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Suppression of feline immunodeficiency virus infection *in vivo* by 9-(2-phosphonomethoxyethyl)adenine

(acquired immunodeficiency syndrome/antiviral chemotherapy)

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ABSTRACT **The acyclic purine nucleoside analogue 9-(2-phosphonomethoxyethyl)adenine [PMEA; formerly referred to as 9-(2-phosphonylmethoxyethyl)adenine] is a potent and selective inhibitor of human immunodeficiency virus replication *in vitro* and of Moloney murine sarcoma virus-induced tumor formation in mice. In the latter system PMEA has stronger antiretroviral potency and selectivity than 3'-azido-3'-thymidine (AZT). We have now investigated the effect of the drug in cats infected with the feline immunodeficiency virus (FIV). *In vitro*, PMEA was found to efficiently block FIV replication in feline thymocytes (50% effective dose, 0.6 μM). When administered to cats at doses of 20, 5, or 2 mg/kg per day, PMEA caused a dose-dependent suppression of FIV replication and virus-specific antibody production. Seropositive field cats with signs of opportunistic infection (gingivitis, stomatitis, and diarrhea) showed clinical improvement during PMEA therapy (5 mg/kg per day) and recurrence of the disease after treatment was discontinued. Thus, FIV infection in cats is an excellent model to test the efficacy of selective anti-human immunodeficiency virus agents *in vivo*.**

Acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus (HIV), a retrovirus of the lentivirinae subfamily (1, 2). So far, only 3'-azido-3'-deoxythymidine (AZT; Zidovudine) has unambiguously demonstrated clinical benefit in the treatment of AIDS patients (3–5). Despite toxic side effects like megaloblastic anemia and evidence that AZT resistance is developing among strains of HIV-1, the drug is valuable since it has the capacity of extending the lives of AIDS patients (6). The development of AZT-resistant strains of HIV-1 is troubling; however, cross-resistance has been observed to only one other nucleoside, 3'-azido-2',3'-dideoxyuridine, which is closely related to AZT. Therefore, other efficacious antiretroviral drugs have to be developed; resistance can then be avoided or mitigated by alternating drug use. Many compounds that possess the same mode of action as AZT have been evaluated *in vitro*, and some compounds reported to be potent and selective inhibitors of HIV replication *in vitro* have passed phase II clinical trials.

We have found (7) that the acyclic adenosine derivative 9-(2-phosphonomethoxyethyl)adenine [PMEA; formerly referred to as 9-(2-phosphonylmethoxyethyl)adenine] inhibits HIV-induced cytopathogenicity in human T-lymphocyte MT-4, H9, and ATH8 cells. The effective concentration (1.6–2 μM) of the drug is far below the cytotoxic threshold for the host cells (40–67 μM) (7). PMEA also inhibits simian immunodeficiency virus replication in MT-4 cells, simian AIDS-related virus-induced giant cell formation in Raji cells, and transformation of murine C3H embryo fibroblasts by Moloney murine sarcoma virus (7–9). Over a wide range of doses (1–50 mg/kg per day) PMEA caused a 90–100% protection of mice against Moloney murine sarcoma virus-induced tumor formation and associated mortality, Friend leukemia virus-induced splenomegaly in BALB/c mice, and LP-BM5-induced immunosuppression and mortality in mice (8, 10–12).

Until recently, a major problem in evaluating antiretroviral compounds with proven activity against HIV *in vitro* was the lack of a natural immunosuppressive lentivirus infection model in animals. Pedersen *et al.* (13) have discovered a lentivirus, feline immunodeficiency virus (FIV), that causes a condition in cats that is very similar to AIDS in humans. FIV infection meets criteria of an animal model for AIDS; FIV is genetically similar to HIV (14–16) and causes a disease with a similar pathogenesis (13, 17). The reverse transcriptase (RT) of FIV is similar to that of HIV-1 in its sensitivity to several antiretroviral compounds, including AZT and phosphonoacetate (18). FIV replicates preferentially in T lymphocytes, macrophages, and neural cells (refs. 19–21; H.E., unpublished data). In cats the infection is characterized by an initial asymptomatic phase of several months or even years during which virus can be demonstrated (19). Subsequently, clinical signs may develop consisting of anorexia, weight loss, stomatitis, gingivitis, rhinitis, diarrhea, pustular dermatitis, anemia, and generalized lymphadenopathy (22, 23). Ultimately, the infected animals may die of opportunistic infections.

