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Immunomodulatory activity of 9-(2-phosphonylmethoxyethyl)adenine (PMEA), a potent anti-HIV nucleotide analogue, on in vivo murine models

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Summary

In order to evaluate the influence of antiviral nucleoside analogues upon the natural immune system, we investigated the immunomodulatory activity of 9-(2-phosphonylmethoxyethyl)adenine (PMEA), a nucleotide analogue with potent anti-HIV and anti-herpes activity, in a murine system. C57BL/6 mice were inoculated intraperitoneally with 10, 25 and 50 mg PMEA/kg. Mononuclear cells were isolated from their spleens, and some natural immune functions were evaluated. The results show that PMEA significantly increases the levels of natural killer (NK)-cell cytotoxicity. We also found that α/β IFN production was substantially increased in PMEA-treated mice, while both IL-1 and IL-2 production was decreased. Thus, PMEA can increase some natural immunity functions, such as NK activity and IFN production. These results suggest that PMEA might be active in vivo against HIV and herpes viruses both as an immunomodulator and as an antiviral compound.

Nucleotide analog; 9-(2-Phosphonylmethoxyethyl)adenine; NK cytotoxicity; Interferon; Interleukin-1; Interleukin-2

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Introduction

An optimal approach toward the therapy of virus-induced diseases requires drugs potentially able to disrupt viral replication and to enhance host innate immune responses (Masek, 1989). In fact, it is very well known that most viruses could down-modulate some immune functions (Bukowski et al., 1983; Tonietti et al., 1983; Del Gobbo et al., 1990). Moreover, the disruption of the immune system is a peculiar feature of infection by human immunodeficiency virus, the causative agent of AIDS (Yarchoan and Broder, 1989). Finally, the restoration of natural immune response could be of some importance in immunosuppressed patients (Herberman and Ortaldo, 1981; Welsh, 1986), and is one of the goals of immunochemotherapy. Recently, it has been reported that some phosphonated adenine derivatives have an inhibitory effect on a wide spectrum of DNA viruses including herpesviruses (De Clercq et al., 1986; De Clercq, 1989). Besides this antiviral activity, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), a prototype of such purine derivatives, has also shown a marked activity against HIV and other retroviruses (De Clercq et al., 1988; Pauwels et al., 1988; Balzarini et al., 1989,1990). In contrast, little information is now available on the modulation of host natural and reactive immune responses induced by these and other antiviral compounds (Masek, 1989; Smee et al., 1990). Since there is evidence that nucleoside analogues can modulate some immune functions (Nagahara et al., 1990), we undertook an investigation to assess the immune modulatory activity of PMEA in natural immune systems. Our data suggest that PMEA induces interferon (IFN) production as well as enhancement of natural killer- (NK) cell activity in a murine model.

Materials and Methods

Mice

C57BL/6 mice, four to five weeks old, provided by Charles River (Calco, Co, Italy), were used in these studies.

PMEA administration

PMEA, 9-(2-phosphonylmethoxyethyl)adenine was synthesized by Dr. A. Holý and Dr. I. Rosenberg, as described previously (Holý and Rosenberg, 1987) and dissolved in a saline solution. For NK activity, PMEA was injected intraperitoneally (i.p.) in C57BL/6 mice at a dose of 10, 25 or 50 mg/kg once daily for five consecutive days. For the kinetics (NK, IFN, IL-1 and IL2), six different groups of mice were inoculated i.p. once daily with 25 mg PMEA/kg for 1, 2, 3, 4, 5 or 6 consecutive days.

Preparation of spleen cell suspension

At various time-intervals, control and treated mice were sacrificed by cervical dislocation and spleens were removed. The cell suspension was obtained from individual spleens by passage through a sterile syringe and by subsequent filtering through a Nytex mesh. Cells were washed twice and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM), 100 units penicillin/ml and 100 μg streptomycin/ml (all reagents from Flow Laboratories, Irvine, Ayrshire, U.K.). The cell viability, as determined by trypan blue exclusion, was >95%.

NK cytotoxicity assay

The NK cell activity was determined by a standard ^{51}Cr assay (Herberman et al., 1975). To recount briefly, YAC-1 cells, a Moloney leukemia virus-induced mouse T-cell lymphoma, used as target cells, were labeled with ^{51}Na chromate (New England Nuclear, Boston, MA) for 1 h at 37°C, washed and resuspended at 1×10^5 cells/ml. Individual spleen cells from control and PMEA-treated mice were used as effector cells. Effector and target cells (cell ratios 100:1, 50:1 and 25:1) were placed into U-shaped 96-well microtiter plates (Flow Laboratories) in a total volume of 0.2 ml. After 4 h incubation at 37°C, the specific ^{51}Cr release (counts per minute, cpm) was calculated as follows:

$$\text{specific } ^{51}\text{Cr release\%} = \frac{\text{test cpm–baseline cpm}}{\text{total cpm incorporated–baseline cpm}} \times 100$$

Tests were run in quadruplicate, and baseline release never exceeded 10% of total counts incorporated by target cells.

