more natural route of infection (i.e., i.n. inoculation), and that produced a disease more closely resembling the distribution and sequence of infected tissues seen in the natural host. By means of histological examination together with evidence from a previous immunohistological study (Awan et al., in press), the site of virus growth was shown to be restricted to the epithelial surfaces of the respiratory mucosa and closely underlying tissue. Consolidation of the infected murine lungs by a cellular exudate appeared to be a secondary effect consequent upon virus infection of the bronchi and bronchioles. The use of HPMPA reversed the pathological process; clinical signs were reduced and mice survived the infection. Histological examination showed how especially the bronchial epithelia were spared with minimal accumulation of exudate in the lumen of these vessels. A second notable feature of the therapy was the rapid clearance of infected leukocytes from the circulation. The important complications of EHV-1 infection in the horse, namely, neurological signs and abortion, are thought to relate to intense and prolonged viremia (1, 5); thus, the ability to prevent this phase in the horse by chemotherapy could have considerable clinical benefit.

The compound HPMPA was found to inhibit EHV-1 in tissue culture (using rabbit cells) with a concentration of approximately 0.1 μg/ml, which is of similar order to dihydroxypropoxymethyl guanosine reported by Rollinson and White to be sensitive to 0.06 μM. PMEA was found to be approximately 10-fold less active in vitro, and this correlated with relatively poor effect in the murine model. Comparison of drugs in a single model can, of course, be very misleading, since many pharmacological factors including the adsorption, degradation and clearance, and intracellular processing differ markedly among the species (11, 12). Little information is available on the tissue distribution and half-lives of the compounds used in this study, and these data together with information on their metabolism in the horse and potential toxicity, if any, will be required to predict the likely efficacy in the natural host. The mode of action of HPMPA differs from that of dihydroxypropoxylmethyl guanosine in that the latter is activated within cells by the virus thymidine kinase. HPMPA has been reported to act independently of virus thymidine kinase, and thymidine kinase-defective variants of herpes simplex virus were reported to be sensitive to the drug (15). The most likely mechanism of drug action is by direct inhibition of the virus DNA polymerase (20). We have not studied the mode of action of HPMPA against EHV-1, but the isolation of a drug-resistant mutant, AB4 (R1), provides further proof that the compound has a virus-specific site of action. In due course, this mutant will be tested for resistance in vivo.

Notwithstanding these unknown factors, the present data taken together with previous results indicate that there is a potential for chemotherapy or prophylaxis to prevent disease caused by EHV-1. The site of latency, state of the latent virus, and molecular events leading to reactivation and recrudescence of EHV-1 in the horse are yet to be elucidated. Circumstantial evidence has now been obtained which suggests persistence of virus in the murine model. We have yet to prove that latent infections are established and, if so, whether chemotherapy influences establishment, maintenance, or reactivation of virus. These questions are under investigation.

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