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9-(2-Phosphonylmethoxyethyl)Adenine in the Treatment of Murine Acquired Immunodeficiency Disease and Opportunistic Herpes Simplex Virus Infections

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The murine model of acquired immunodeficiency disease was used to evaluate both the antiretroviral and antiherpetic activities of the acyclic nucleotide analog 9-(2-phosphonylmethoxyethyl)adenine (PMEA). The antiretroviral activity of PMEA was compared with that of azidothymidine (AZT) in mice receiving the drug either immediately after infection or at late times in disease progression. Both AZT (oral, 30 mg/kg) and PMEA (parenteral, 25 and 5 mg/kg) were effective in slowing the development of disease when administered daily beginning on the day of infection. In contrast, neither drug alone was effective in modifying disease outcome when administered several weeks after viral infection. Human recombinant alpha interferon (rhuIFNalpha-B/D at 5×10^7 U/kg) was also ineffective when administered late in the course of disease. However, when administered in combination, both alpha interferon and PMEA (25 mg/kg) were able to suppress disease progression even when treatment was initiated as late as 3 weeks postinfection. Mice that were immunocompromised due to LP-BM5 virus infection were highly susceptible to acute (lethal) infection with herpes simplex virus type 1, whereas their immunocompetent littermates were not. PMEA was as effective as acyclovir in the treatment of opportunistic herpes simplex virus type 1 infections in LP-BM5 virus-infected mice. Thus, like AZT, PMEA was effective against retrovirus infection, and, like acyclovir, PMEA was effective against herpes simplex virus type 1 infection. This gives PMEA the unique potential of being useful in the treatment of opportunistic herpes simplex virus infections as well as the underlying retroviral disease.

The discovery and development of drugs effective against the human immunodeficiency virus or against the opportunistic pathogens commonly associated with acquired immunodeficiency syndrome (AIDS) is made difficult by the lack of suitable and convenient animal models. Several conventional murine retrovirus models such as Friend, Rauscher, and Moloney leukemia are currently used to determine the in vivo efficacy of new antiviral agents. Although these animal models are useful in measuring the ability of a drug to prevent or suppress retrovirus replication, they tell us little about its ability to prevent or suppress the development of virus-induced immunosuppression and susceptibility to opportunistic infections.

Infection of C57BL/6 mice with the LP-BM5 retrovirus complex causes an AIDS-like disease, murine AIDS (MAIDS), the pathology of which at least partially resembles that of human AIDS (14, 17, 18, 22). Because of the similarities to human AIDS and the ease with which disease manifestations can be measured, the MAIDS model provides a safe and inexpensive means with which to evaluate the in vivo efficacy of antiretroviral agents and other agents that may be useful in the treatment of opportunistic infections. Moreover, this model could be used to establish a base from which therapeutic strategies to be employed in other more costly feline and primate models could be designed.

A number of 3-hydroxy-2-phosphonylmethoxypropyl (HPMP) and 2-phosphonylmethoxyethyl (PME) derivatives of purine and pyrimidine have been evaluated for their antiviral properties (1, 5–7, 13). In general, HPMP analogs are more active than PME compounds against vaccinia virus, adenovirus, cytomegalovirus, and varicella-zoster virus. In contrast, PME analogs are more effective than HPMP analogs against the human immunodeficiency virus. Nonetheless, HPMP and PME analogs are equally effective against herpes simplex viruses (HSV).

Because of their broad-spectrum activity against DNA viruses and retroviruses, both HPMP and PME analogs offer a wealth of potential applications, particularly in the treatment of AIDS and the opportunistic infections associated with immunosuppression. Recently, Balzarini et al. (1) demonstrated the in vivo antiretroviral activity of one PME analog, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), and De Clercq et al. (5) demonstrated its in vivo anti-HSV activity. This study extends previous in vivo observations and further demonstrates the therapeutic potential of PMEA in a retroviral model of immunosuppression and opportunistic HSV type 1 (HSV-1) infection.