Interferon titration and interferon neutralization assay

Control and PMEA-treated mice were bled 24 h after the last injection, and IFN-levels were assessed by cytopathic effect reduction (Van Damme et al., 1987). To recount briefly, mouse serum samples from control and PMEA-treated mice, two-fold serially diluted, were incubated in 96-well microplates with murine L929 fibroblasts at 37°C. After 1 day, an optimal infectious dose of vesicular stomatitis virus (VSV) was added to each well; after 24 h, virus-induced cytopathic effect (CPE) was assessed with crystal violet dye.

This assay was calibrated with standard mouse α/β IFN (Sigma, St. Louis, MO, USA) international units (IU) of IFN were referred to a standard preparation of mouse α-β IFN. IFN titers were expressed as the maximum dilution of samples which inhibits CPE by 50%. All samples were assayed in duplicate at least three times. The type of IFN produced was determined by a neutralization assay (Hoss et al., 1989). The neutralization assays were performed by mixing test serum samples from control and PMEA-treated

mice with polyclonal rabbit anti-mouse α/β IFN antibodies (Calbiochem, La Jolla, CA, USA) for 2 h at 4°C. The antibody concentration used was optimal for neutralization assay. After a 2-h incubation at 4°C, the IFN activity in test samples with/without anti-IFN Ab was assayed on monolayers of L-929 cells, as described above.

Interleukin-2 assay

The production of IL-2 was induced by stimulating, 'in vitro', individual spleen cells (1×10^7/ml in 24-well plates) from control and PMEA-treated mice with 2 μg/ml Con A and 10 ng/ml phorbol myristic acetate (PMA; Sigma, St. Louis, MO, USA). Con A and PMA concentrations were optimal for maximum IL-2 production. After 48 h incubation at 37°C, the supernatant was collected and stored at −20°C until titration.

IL-2 titration assay was based upon the method of Gillis et al. (1978). In brief, the culture supernatants, 2-fold serially diluted from 1:2 to 1:256, were added into 96-well microtiter plates containing a cell suspension of an IL-2 dependent cytotoxic T-lymphocyte line (CTLL; 1×10^5/ml). All assays were performed in triplicate and incubated for 24 h at 37°C. Positive and negative controls were run in each experiment, according to the method. After 20 h incubation, 1 μCi of [^{3}H]thymidine (^{3}H-TdR, specific activity 86 μCi/ml; Amersham International, Amersham, Bucks, U.K.) was added and 4 h later cells were harvested by a semiautomatic cell harvester. The radioactivity (cpm) was measured in a β-counter. IL-2 activity was calculated as [^{3}H]thymidine incorporation by the IL-2-dependent CTLL clone, and the number of units was expressed as the reciprocal of supernatant dilution producing 50% maximal ^{3}H-TdR incorporation with reference to a standardized IL-2 preparation. The number of units were calculated using the computer program previously described by Sette et al. (1986).

Interleukin-1 assay

IL-1 assay was performed according to the method of Krakauer et al. (1982). In brief, macrophages from spleen cells of control and PMEA-treated mice were separated by adherence on plastic dishes for 1 h at 37°C, followed by removal of non-adherent cells. Macrophages were then detached by vigorous washing followed by gentle scraping, counted, and plated in 24-well plates, 1×10^6 cells/well in medium with the addition of 20 μg/ml LPS (F. Coli 0111:B4, Sigma, St. Louis, MO, USA). After 48 h, macrophage supernatants were harvested and serially diluted in 96-well plates containing 1×10^6 thymocytes from 1–2-month-old C3H/HeJ mice and 3 μg PHA/ml (Sigma). All assays were performed in triplicate and incubated for three days at 37°C. Eighteen h before harvesting, thymocytes were pulsed with 0.5 μCi [^{3}H]thymidine/well, then harvested and counted as described above ([^{3}H]thymidine, specific activity 86 μCi/ml). IL-1 activity was calculated as [^{3}H]thymidine incorporation by the

thymocytes, and the number of units was expressed as the reciprocal of supernatant dilution producing 50% maximal ^{3}H-TdR incorporation with reference to a standardized IL-1 preparation (IL-1 alfa, Boehringer-Mannheim, F.R.G.). The calculation of unit number was performed by a computer program (Sette et al., 1986).