Feline leukemia virus infection has been used as a model for antiretroviral chemotherapy studies *in vitro* and *in vivo* (24, 25); however, the use of a lentivirus, rather than of an oncovirus, would be preferred as an animal model for AIDS; FIV fulfills this requirement.

In the present study we report that the anti-HIV drug PMEA interferes with the replication of FIV *in vitro* and *in vivo*; furthermore, PMEA treatment was found to cause clinical improvement of diseased cats in the field.

MATERIALS AND METHODS

Virus and Cells. The virus strains FIV-48 and FIV-113 were isolated from peripheral blood mononuclear cells of seropositive field cats with severe stomatitis and gingivitis. Virus stocks were prepared by the cocultivation method using feline thymocytes collected from specific pathogen-free cats

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Abbreviations: HIV, human immunodeficiency virus; FIV, feline immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; PMEA, 9-(2-phosphonomethoxyethyl)adenine; AZT, 3'-azido-3'-deoxythymidine; RT, reverse transcriptase; IFA, immunofluorescence assay.

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(Harlan, Zeist, The Netherlands) stimulated with concanavalin A at 5 μg/ml and cultured in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum supplemented with recombinant interleukin 2 (at 100 international units/ml). Virus yields were determined by assaying the RT activity in the culture supernatants. Crandell feline kidney cells (CRFK) persistently infected with the isolate FIV-113 were used for large-scale antigen production. The procedures to concentrate and purify virus have been described (26).

Antibody Assays. FIV-specific antibodies were assayed using an indirect immunofluorescence assay (IFA) on FIV-infected CRFK cells and a standard laboratory ELISA based upon sucrose-gradient purified virus as antigen source. The standard ELISA was as sensitive as the commercial FIV ELISA (Idexx, Portland, ME).

Inhibitory Effect of PMEA on FIV Replication in Feline Thymocytes. The anti-FIV activity of AZT and PMEA was determined in feline thymocytes. Mitogen-stimulated thymocytes were seeded at 1×10^6 cells per ml into 1.6-cm^2 wells of a 24-well tissue culture plate containing various concentrations of the test compounds. After a 1-hr incubation at 37°C, cells were infected with FIV (equivalent to 6×10^5 cpm of RT activity) for 60 min. Then the medium was replaced by 1 ml of fresh culture medium containing various concentrations of the compounds to be tested. After 4 and 6 days of culture, the RT activity in the culture supernatants and the number of viable cells (trypan blue exclusion) were determined in parallel for both mock- and FIV-infected cells. The 50% effective dose (ED_{50}) was defined as the concentration of compound that reduced the RT activity by 50%, whereas the 50% cytotoxic dose (CD_{50}) corresponded to the concentration of compound that reduced the number of viable mock-infected cells by 50%.

Inhibitory Effects of PMEA on FIV Replication in Cats. Two out of four cats per group were treated intramuscularly twice a day at 12-hr intervals with a dose of 1, 2.5, or 10 mg of PMEA per kg body weight for 35 days. PMEA was emulsified in 5% (wt/vol) glucose. One hour after the first drug administration both treated and control animals were subcutaneously and intraperitoneally infected with 1 ml of virus strain FIV-48 (equivalent to 2×10^6 cpm of RT activity). Peripheral blood lymphocytes were collected weekly and assayed for infectious virus by the cocultivation method. Seroconversion was determined by ELISA and by IFA on persistently infected CRFK cells. In addition, several hematological/biochemical parameters (liver enzymes, hematocrit, urea, and creatinine) and plasma PMEA levels were monitored weekly. Animals treated with PMEA at 5 mg/kg per day were immunized by intramuscular injection with an inactivated pseudorabies vaccine (Geskypur, Rhône Mérieux, Lyon, France) at day 0 and day 28 of the experiment. Serum samples were assayed for antibodies by IFA on pseudorabies virus-infected Ratec (rat embryonic) cells.

