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# 9-(2-Phosphonylmethoxyethyl) adenine increases the survival of influenza virus-infected mice by an enhancement of the immune system

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**Abstract**

PMEA (9-(2-phosphonylmethoxyethyl)adenine) is a potent inhibitor of DNA viruses and retroviruses able to enhance natural immune functions such as natural killer cell activity and interferon production. The results reported in this paper show that the treatment with PMEA significatively decreased the mortality of mice challenged with influenza A/PR8 virus (an RNA virus, non sensitive to the antiviral effect of PMEA) compared to untreated, infected controls (median survival 8.64 days and 7.61 days, respectively), and reduced lung weight and consolidation (two surrogate markers of virus infection). Furthermore, virus titer obtained from lung homogenates was substantially decreased in PMEA-treated mice compared to controls. Finally, enhancement of natural killer cell activity was achieved in PMEA-treated A/PR8-infected mice compared to A/PR8-infected controls. Overall, results suggest that PMEA decreases the influenza virus-related mortality and morbidity through the enhancement of some immune functions, and that this effect might be additive or even synergystic with the direct inhibitory effect of DNA viruses or retroviruses induced by PMEA itself. This supports the importance of evaluating this drug in patients with diseases related to herpesviruses or to human immunodeficiency virus.

*Keywords:* A/PR/8/34 influenza virus; PMEA; Immunomodulation; Natural killer

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## 1. Introduction

PMEA (9-(2-phosphonylmethoxyethyl)adenine) is a prototype of a new class of compounds named acyclic nucleoside phosponates (ANPs), shown to be potent inhibitors of the replication of DNA viruses (i.e., herpesviruses) and retroviruses (Balzarini et al., 1991c; De Clercq et al., 1986; De Clercq et al., 1991). Inhibition of the replication of human immunodeficiency virus (HIV) in macrophages can be achieved at PMEA-concentrations > 1000-fold lower than those that are toxic for the cells (Balzarini et al., 1991b). Furthermore, at non toxic concentrations PMEA potently inhibits both the replication of animal retroviruses and the therewith associated diseases in various in vivo experimental models (Balzarini et al., 1989; Balzarini et al., 1990a; Balzarini et al., 1990b; Egberink et al., 1990) PMEA blocks retrovirus replication by inhibiting either the DNA-dependent or the RNA-dependent-DNA polymerase activity, and terminating the formation of the viral DNA chain (Balzarini et al., 1991a; Foster et al., 1991; Holý et al., 1990).

In addition to its direct antiviral activity, PMEA (and a number of other ANP derivatives) also enhances some parameters of natural immunity, like natural killer (NK) cell activity and interferon (IFN) production (Caliò et al., 1994; Del Gobbo et al., 1991) which is important in the host's defense against infection by most viruses, including HIV (Bonagura et al., 1992; Mansour et al., 1990). This immunomodulating activity suggests that the in vivo antiviral efficacy of PMEA may be at least partially achieved by enhancing the immune response against the virus challenge. This hypothesis, however, is difficult to prove, due to the direct antiviral activity of PMEA that may mask immuno-mediated antiviral activity.

Therefore, we have used an in vivo mouse model based on influenza A/PR8/34 virus infection. This virus causes a profound immunosuppression (mainly characterized by the impairment of natural and cellular-mediated immunity) and lethal pneumonitis (Ada and Jones, 1986; Del Gobbo et al., 1990; Hashimoto et al., 1983). RNA viruses are completely insensitive to the direct antiviral effect of PMEA. Thus it is conceivable that any delay of virus-induced mortality and/or reduction of virus replication be related to an indirect, immuno-mediated, antiviral effect of PMEA in the influenza A/PR8/34 virus model.

## 2. Materials and methods

A/PR/8/34 influenza virus ($H_1N_1$) (hereinafter called A/PR8) was grown in the allantoic cavity of 10-day-old embryonated eggs. Forty eight hours later, the allantoic fluid was harvested, titrated by the hemagglutination test using chicken red blood cells, and stored at $-70°$ until used.