MATERIALS AND METHODS

Virus and animals. LP-BM5 virus was kindly provided by Robert Yetter, Veterans Administration Hospital, Baltimore, Md., and was maintained in a persistently infected SC-1 cell line (TC 2110). Working stocks of virus were prepared by overlaying TC 2110 cells with rapidly growing uninfected SC-1 cells maintained in Eagle minimal essential medium supplemented with 5% fetal calf serum, penicillin, and streptomycin. These cultures were incubated for 24 h; supernatant fluids harvested, centrifuged at $1,000 \times g$ for 30 min, and filtered through a 0.22-μm-pore-size membrane filter. Virus stocks were stored at −80°C for up to 6 months

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before use. Female C57BL/6 mice (28 to 33 days old; CIBA-GEIGY, Sisseln, Switzerland) were infected intraperitoneally with 0.5 ml of undiluted virus. Complete descriptions of the LP-BM5 virus complex and the immunosuppression that occurs after infection have been reported elsewhere (10–12, 14, 17, 18).

A nonneurovirulent strain (VR3) of HSV-1 was obtained from the laboratory of A. Nahmias (Emory University, Atlanta, Ga.) and propagated in Vero cells. Virus stocks contained 2.5×10^7 PFU/ml and were stored at −80°C. Complete descriptions of this virus and the marine model of pneumonitis that is induced in young (4-week-old) mice after intranasal instillation have been reported elsewhere (9, 20); older mice are resistant to infection with this virus unless they are immunosuppressed. A neurovirulent strain of HSV-1 (McIntyre) was obtained from the American Type Culture Collection (ATCC VR-539) and propagated in human embryonic foreskin cells (7000; Flow Laboratories, Inc., McLean, Va.). Culture supernatants were harvested at 72 h after infection, clarified by centrifugation (1,000 × *g* for 20 min), and stored at −80°C. The virus titer, as assessed on Vero cells, was 10^8 PFU/ml.

Drugs. Hybrid recombinant human alpha interferon (rhuIFNalpha-B/D; CGP 35269) was produced in *Saccharomyces cerevisiae* and purified as previously described (15). This interferon was biologically active in a variety of animal species, including mice, and was stored carrier free in phosphate-buffered saline (PBS) at 4°C at a concentration of 0.2 mg/ml (3×10^7 IU).

Azidothymidine (AZT) was synthesized in the Chemistry Department of CIBA-GEIGY, Basel, Switzerland, and dissolved in distilled water at a concentration of 0.2 mg/ml. This solution replaced distilled water for drinking in those animals receiving AZT. Water consumption for each cage was recorded, and the average dose of AZT that each mouse received was calculated from this information. Mice receiving AZT consumed approximately 3 ml per day.

PMEA was kindly provided by A. Holý (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia.). This drug was dissolved in PBS before intraperitoneal injection. Acyclovir (Burroughs Wellcome Co., Research Triangle Park, N.C.) and ribavirin (Viratek, Palo Alto, Calif.) were also dissolved in PBS before use.

Mitogen response of splenocytes to ConA. A rapid colorimetric assay for cell proliferation was used to evaluate the response of splenocytes to stimulation by concanavalin A (ConA). The assay (19) is based on the ability of the dehydrogenases present in the mitochondria of living cells to cleave the tetrazolium ring of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. Briefly, 2×10^5 splenocytes were cultured for 48 h in the presence of 4 μg of ConA. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was then added, and the products of cleavage were determined colorimetrically on a Dynatech Microelisa reader at a wavelength of 570 nm.