Six FIV-infected field cats (four European short hair and two Abyssinians), which suffered from a variety of opportunistic infections, were intramuscularly injected with PMEA at 2.5 mg/kg twice a day for 21 days.

Pharmacokinetics of PMEA. The pharmacokinetics of PMEA was determined in two 4-month-old kittens. Animals were injected intramuscularly with a single dose of either 5 or 10 mg of PMEA per kg of body weight emulsified in 5% glucose. The PMEA concentration in serum samples collected was determined by reverse-phase HPLC analysis (9).

Table 1. Inhibitory effects of PMEA and AZT on FIV replication in feline thymocytes and HIV-induced cytopathogenicity in MT-4 cells

	FIV-infected thymocytes		HIV-infected MT-4 cells	
Compound	ED_{50}, μM	CD_{50}, μM	ED_{50}, μM	CD_{50}, μM
PMEA	0.60	80	2.0	67
AZT	0.05	120	0.004	8

In FIV-infected thymocytes, ED_{50} was defined as the concentration of compound that reduced the RT activity by 50%. For both thymocytes and MT-4 cells (ref. 8) CD_{50} corresponded to the concentration of compound that reduced the number of viable mock-infected cells by 50%. In HIV-infected MT-4 cells, ED_{50} was defined as the concentration of compound that protected HIV-infected cells by 50%.

RESULTS

Anti-FIV Effect of PMEA *in Vitro*. Comparative assays of the anti-FIV activity of PMEA and AZT were performed *in vitro* by using feline thymocytes (Table 1). Both drugs inhibited FIV replication in thymocytes, the 50% effective dose (ED_{50}) of AZT being 0.05 μM, as compared to 0.60 μM for PMEA; these data are close to values reported for FIV in productively infected CRFK cells (18) and for HIV-infected

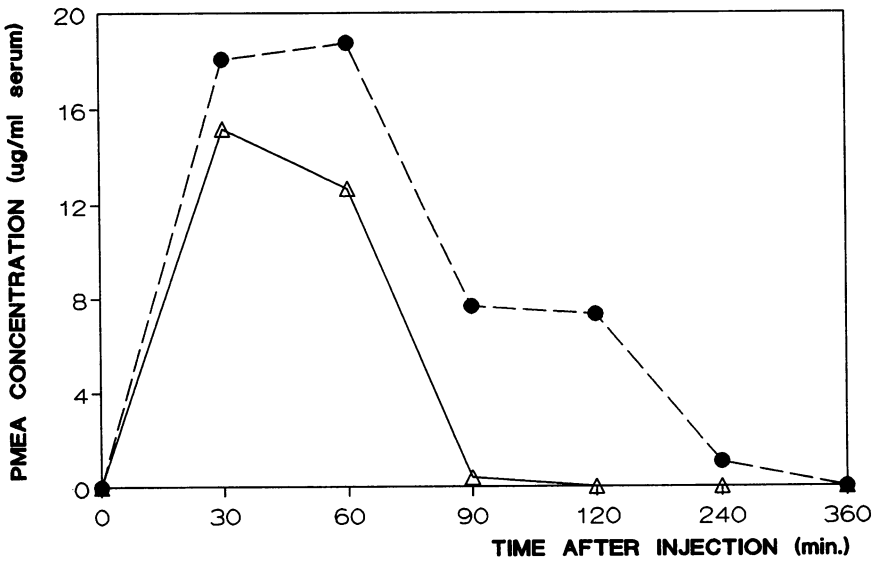

FIG. 1. Pharmacokinetics of PMEA determined in two 4-month-old kittens after intramuscular injection of a single dose of 5 mg/kg (△) or 10 mg/kg (●) of PMEA.