Results

Effects of PMEA treatment on the NK cell activity

C57BL/6 mice were inoculated i.p. once daily with 9-(2-phosphonyl-methoxy-ethyl)adenine (PMEA) at 10, 25, 50 mg/kg for five consecutive days (see Material and Methods). Twenty-four h after the last injection, spleens were removed and assayed for NK cell activity. Our data show that PMEA administration induces a substantial increase in natural killer (NK)-cell activity of all groups of mice treated, compared with controls (Fig. 1). The greatest enhancement of NK-cell activity is observed with PMEA at 25 mg/kg, and is similar to that obtained with poly I:C (Sigma), an inducer of endogenous production of interferon, and with mouse α/β IFN itself. This concentration of PMEA indeed increases the NK cell cytotoxicity about 2-fold compared with the untreated control mice ($P<0.001$). In order to study the kinetics of NK-cell activation, we chose the most effective PMEA dose (25 mg/kg). For this purpose, six different groups of C57BL/6 mice were inoculated i.p. once daily with 25 mg/kg for 1, 2, 3, 4, 5 or 6 consecutive days; 24 h after the last injection in each group we assayed their spleen cells

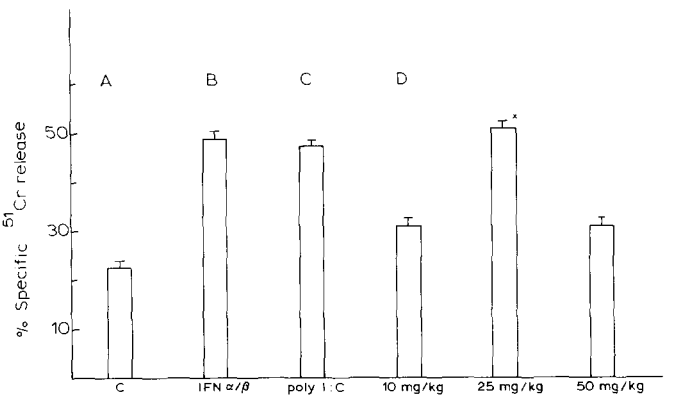

Fig. 1. NK cell cytolytic activity in groups of C57BL/6 mice. A: control; B: inoculated i.p. 24 h before the assay with 30 000 U/ml of mouse IFN α/β; C: inoculated i.p. 24 h before the assay with poly I:C at 5 mg/kg; D: inoculated i.p. once daily with PMEA at 10, 25 and 50 mg/kg for 5 consecutive days. The NK cell cytolytic activity is expressed as % specific ^{51}Cr release at effector: target-cell ratio of 100:1. The results express the means of individual spleen cell NK activity of ten mice for each experimental group. Each experiment was repeated three times. *Statistical analysis was performed with Student's t-test ($P<0.001$).

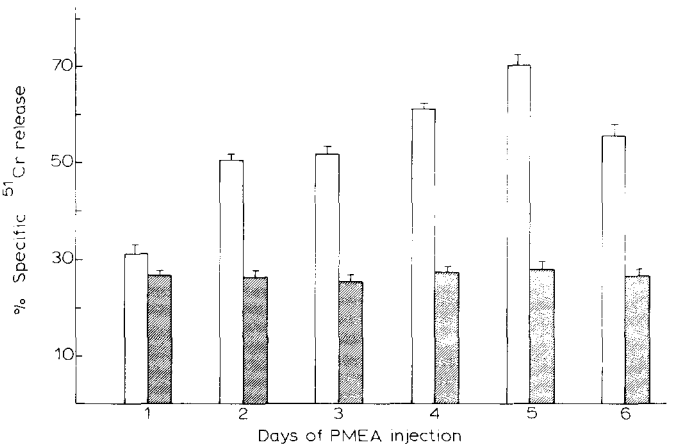

Fig. 2. Kinetics of NK cell cytolytic activity in six different groups of C57BL/6 mice inoculated i.p. once daily with PMEA at 25 mg/kg for 1, 2, 3, 4, 5 or 6 consecutive days. Twenty-four h after the last injection, the control and each group of PMEA-treated mice were killed and individual spleen NK cell activity was tested. Open bars, PMEA-treated mice; shaded bars, controls. The NK cell cytolytic activity is expressed as % specific ^{51}Cr release at effector: target-cell ratio of 100:1. The results express the means of individual spleen NK cell activity of ten mice for each experimental group. Each experiment was repeated three times. Statistical analysis was performed with Student's t-test ($P<0.05$ in all groups starting from day 2).