Determination of LP-BM5 titers in spleen homogenates. Virus titers in the supernatants of 10% spleen homogenates were determined by a focal immunoassay based on the method of Chesebro and Wehrly (3) (personal communication). Briefly, SC-1 cells were seeded in 24-well tissue culture plates at a density of 4×10^4 to 5×10^4 per well. DEAE-dextran (10 μg/ml) in serum-free medium was added 18 h later, and the monolayers were incubated for an additional 30 min. After monolayers were washed with serum-free medium, they were infected with virus diluted in RPMI medium containing 0.1% fetal calf serum and incubated for 2 h. Complete medium containing 10% fetal calf serum was added to infected monolayers, and the cells were incubated at 37°C for 3 to 4 days. At the end of this incubation period, culture medium was removed, and the monolayers were incubated with undiluted culture supernatant from a hybridoma-secreting rat immunoglobulin G2a reactive against gp70 of murine leukemia viruses (hybridoma 83A25 kindly provided by Leonard Evans and Bruce Chesebro, Rocky Mountain Laboratory, National Institutes of Health, Hamilton, Mont.).

After incubation with the primary anti-gp70 hybridoma antibody, cell monolayers were washed with PBS–1% fetal calf serum and fixed with absolute methanol. Fixed monolayers were incubated with peroxidase-conjugated anti-rat immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) for 45 min at room temperature and washed three times with Tris-buffered saline (pH 7.5) containing 0.002 M EDTA. A solution containing aminoethylcarbazole and hydrogen peroxide was then added, and plates were held in the dark for 30 min (21). Cell monolayers were washed with water, air dried, and examined with a dissecting microscope. Foci were counted, and the results are expressed as focus-forming units per gram of spleen.

Superinfection with HSV-1 and treatment with antiviral agents. Mice at 60 to 80 days after LP-BM5 infection were infected with either HSV-1 strain McIntyre by intraperitoneal injection of 5×10^5 PFU per mouse or with HSV-1 strain VR3 by instilling 1×10^5 PFU into the external nares of ether-anesthetized mice. Antiviral agents (acyclovir, ribavirin, or PMEA) were administered intraperitoneally on the day of superinfection and on days 2, 4, and 6 after infection. Deaths were recorded, and the data were analyzed by Wilcoxon rank analysis (one-tailed test). Each treatment group consisted of at least 10 mice.

RESULTS

Therapeutic activity of rhuIFNalpha-B/D, PMEA, and AZT in the treatment of MAIDS: effects of early drug intervention. C57BL/6 mice infected with the LP-BM5 virus complex developed (i) pronounced splenomegaly, (ii) mitogen-unresponsive splenocytes, (iii) detectable levels of infectious virus in spleens, and (iv) susceptibility to superinfection with HSV-1. Although treatment with AZT or PMEA beginning on the day of virus infection and continuing for either 21 or 60 days could not prevent these changes from occurring, both drugs slowed the progression of immunosuppression and reduced susceptibility to HSV-1 superinfection (Table 1). Recombinant alpha B/D interferon did not have a demonstrable effect on the immunological parameters studied but did have a modest effect on susceptibility to HSV-1 superinfection.

Therapeutic activity of PMEA alone or in combination with interferon: effects on established disease. Treatment of LP-BM5 virus-infected mice with PMEA or AZT starting 23 days after infection did not result in a significant improvement in either the immunological or virological parameters studied (Table 2). Moreover, mice receiving drug therapy at this late stage of the disease were virtually as susceptible as untreated controls to experimental superinfection with HSV-1. In contrast, mice receiving both PMEA and rhuIFNalpha-B/D had reduced levels of infectious virus in their spleens and were markedly resistant to HSV-1 infection. Treatment with this combination had no effect on spleen enlargement or mitogenic responsiveness.

TABLE 1. Effect of continuous (days 0 to 59) or early (days 0 to 22) therapy with PMEA, AZT, or recombinant human interferon on the development of immunosuppression in LP-BM5 virus-infected mice