Table 2. Inhibitory effect of PMEA treatment on the replication of FIV in peripheral blood lymphocytes of cats experimentally infected wtih FIV

Exp.	Animal	PMEA dose, mg/kg per day	RT activity, cpm	Virus isolation	Sero-conversion
I	1	20	161	42	56
	2	20	65	56	70
	3	0	2,875	14	21
	4	0	12,907	14	21
II*	1	5	280	14	35 (21)
	2	5	10	14	42 (28)
	3	0	6,635	14	21 (21)
	4	0	1,369	14	21 (21)
III	1	2	83	14	42
	2	2	79	14	42
	3	0	135	14	21
	4	0	11,482	14	21

PMEA was administered intramuscularly twice a day at 12-hr intervals from 0 to 35 days after infection. RT activity was calculated per 10^5 viable peripheral blood lymphocytes isolated 35 days after the onset of the experiment, stimulated with concanavalin A, and cultured in the presence of human recombinant interleukin 2 (100 international units/ml) for 13 days. For virus isolation, data are the day after infection when lymphocytes were first found infected. Seroconversion was determined by IFA and ELISA and data are expressed as days after injection. The day that antibodies against pseudorabies virus were first detected is indicated in parentheses.
*Animals in experiment II were immunized with inactivated pseudorabies vaccine.

MT-4 cells (Table 1; ref. 8). The concentration of compound that reduced the number of viable mock-infected thymocytes by 50% (CD_{50}) was 120 μM for AZT and 80 μM for PMEA.

Pharmacokinetics of PMEA. The half-life of PMEA *in vivo* was determined after intramuscular injection of a single dose of 5 or 10 mg of PMEA per kg of body weight, respectively (Fig. 1). Maximum plasma levels were measured 30–60 min after injection, after which the plasma drug values decreased very rapidly, depending upon the dose injected. After administration of PMEA at a dose of 10 mg/kg, plasma drug values had declined to zero by 6 hr after infection. It was decided to study the effect of PMEA in FIV-infected cats after injection of the compound at 12-hr intervals.

Prophylactic Effect of PMEA on FIV Infection. Cats were treated with PMEA doses of 20, 5, or 2 mg/kg per day from day 0 until 35 days after infection. Blood samples were collected and assayed for the presence of virus, specific antibodies, several hematological/biochemical parameters (liver enzymes, hematocrit, urea, and creatinine), and plasma PMEA levels. From lymphocytes of cats treated with PMEA at 20 mg/kg for 35 days virus could not be isolated until 42 days after infection (Table 2). Release of FIV from the lymphocytes was measured based upon RT activity detected in the supernatant of the cells after a 13-day incubation period. Irrespective of the variation between the individual animals (experiment III, animals 3 and 4), suppression of RT activity at all PMEA dosages (20, 5, or 2 mg/kg per day) was observed (Table 2). Sera collected at weekly intervals were used to assess the effect of PMEA on the FIV-specific antibody response. PMEA treatment also delayed the FIV-specific antibody response whether it was used at a dose of 20, 5, or 2 mg/kg per day. This delay must be attributed to a suppressive effect of PMEA on FIV replication, since PMEA did not directly interfere with the immune system: upon vaccination with pseudorabies virus, specific antibodies became detectable in PMEA (5 mg/kg per day)-treated cats at about the same time as in the untreated animals. Unlike AZT, which causes severe anemia and leukopenia in human (6) and may cause signs of idiosyncratic hepatotoxicity in cats (25), PMEA did not affect the hematological parameters in cats except for a slight anemia at a dose of 20 mg/kg per day. This anemia disappeared after discontinuing PMEA therapy.

Therapeutic Effect of PMEA on FIV Infection. The therapeutic effect of PMEA was evaluated in experimentally infected asymptomatic cats and in seropositive diseased field cats. In experimentally infected asymptomatic cats treated with PMEA at a dose of 5 mg/kg per day, the release of virus from peripheral blood lymphocytes was again delayed whereas antibody titers were not affected at all doses used (2, 5, or 20 mg/kg per day) (data not shown). When compared to the untreated animals, a 50- to 230-fold reduction of RT activity was observed. The RT activity had been calculated per 1×10^5 viable peripheral blood lymphocytes isolated at day 21 after onset of the therapy; the cells had been stimulated with concanavalin A and maintained on interleukin 2 for 13 days.