PMEA was synthesized by Dr. A. Holý and Dr. I. Rosenberg (1987) as previously described, and was dissolved in a sterile apyrogenic saline solution just before use. The in vitro testing of the antiviral activity of PMEA against A/PR8 virus was performed by using the MDCK (Madin-Barby canine kidney) cell line (sensitive to the cytopathic effect of influenza virus). MDCK cells were challenged with $10^3$ cytopathic effect

(CPE) 50% units/ml of A/PR8 influenza virus in the presence of various concentrations of PMEA (given either 1 h before, or at the time of virus challenge). The A/PR8-induced cytopathic effect was assessed by crystal violet dye staining followed by spectrophotometrical measurement.

In vivo studies were performed by using 4–5 weeks old C57Bl/6 mice (Charles River, Calco, CO). Each mouse was inoculated intranasally (i.n.) under light ether anaesthesia with 50 $\mu$l of A/PR8 virus suspension containing $5 \times 10^3$ CPE 50% units/ml, a virus dose sufficient to induce a profound immunosuppression and a massive lethal effect. Treatment with PMEA was started 2 days before virus challenge, and continued with an alternating daily schedule until the end of the experiment. A dose of 25 mg/kg/day of PMEA was chosen on the basis of previous experiments, showing that such concentration consistently induces the greatest enhancement of NK cell function and the production of $\alpha/\beta$ IFN (Caliò et al., 1994; Del Gobbo et al., 1991). Control mice were injected either with nothing or with the same diluent of PMEA, and identical results were obtained. Survival was assessed daily in both PMEA-treated and untreated groups of mice (70 mice in each group). Mice surviving for more than 24 days after virus challenge were considered as being cured.

For the assessment of additional markers of virus infection, 30 mice for each group (other than the 70 mice for groups used for the assessement of survival) were sacrificed at 3 and 5 days after infection (15 at each time point) and the weight and consolidation (inflammation/cell infiltration) of the lungs were measured. Lung scores were expressed as the percentage of the lung displaying signs of consolidation, with 0 being normal and 4 indicative for the 100% consolidation. As an additional parameter, the virus titer in the lungs was also assessed. To this end, lungs were homogenized, centrifuged, and the supernatants were titrated in MDCK cell cultures as described above.

NK cytotoxic activity was assayed by the method of Herberman et al. (1975) and modified by Del Gobbo et al. (1990). Briefly, spleen cells from controls, virus-infected and PMEA-treated, virus-infected mice (effector cells) were incubated for 4 h at 37°C with [$^{51}$Cr]YAC-1 cell (target cells). All tests were performed using four effector: target cell ratios (from 100 : 1 and below). The specific $^{51}$Cr-release was calculated according to a previously described method by Del Gobbo et al., 1990.

Statistical analysis was performed by the Student's *t*-test. The survival data were analyzed using the algorithm of Lee and Desu (Lee and Desu, 1972). The overall accepted level of significance was 0.05.

## 3. Results

In a first series of in vitro experiments, we consistently found that PMEA is completely inactive against the influenza A/PR8-induced cytopathic effect in MDCK cells at concentrations (40 $\mu$M) up to those that were toxic for most cells including lymphocytes, and far above those achievable upon in vivo treatment (Table 1). This is in agreement with data previously reported on the lack of antiviral activity of PMEA against various RNA viruses other than PR8 (De Clercq et al., 1986).

Table 1
Lack of antiviral activity of PMEA against A/PR8 virus

| %Cytopathic effect | | |
|---|---|---|
| PMEA concentrations ($\mu$M) | PMEA added 1 h before PR8 infection | PMEA and PR8 added together |
| 40 | 100 | 97 |
| 4 | 97 | 100 |
| 0.4 | 98 | 95 |

The antiviral activity of PMEA was measured by assessing the virus-induced cytopathic effect (CPE) 50% U/ml in MDCK cell cultures as described in Section 2.

Based on these data, we then evaluated the effect of PMEA treatment on A/PR8 virus-infected C57Bl/6 mice. As shown in Fig. 1, 25 mg/kg PMEA induced a statistically significant increase of the median survival, from 7.61 days in the untreated A/PR8-infected mice, to 8.64 days for the PMEA-treated, A/PR8-infected mice ($P = 0.0053$). The number of cured mice was also substantially greater in the PMEA-treated group than in the untreated group (15.7% vs. 5.7%) (Fig. 1).