Group and treatment[a]	Mean spleen wt (mg) ± SD[b]		Splenocyte mitogen response[c]		Splenic virus titer[d]		HSV-1 challenge (% survivors) ($n \geq 9$)
	$t = 23$[e]	$t = 60$	$t = 23$	$t = 60$	$t = 23$	$t = 60$	
1. Immunocompetent	59 ± 21	79 ± 45	2.15	2.19			100
2. Immunosuppressed (no drug therapy)	198 ± 55	520 ± 87	1.60	1.04	1,130	8,120	8
3. AZT[f] (30 mg/kg daily), days 0 to 22	87 ± 38	383 ± 53	1.74	1.11	615	1,650	60 ($P \leq 0.05$)[g]
4. PMEA[h] (25 mg/kg daily)							
Days 0 to 22	59 ± 16	325 ± 9	1.93	1.14	2,390	7,680	80 ($P \leq 0.005$)[g]
Days 0 to 59	109 ± 13	195 ± 95	1.97	1.47	1,430	1,540	90 ($P \leq 0.005$)[g]
5. PMEA (5 mg/kg daily), days 0 to 22	141 ± 55	394 ± 81	1.79	1.02	1,300	4,150	50 ($P \leq 0.05$)[g]
6. rhuIFN[i] (5×10^7 IU/kg 3 times weekly), days 0 to 22	157 ± 61	444 ± 34	1.47	1.03	3,820	4,130	40

[a] Four-week-old C57BL/6 mice were placed in the six indicated groups (16 mice per group). Except for group 1, which was sham infected, all mice were infected (intraperitoneally) with LP-BM5 virus. Mice in group 2 received daily injections of PBS, and mice in groups 3 through 6 received the treatments indicated. All groups were assayed for spleen weight, splenocyte mitogen response, splenic virus titers, and susceptibility to superinfection with HSV-1.
[b] $n = 3$.
[c] ConA response (stimulation index) in pooled splenocytes; three mice per group.
[d] Focus-forming units per gram of spleen as measured by immunoperoxidase staining; pooled spleens of three mice.
[e] t, Time in days after infection; the last dose administered at least 24 h before assay.
[f] AZT was administered in drinking water; 30 mg/kg is an average dose based on the observation that on the average mice consume 3 ml of water per day.
[g] Compared with infected controls; Wilcoxon rank analysis using a one-tailed test.
[h] PMEA was dissolved in PBS and administered intraperitoneally.
[i] Recombinant human interferon alpha B/D hybrid was dissolved in PBS and administered subcutaneously.

Treatment of opportunistic HSV-1 infections in mice immunosuppressed by LP-BM5 virus infection. LP-BM5-immunosuppressed mice were susceptible to experimental infection with HSV-1 2 months after their initial retrovirus infection, whereas immunocompetent littermates were fully resistant.

Acyclovir (Fig. 1) and PMEA (Fig. 2) were equally effective in prolonging the mean survival time of immunosuppressed mice experimentally infected (intraperitoneally) with a neurovirulent strain of HSV-1 (McIntyre) that ultimately disseminated to the brain; however, ribavirin was inactive against this infection (Fig. 1). PMEA was also highly effective in the treatment of pulmonary HSV-1 infections (Fig. 3).

DISCUSSION

Few animal models exist that are useful for evaluating antiviral agents that may be effective in the treatment of human AIDS. Most of the animal models (primate, bovine, or feline) considered relevant are too expensive for routine drug evaluation and too difficult to handle and maintain. The MAIDS model offers the advantage of being inexpensive and easy to handle. The classical AIDS drug AZT was quite effective in controlling virus replication and immunosuppression when therapy was initiated on the day of LP-BM5 virus infection. In contrast, AZT was not effective when given to mice with established disease (23 days postinfection). Thus, our observations on the therapeutic effects of AZT in MAIDS are similar to those with AZT in human AIDS (4) and in other murine retroviral models (23).

Both in vitro and in vivo antiretroviral and antiherpesviral activities of PMEA have been reported (1, 5–7). The present study has used the MAIDS model to demonstrate that PMEA was as effective as AZT in delaying immunosuppression, as evidenced by the increased resistance to HSV-1 superinfection. Like AZT, PMEA was not effective in reversing or slowing the course of established disease.