The drug had a pronounced effect on the opportunistic infections accompanying FIV persistence in field cats when administered at a dose of 5 mg/kg per day. Five out of six treated cats recovered from a variety of severe symptoms (stomatitis, gingivitis, and diarrhea) or showed general clinical improvement (Table 3). Cat 5 with a history of stomatitis (Fig. 2*A*) that had not responded to prolonged corticosteroid treatment completely recovered when treated with PMEA (Fig. 2*B*). Recurrence of the symptoms was noted 2 months after the first therapy had been discontinued. The cat recovered from stomatitis and showed general clinical improvement when the same regimen (3 weeks) of PMEA treatment was applied. Symptoms reappeared 8 months after the sec-

Table 3. Effect of PMEA on the clinical signs of FIV-infected field cats

Cat	Age, years	Breed	Sex	Duration of therapy, weeks	Clinical sign(s)		
					Before PMEA treatment	Time	After PMEA treatment
1	13	ESH	NM	2	Lethargy/emaciation/diarrhea/dehydration	4 weeks	Improved activity/weight gain/feces normal (see text)
2	8	ESH	NM	2	Lethargy/emaciation/lameness	3 months	Improved activity/weight gain/ lameness disappeared
3	4	Abyss	F	3	Chronic diarrhea/emaciation/retardation	>1 year	Feces normal/weight gain
4	14	Abyss	NM	3	Emaciation/gingivitis/stomatitis	6 months	Weight gain/stomatitis disappeared
5	NK	ESH	NM	3	Upper respiratory symptoms/stomatitis (resistant to therapy with antibiotics, corticosteroids, and gestagens)	1 year	Complete recovery (see text and Fig. 2.)
6	1	Abyss	M	3	Emaciation/growth retardation/upper respiratory tract disease	6 months	No clinical improvement/ euthanasized

The column labeled time gives the duration of clinical signs prior to PMEA treatment. M, male; F, female; NM, neutered male; Abyss, abyssinian; ESH, European short hair; NK, not known.