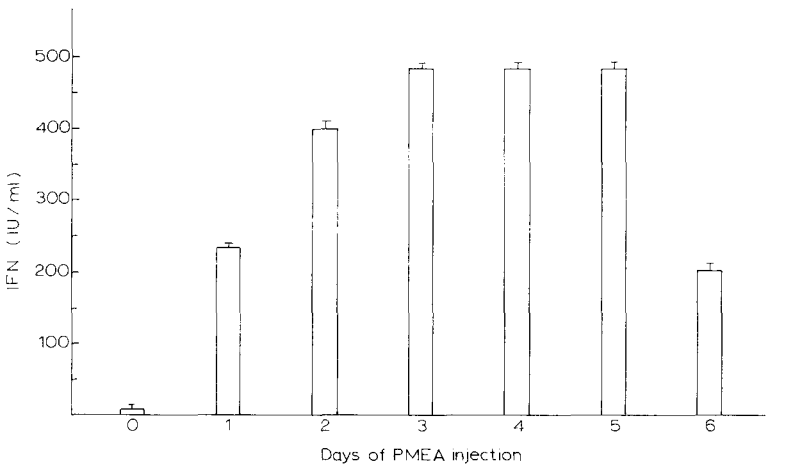

Fig. 3. Kinetics of interferon (IFN) induction in serum of six different groups of C57BL/6 mice treated once daily with PMEA at 25 mg/kg for 1, 2, 3, 4, 5 or 6 consecutive days. Twenty-four h after the last injection, sera from the control and each group of PMEA-treated mice were assayed by reduction of cytopathic effect. IFN titer is expressed in IU/ml referred to mouse α/β IFN standard preparation (see Materials and Methods). Each experiment was performed in duplicate at least three times. In agreement with the literature, no IFN activity was detected in serum samples from control mice at day 0 or at any following day.

for NK cell activity. As shown in Fig. 2, a single administration of PMEA does not significantly induce a modification of NK activity tested 24 h after the injection. Conversely, a substantial increase of such activity has been found in all groups treated with PMEA for at least two consecutive days.

Induction of IFN production by PMEA treatment

Since it is well known that IFN is a major inducer of NK activity (Djeu et al., 1973; Herberman et al., 1979), we considered the possibility that the enhancement of NK-cell cytotoxicity observed in PMEA-treated mice may be due to the induction of IFN production. In order to examine this hypothesis, six different groups of mice inoculated i.p. once daily with 25 mg/kg PMEA for 1, 2, 3, 4, 5 or 6 consecutive days were bled 24 h after the last injection, and IFN levels were evaluated by the inhibition of a cytopathic effect induced by VSV (see Material and Methods). Figure 3 shows the kinetics of IFN production of mice treated with PMEA. As early as 24 h after one injection, PMEA was able to induce IFN production in mouse serum (240 IU/ml). The maximum IFN induction (480 IU/ml) occurred after the third injection, and its titer declined to 200 IU/ml after the sixth. Thus, these results suggest that PMEA administration is active both in promoting NK-cell activity, and in inducing the production of IFN. The reduction of IFN production after six consecutive administrations of PMEA could be explained by the hyporesponsivity often seen with other IFN inducers. Moreover, our data do not exclude the possibility that the enhancement of IFN production is caused by a late effect of

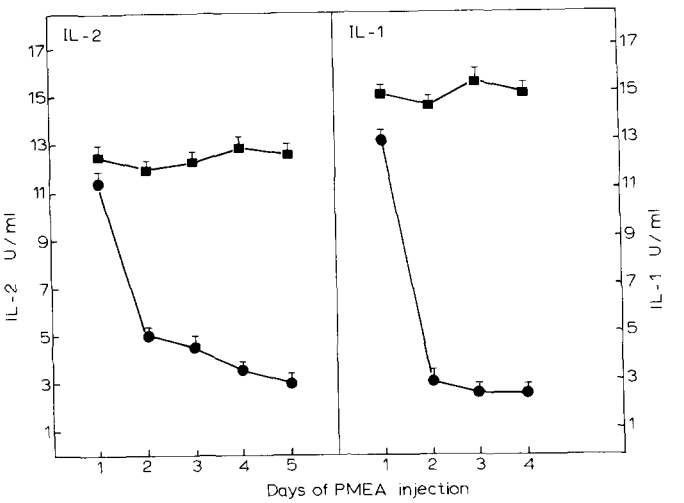

Fig. 4. Kinetics of IL-2 and IL-1 activity in different groups of C57BL/6 mice inoculated once daily with PMEA at 25 mg/kg for five and four consecutive days, respectively. IL-2 and IL-1 production was assayed according to the probit analysis method of Sette et al. (1986). IL-2 and IL-1 activity from control (■) and PMEA-treated mice (●) is expressed in U/ml. Each experiment was repeated three times. Statistical analysis was performed with Student's *t*-test ($P<0.05$ in all groups, starting from day 2)

the first (and second) administration, rather than being related to the continuous administration of PMEA.