We previously reported the synergistic response observed when rhuIFNalpha-B/D was used with AZT in the treatment of MAIDS (8). In this study, rhuIFNalpha-B/D used in combination with PMEA was more effective than either drug alone when administered late in the course of MAIDS. Although the mechanism by which rhuIFNalpha-B/D complements PMEA is not clear, it is quite likely that both drugs exert their virustatic effects at different points in the viral replicative pathway. Thus PMEA may interfere with the synthesis of viral nucleic acid, whereas interferon may

TABLE 2. Effect of delayed drug therapy with PMEA, AZT and/or recombinant human interferon on the development of immunosuppression in LP-BM5 virus-infected mice[a]

Group and treatment	Mean spleen wt (mg) ± SD		Splenocyte mitogen response		Splenic virus titer		HSV-1 challenge (% survivors) ($n \geq 9$)
	$t = 23$	$t = 60$	$t = 23$	$t = 60$	$t = 23$	$t = 60$	
1. Immunocompetent	59 ± 21	79 ± 45	2.15	2.19			100
2. Immunosuppressed (no drug therapy)	198 ± 55	520 ± 87	1.60	1.04	1,130	8,120	8
3. AZT (30 mg/kg daily), days 23 to 59	167 ± 35	490 ± 42	1.65	1.05	1,660	1,140	11
4. PMEA (25 mg/kg daily), days 23 to 59	236 ± 33	587 ± 111	1.58	0.97	2,400	4,090	20
5. rhuIFN (5×10^7 U/kg 3 times weekly), days 23 to 59	222 ± 20	602 ± 172	1.31	0.94	3,160	2,310	0
6. PMEA (25 mg/kg daily) + rhuIFN (5×10^7 U/kg 3 times weekly) days 23 to 59	169 ± 42	431 ± 147	1.72	1.04	3,750	456	70 ($P \leq 0.005$)

[a] See footnotes *a* through *i* of Table 1.

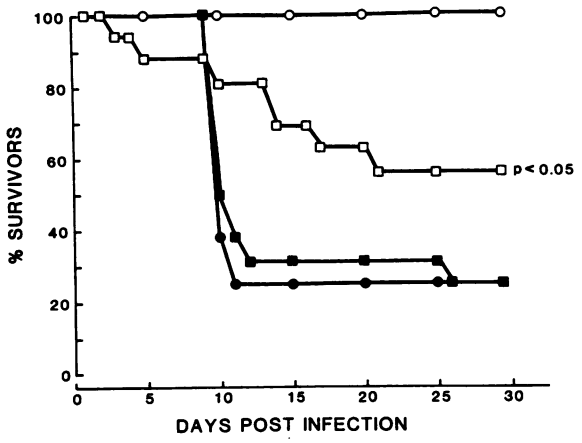

FIG. 1. Survival of LP-BM5 virus-infected mice after challenge (65 days after LP-BM5 virus infection) with a neurotropic strain of HSV-1 (McIntyre) and treatment with acyclovir or ribavirin. Mice were infected intraperitoneally (5×10^5 PFU) and treated with acyclovir (200 mg/kg; intraperitoneally) or ribavirin (500 mg/kg, intraperitoneally) beginning on the day of virus infection and again on days 2, 4, and 6 after infection. Symbols: ○, immunocompetent controls; ●, immunosuppressed controls (no drugs); ■, ribavirin-treated mice; □, acyclovir-treated mice. Statistics were evaluated (10 mice per group) by Wilcoxon rank analysis.

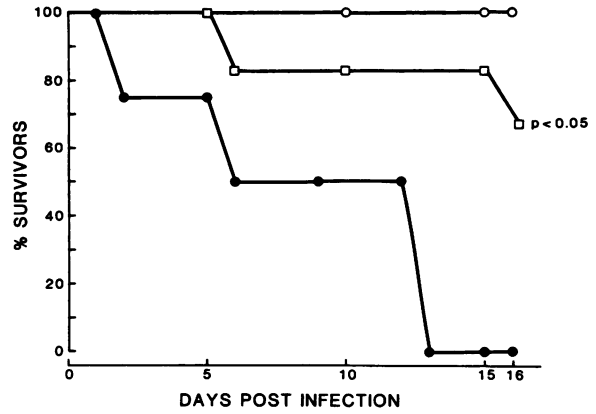

FIG. 3. Survival of LP-BM5 virus-infected mice after challenge (75 days after LP-BM5 virus infection) with a pneumonic strain (VR3) of HSV-1 and treatment with PMEA. Mice were infected intranasally with 10^5 PFU and treated with 100 mg of PMEA per kg beginning on the day of virus infection and again on days 2, 4, and 6 after infection. Symbols: ○, immunocompetent controls; ●, immunosuppressed controls (no drug); □, PMEA-treated mice. Statistics are evaluated (10 mice per group) by Wilcoxon rank analysis.