Figs. 2 and 3 summarize the data related to the weight and the consolidation of the lungs, two surrogate markers for the in situ replication of the influenza virus. As shown in Fig. 2, lung weight 3 days after virus challenge was substantially increased in A/PR8-infected mice compared to uninfected controls, yet the treatment with PMEA

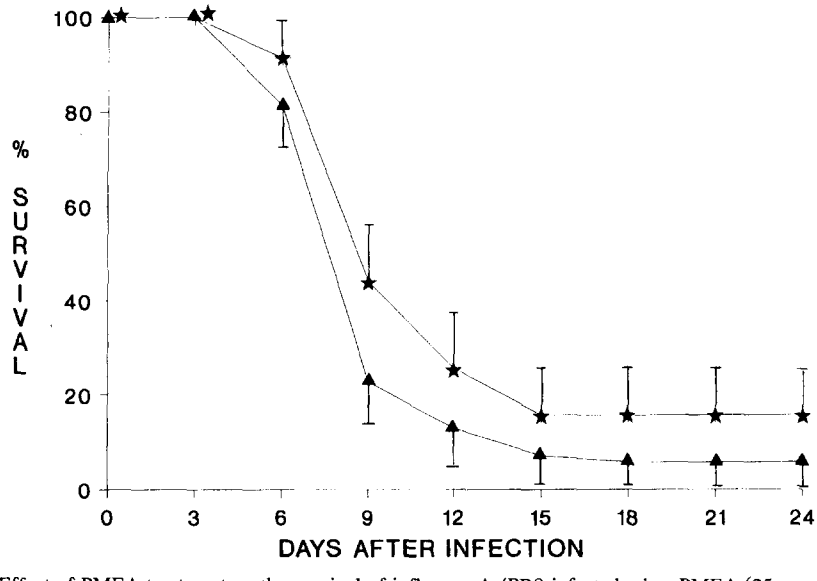


Fig. 1. Effect of PMEA treatment on the survival of influenza A/PR8-infected mice. PMEA (25 mg/kg) was administered intraperitoneally at 2 days before virus infection ($5 \times 10^3$ CPE 50% U/ml). Thus, treatment continued every other day. ▲, untreated A/PR8-infected mice; ★, PMEA-treated A/PR8-infected mice. Statistical difference between the two curves (algorithm of Lee and Desu): $P = 0.0053$.

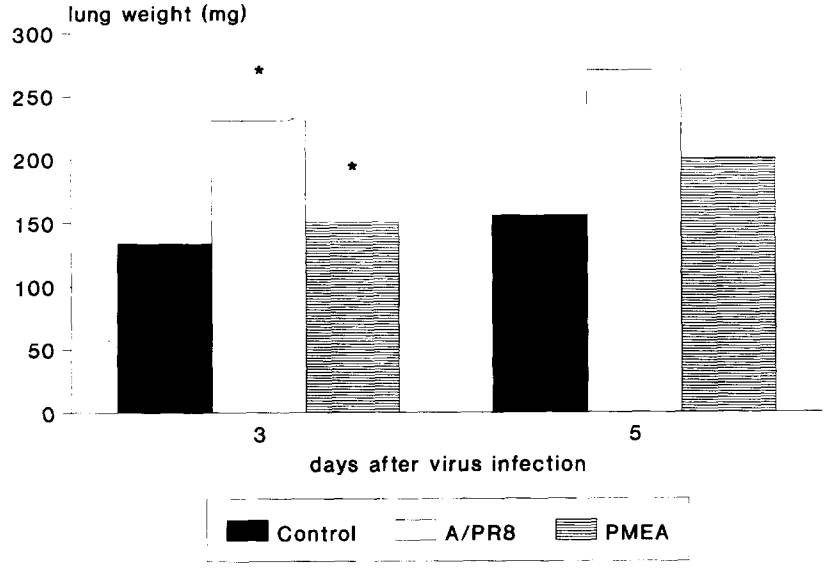


Fig. 2. Weight increase of lungs in A/PR8-infected mice treated or not with 25 mg/kg PMEA. Infection and PMEA treatment were done as described in the legend of Fig. 1 and in Section 2. Black bars, untreated uninfected mice; white bars, untreated A/PR8-infected mice; striped bars, PMEA-treated A/PR8-infected mice. Statistical difference between the two bars (*) (algorithm of Lee and Desu): $P < 0.01$.