Experiments were then performed to determine the type of IFN produced. Serum samples from PMEA-treated mice were incubated with polyclonal anti-mouse α/β IFN antibodies for 2 h at 4°C, or control medium and residual IFN titers were assayed by CPE assay (see Materials and Methods). Rabbit anti-mouse α/β IFN immunoglobulins significantly reduce the IFN activity of serum samples from PMEA-treated mice from 480 IU/ml to 100 IU/ml. Thus, our data suggest the greatest part of IFN produced in PMEA-treated mice is α/β.

Effect of PMEA on IL-1 and IL-2 production

To assess the spectrum of immunomodulatory activity of PMEA, we evaluated the production of IL-1 and IL-2 in mice treated with 25 mg PMEA/kg. As shown in Fig. 4, a substantial decline of both IL-2 and IL-1 was detected in spleen cells from PMEA-treated mice compared to controls. This reduction was found starting after two injections of PMEA, and was still evident up to five days of continuous PMEA administration. Thus, at least in our hands, the immunomodulatory activity of PMEA seems to be restricted to the natural killer activity and to IFN production, the latter being a potential inducer of NK activity.

Discussion

PMEA is an adenine derivative that shows potent antiviral activity against HIV and most herpesviruses (De Clercq et al., 1986; Pauwels et al., 1988; De Clercq, 1989). For this reason, PMEA is a good candidate for clinical trials in patients with HIV infection. Our study aimed to assess whether PMEA can modulate some immune responses such as natural immunity that are depressed in HIV-infected patients (Yarchoan and Broder, 1989). The results indicate that PMEA injection in C57BL/6 mice induces a significant enhancement of NK-cell activity and the production of α/β-interferon, while it substantially reduces the production of IL-1, and IL-2.

PMEA is most active in promoting NK-cell activity at 25 mg/kg. This concentration is able to increase NK-function more than two-fold. Moreover, this concentration of PMEA induces an optimal IFN production. In fact the results in Figs. 2 and 3 show that the kinetics of NK activity appear to be correlated with the highest titers of IFN produced in groups of PMEA-treated mice.

The enhancement of NK-cell activity is a stepwise process controlled by a series of lymphokines released by lymphoid cells. The role of IFNs and IL-2 in this process is rather well defined (Kabelizt et al., 1985; Djeu et al., 1979; Herberman et al., 1979). Furthermore, data from several authors describe a direct role of some nucleoside analogues in IFN induction and in natural killer-

cell activation in virus-infected animals (Skulnick et al., 1985; Richard et al., 1987; Smee et al., 1989, 1990; Nagahara et al., 1990). In the case of PMEA, we found a substantial increase of α/β IFN production after 24 h treatment. It should be noted that such a time-interval between stimulation and IFN production is somewhat longer than that described for other drugs in different experimental conditions (i.e., virus infection; Smee et al., 1990). In fact, no IFN production had been detected 2, 3 and 6 h after PMEA treatment; similarly, no further increase of IFN production had been achieved 24 h after a single administration of high doses of PMEA (50–100 mg/kg). More experiments are then required to further investigate the fine mechanisms related to this phenomenon. Nevertheless, IFN production after PMEA administration is a reproducible event under our experimental conditions. The production of IL-1 and IL-2, two other cytokines related to cellular immunity, is substantially suppressed by PMEA. Thus, in the absence of other experimental evidence, and in agreement with results obtained by others (Skulnick et al., 1985, Smee et al., 1990), our data strongly suggest that enhancement of NK activity is related to the production of α/β but not gamma-IFN, production of the latter being usually up-modulated by IL-2 (Farrar et al., 1981; Handa et al., 1983; Giovarelli et al., 1988; Landolfo et al., 1988).

Taking all data into consideration, PMEA could be able to affect viral replication also through an indirect mechanism mediated by natural immunity. This can lead to a potential advantage of PMEA toward other antiviral drugs, such a azidothymidine, that are somewhat immunosuppressive in a long-term treatment (Yarchoan et al., 1986; Yarchoan and Broder, 1987). Further studies are now required to better define the role that PMEA can have in the in vivo treatment of viral diseases, such as those related to HIV infection, that directly affect the cells of the immune system and lead to profound immunosuppression.

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