augment immune surveillance and induce the synthesis of cellular proteins that inhibit viral replication.

As a consequence of the marked immunosuppression caused by infection with LP-BM5 virus, mice develop susceptibility to experimental superinfection with HSV-1 (8) or ectromelia virus (2). This feature has allowed us to determine the efficacy of PMEA against opportunistic infections in immunocompromised mice. Both PMEA and acyclovir afforded mice a significant degree of protection to infection with the McIntyre strain of HSV-1, whereas ribavirin was inactive. PMEA was also effective against the VR3 strain. Thus PMEA has the unique advantage of being useful in the treatment of opportunistic HSV infections as well as the underlying retroviral cause of immunosuppression. This feature of PMEA may be of great value in view of the observation that herpesviruses may act as cofactors in the onset and progression of human AIDS (16).

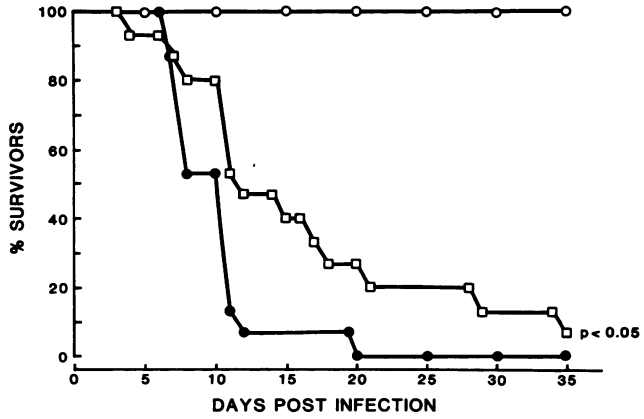

FIG. 2. Survival of LP-BM5 virus-infected mice after challenge (75 days after LP-BM5 virus infection) with HSV-1 strains McIntyre and treatment with PMEA. Mice were infected intraperitoneally (5×10^5 PFU) and treated with 100 mg of PMEA per kg beginning on the day of virus infection and again on days 2, 4, and 6 after infection. Symbols: ○, immunocompetent controls; ●, immunosuppressed controls (no drug); □, PMEA-treated mice. Statistics were evaluated (10 mice per group) by Wilcoxon rank analysis.

Although a number of disease parameters (e.g., spleen weight, mitogenic response of splenocytes, viral titers, and susceptibility to superinfection) have been used to monitor drug efficacy in the MAIDS model, it is difficult to conclude which parameter is the most relevant. For instance, we previously reported (8) that AZT treatment protects against HSV-1 superinfection but produces only a slight enhancement of mitogenic response of splenocytes, similar to that observed in the current study. The lack of an effect of AZT on mitogen responses may be due to the drug itself being toxic to lymphocyte subpopulations that are responsive to ConA. Alternatively, diminished ConA responses may be caused by virus-induced proteins not associated with infectious virus. Nonetheless, the fact that death in AIDS patients usually results from opportunistic infections suggests that resistance to superinfection may be the most important determinant of drug efficacy.

In conclusion, this study has demonstrated the value of the broad-spectrum antiviral agent PMEA on both early retroviral replication and opportunistic infections that appear late in the course of immunosuppression. Moreover, our data support the concept that the MAIDS model, in combination with experimentally induced bacterial or viral superinfection, provides a suitable alternative to drug evaluation in more expensive and less convenient animal models of retrovirus-induced immunosuppression.

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