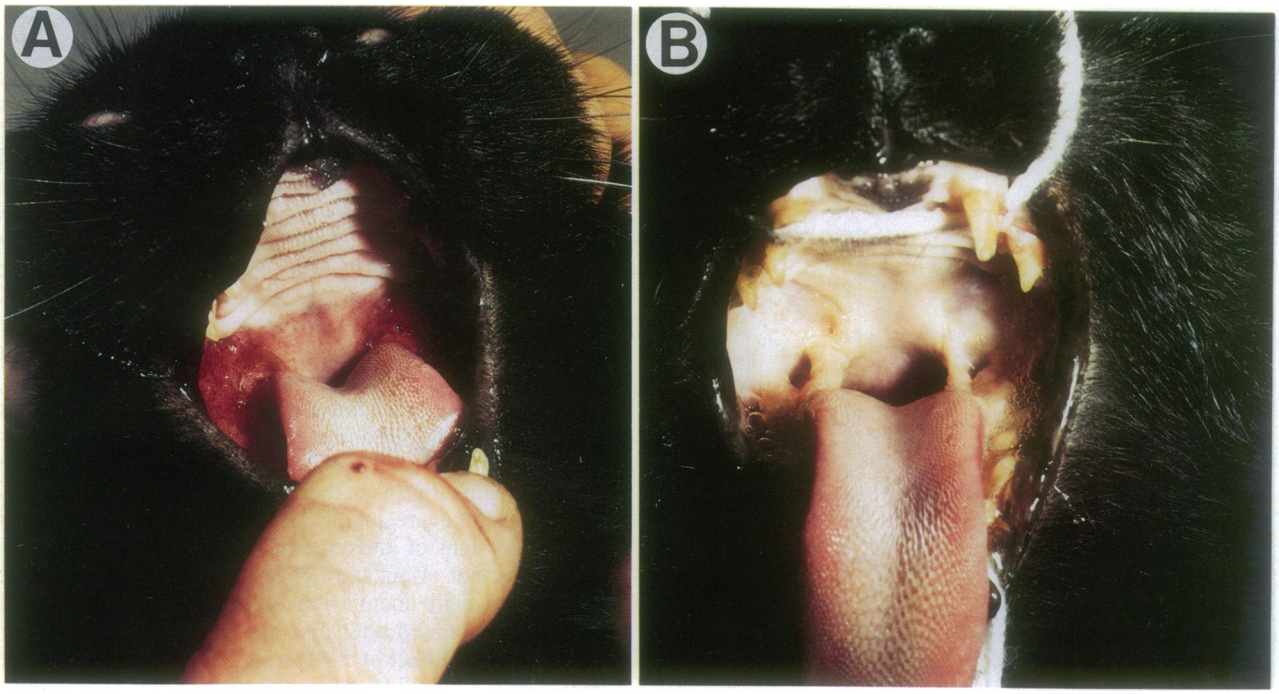

FIG. 2. Therapeutic effect of PMEA on opportunistic pharyngitis in a field FIV-infected cat. (*A*) Before treatment, inflammation and ulceration of the oropharynx. (*B*) After 21 days of treatment (5 mg/kg per day intramuscularly).

ond therapy had been discontinued and the cat again responded well to a third PMEA treatment (3 weeks).

Six weeks after the therapy had been discontinued cat 1 also showed recurrence of the symptoms (Table 3). The cat responded well to a second PMEA treatment. Four out of five cats are still healthy 9 months after termination of therapy (Table 3).

DISCUSSION

FIV infection of cats meets many criteria of an animal model for HIV infection of man; as demonstrated in this report, it can be used to study the efficacy of antiretroviral agents in the treatment of immunosuppressive lentivirus infections *in vivo*.

In the Moloney murine sarcoma virus infection model, PMEA has a stronger *in vivo* antiretrovirus potency and selectivity than several other compounds including AZT (8, 9). Here, we demonstrate that PMEA has proved at least as efficacious as AZT in suppressing FIV infection *in vitro* (18); it also had a marked effect on FIV-associated symptoms in cats. Adverse reactions (e.g., megaloblastic anemia), described after AZT treatment of humans (6), were observed for PMEA only at high doses (20 mg/kg per day). The question whether PMEA treatment leads to drug-resistant strains of FIV has still to be addressed; in our patients, the drug still had a pronounced effect after the second recurrence of symptoms in cat 5.

PMEA also inhibits herpes simplex virus infections *in vivo* (12, 27). Feline caliciviruses and feline herpesvirus are associated with oropharyngeal disease in cats (28). The oropharyngeal symptoms of chronic infections are observed in about 50% of FIV cats in North America (19) and Japan (29). Feline herpesvirus occurs in between 25% and 80% of healthy cats as a latent infection (30) and its activation by a immunosuppression has been established (31). It would therefore appear that the clinical improvement seen after PMEA treatment has been due to a combined effect of inhibiting FIV replication with its immunosuppressing consequences as well as feline herpesvirus. The dual antiviral activity of PMEA may broaden its therapeutic usefulness in controlling the opportunistic infections; this has been seen in FIV-infected field cats and may prove valuable in the human AIDS pathogenesis.

Previous studies have shown that radiolabeled PMEA, besides accumulating in the liver and kidneys, crosses the blood-brain barrier (8, 27). This latter property is important in view of the propensity of lentiviruses, in particular HIV and FIV, to infect and damage the central nervous system.

In conclusion, we report an immunosuppressive natural lentivirus infection that can be influenced by chemotherapy. The compound PMEA deserves further investigation as a drug for treatment of HIV infections in humans; FIV infection in cats can be considered as a useful and readily available model for *in vivo* screening of antilentivirus compounds.

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