decreased lung weight virtually to the levels of uninfected controls (infected untreated A/PR8 infected mice $235 \pm 30$ mg; infected, PMEA-treated A/PR8 infected mice $150 \pm 20$ mg; uninfected mice $130 \pm 20$ mg; $P < 0.01$ between PMEA-treated and untreated infected mice). This difference was still detectable at 5 days after virus challenge, even though no longer statistically significant. Similarly, lung consolidation 3 days after virus challenge was significantly reduced in A/PR8-infected PMEA-treated mice compared to the untreated infected mice (13% and 40%, respectively, $P < 0.02$). Again, this difference was clearly evident but not statistically significant 5 days after virus challenge (Fig. 3). As an additional control, treatment of uninfected mice with PMEA did not affect the weight or the consolidation of the lungs (data not shown). Thus, the treatment with PMEA improves two surrogate markers of the influenza A virus infection, such as weight and consolidation of the lungs, which are typically related to the inflammation and infiltration of the lungs caused by virus replication.

As a confirmatory test, we assessed whether the increased survival rate of PMEA-treated mice was related to a concomitant decrease of virus load in the lungs. Indeed, the virus titer 3 days after infection is clearly reduced in the lungs of PMEA-treated, A/PR8 infected mice compared with that found in untreated A/PR8-infected mice ($0.75 \pm 0.05$ vs. $1.28 \pm 0.09$, CPE 50% U/lung, respectively). This difference was still evident, although not statistically significant, 5 days after virus infection (data not shown).

Since PMEA has no direct antiviral effect upon influenza A/PR8 virus infection, the results suggest that the decrease of virus titer, and the consequent reduction of lung damage, be related to an indirect effect of PMEA upon virus replication, i.e., a

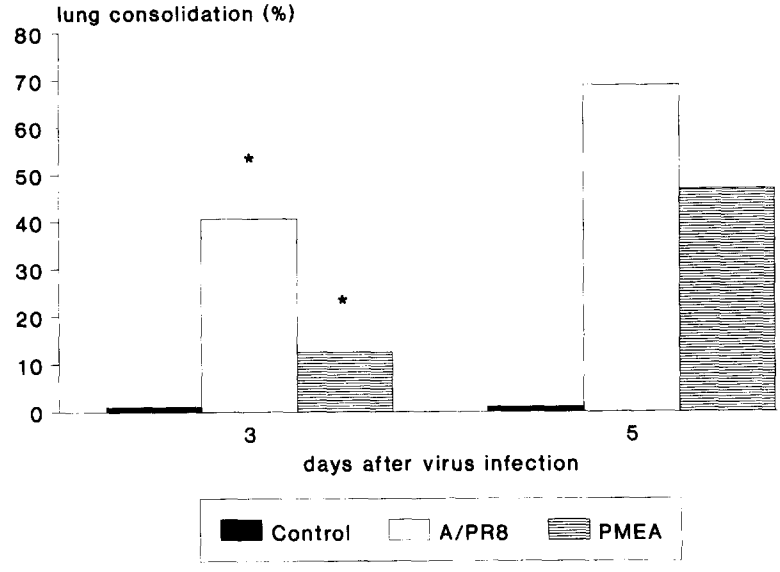


Fig. 3. Lung consolidations in A/PR8-infected mice treated or not with 25 mg/kg PMEA. Infection and PMEA treatment were done as described in the legend of Fig. 1 and in Section 2. Black bars, untreated uninfected mice; white bars, untreated A/PR8-infected mice; striped bars, PMEA-treated A/PR8-infected mice. Statistical difference between the two bars (*) (algorithm of Lee and Desu): $P < 0.02$.

modulating effect on natural immunity. Indeed, as shown in Fig. 4, PMEA-treated mice have a sustained increase of NK cell activity at least up to 7 days after virus challenge, while in agreement with previous data of our group (Del Gobbo et al., 1990), the NK cell function dramatically decreases overtime in untreated mice challenged with A/PR8 virus ($33.97 \pm 6.3\%$ vs. $20.55 \pm 2.2\%$ of specific lysis in PMEA-treated A/PR8-infected mice and untreated A/PR8-infected mice, respectively).

## 4. Discussion

The data reported in this paper show that the enhancement of the immune system induced by PMEA is able to improve the course of infection induced by an RNA virus totally insensitive to the antiviral effect of this drug. The effect of PMEA upon some surrogate markers of infection is statistically significant only in the first days after infection, yet is sufficient to delay virus-related mortality, and to increase the percentage of cured mice. Although there is no straightforward evidence, overall data suggest the hypothesis that this immunomodulatory effect of PMEA be additive, or perhaps synergistic, to its direct inhibitory activity upon the replication of DNA viruses and retroviruses. This might give a selective therapeutic advantage to this compound over most currently used antiviral drugs which are often toxic to the bone marrow or to the immune system (Chen and Cheng, 1989; Heagy et al., 1991; Phillips et al., 1991; Shaw et al., 1991; Zhu et al., 1991).

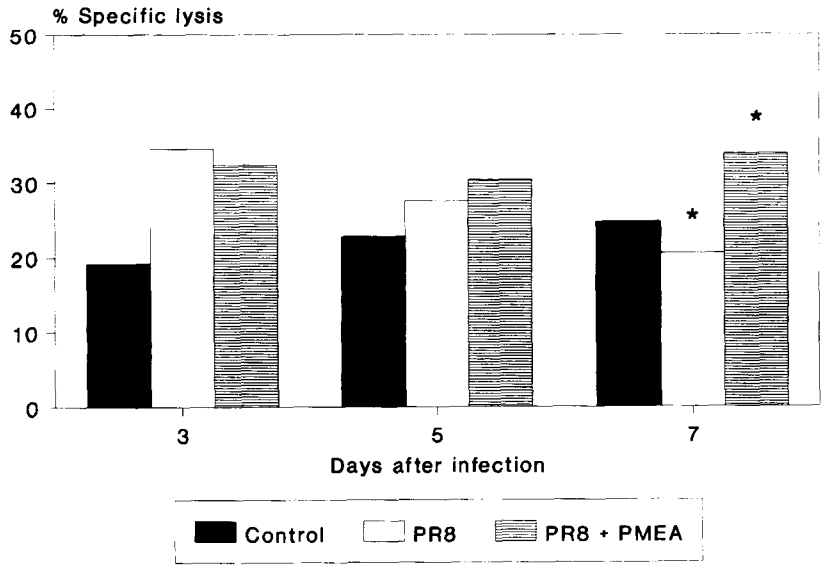


Fig. 4. Effect of PMEA treatment on NK cell cytotoxic activity of influenza A/PR8 infected mice. Infection and PMEA treatment were done as described in the legend of Fig. 1 and in Section 2. Effector target ratio: 100:1. Black bars, control mice; white bars, untreated A/PR8-infected mice; striped bars, PMEA-treated A/PR8-infected mice. Asterisks indicate the statistical difference between the two bars (Student's *t*-test): $P = 0.003$.

The mechanism(s) underlying the inhibition of influenza A/PR8 virus replication by PMEA are not completely understood. Previous experiments have shown that PMEA stimulates the production of $\alpha/\beta$ interferon in treated mice (Del Gobbo et al., 1991). In order to assess this point, we performed additional experiments in which mice infected with A/PR8 influenza virus were continuously treated with doses of interferon able to maintain serum concentrations of this cytokine similar to those induced by PMEA (i.e., about 1000 U/ml). The results show that both average survival and number of cured mice in the group treated with interferon were intermediate between those obtained in infected mice treated or untreated with PMEA. Similarly, lung weight and consolidation, as well as virus titer in the lungs, were intermediate between PMEA-treated and untreated mice (Del Gobbo et al., unpublished data). These results suggest that the PMEA-associated induction of interferon may play a role in the inhibition of virus infection, yet other factors, perhaps related to the activation of a cytokine network, may be at least as important.

Regarding the sustained enhancement of natural killer activity induced by PMEA in infected mice, whether this effect is one of the causes of virus inhibition in our animal model, or is just an effect of the interferon production induced by PMEA, remains to be elucidated. Nevertheless, two recent papers describe the ability of two immunomodulating substances to inhibit virus-induced mortality by enhancing the functions of macrophages and natural killer cells (Ikeda et al., 1993a; Ikeda et al., 1993b). It is then conceivable that PMEA may act in a similar fashion by enhancing the activity of natural

killer cells (and perhaps other cells) and thus inducing the inhibition of virus infection. Further studies are required to address this point.

Taken together, overall data suggest that the characteristics of PMEA (direct antiviral effect, immunomodulatory activity, long intracellular half-life, and long-lasting antiviral efficacy) are such that the compound deserves clinical evaluation in patients with a variety of virus infections, and in particular in AIDS patients with intercurrent virus infections.

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