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Enhancement of natural killer activity and interferon induction by different acyclic nucleoside phosphonates

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Summary

Acyclic nucleoside phosphonate (ANP) analogues are a class of compounds with potent activity against herpesviruses and/or retroviruses. Our preliminary experiments have shown that 9-(2-phosphonylmethoxyethyl)adenine (PMEA), a prototype of the ANP family, enhances some parameters of natural immunity. In this paper we have evaluated the effect of different schedules of administration of PMEA and other ANP analogues of clinical interest upon natural killer (NK) activity and interferon (IFN) production in a mouse model. The results show that PMEA significantly enhances NK activity and interferon production. Other ANP analogues tested in our system, i.e., 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP), and 9-(3-fluoro-2-phosphonylmethoxypropyl)adenine (FPMPA), similarly induced enhancement of natural immunity. The immunomodulating effect of PMEA was even more pronounced with a single administration compared to repeated administrations of the drug. Dose-dependent enhancement of NK activity and IFN production could also be demonstrated during chronic administration of PMEA (more resembling to what will be the schedule of administration of this drug in patients). Overall, the data here presented suggest that the enhancement of some natural immune functions induced by ANP analogues may add to the direct antiviral activity of these drugs against retroviruses and herpesviruses,

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and thus may be able to increase the host resistance against viral infections.

PMEA; Natural killer; Interferon; Natural immunity; Antivirals

Introduction

A rational approach to the chemotherapy of human immunodeficiency virus (HIV) infection must be based upon the development of drugs that selectively inhibit virus infection without causing further immunosuppressive effects to the host (Masek et al., 1989). Based on these criteria, various compounds capable of inhibiting HIV replication in vitro have been found. However, only the 2′-3′-dideoxynucleoside analogues, such as 3′-azido-2′,3′-dideoxythymidine (AZT) 2′,3′-dideoxyinosine (DDI) and 2′,3′-dideoxycytidine (DDC), have shown an appreciable antiviral activity in HIV-infected patients, yet causing toxicity to various organs, including the immune system (Inoue et al., 1989; Eriksson and Schinazi, 1989; Spector et al., 1989; Yarchoan et al., 1989a; Yarchoan et al., 1989b). The toxicities have limited the maximum tolerable dose of these drugs, and thus presumably decreased their overall clinical efficacy.

Recent studies have reported that a new class of compounds, named acyclic nucleoside phosphonates (ANP) analogues, are potent inhibitors of both herpes and retrovirus replication in vitro. One of the leading compounds of this class, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), has shown marked activity against retroviruses (including HIV) and DNA viruses in various cellular systems at concentrations far below those that are cytotoxic (De Clercq et al., 1987; Balzarini et al., 1989; Balzarini et al., 1990a; Balzarini et al., 1991a; Balzarini et al., 1993). Further research has also shown its potent antiviral efficacy in animal models (Gangemi et al., 1989; Balzarini et al., 1990a; Balzarini et al., 1990b; Balzarini et al., 1991d; Egberink et al., 1990), thus suggesting that PMEA and other ANP analogues are good candidates for clinical trials in patients infected by HIV and/or herpesviruses.

Preliminary studies of our group have recently shown that PMEA administration enhances some parameters of the natural immunity in murine models, while other compounds, including AZT, fail to do so or are even immunosuppressive. In particular, we have observed that five consecutive PMEA injections at concentrations non-toxic and known to be active against murine retrovirus infections induce a significant enhancement of NK (natural killer) cell cytotoxicity, together with a relevant increase of interferon (IFN) production (Del Gobbo et al., 1991).

In the present study we extend such preliminary observations by evaluating the immunomodulatory effect of acute and long-term PMEA treatment in vivo, and the immunomodulatory activity of two other ANP analogues drugs, such as (*R,S*)-9-(3-fluoro-2-phosphonylmethoxypropyl)adenine (FPMPA) and 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP), already shown to

be potent inhibitors of virus replication (Naesens et al., 1989; Balzarini et al., 1991b; Balzarini et al., 1991c; Naesens et al., 1993).

Materials and Methods

Mice. C57Bl/6 male mice, 4 to 5 week old, provided by Charles River (Calco, Italy), were used in these studies.

Drug administration. PMEA, PMEDAP and FPMPA were synthesized by Dr. A. Holy' and Dr. I. Rosenberg, as previously described (Holy' and Rosenberg, 1987). All compounds, tested for purity by HPLC analysis, were dissolved in a sterile apyrogenic saline solution just before use. For long-term treatment, PMEA was injected intraperitoneally (i.p.) in two different groups of C57Bl/6 mice (20 mice for each group) at a dose of 5 or 25 mg/kg once daily for 20 consecutive days. For short-term treatment, various groups of mice were inoculated i.p. with a single dose of PMEA, FPMPA or PMEDAP. For PMEA the doses used were 25, 50 and 100 mg/kg; for FPMPA 10, 25, 50, 100 and 200 mg/kg; and for PMEDAP 10, 25, 50 and 100 mg/kg. Control mice were injected with saline at the same time of the drugs.

NK cytotoxicity assay. NK cytotoxic activity was determined by the method of Herberman et al., 1975, modified by Del Gobbo et al., 1990. Briefly, [^{51}Cr]YAC-1 cells (used as a target at 10^4 cells-well in U-bottomed microtiter plates) were incubated for 4 h with the effector cells obtained from spleen cell suspensions from control- and PMEA-treated mice. The preparation of spleen cells was performed according to a previously described method (Del Gobbo et al., 1991). All assays were performed using four effector:target cell ratios (from 100:1 and below). After 4-h incubation at 37°C, the specific ^{51}Cr-release was calculated as follows: specific ^{51}Cr-release % = test cpm − baseline cpm/ total cpm incorporated − baseline cpm × 100.

Baseline ^{51}Cr-release of NK tests, all run in quadruplicate, never exceeded 10% of total counts incorporated by target cells.

Interferon titration. IFN activity in mouse serum samples was determined by cytopathic effect (CPE) reduction method, using L929 cells and vesicular stomatitis virus (VSV), as previously described by Del Gobbo et al., 1991. Briefly, L929 cells were added to microplates containing serial 2-fold dilution of serum samples from control- and PMEA-treated mice, and incubated at 37°C for 24 h. An optimal infectious dose of VSV was then added to each well. After 1 additional day, the cytopathic effect induced by the virus (CPE) was assessed with crystal violet dye. These assays were calibrated with a standard preparation of mouse IFN alpha/beta (Sigma, St. Louis, MO, USA) and IFN titers were expressed in international units (IU). All samples were assayed in quadruplicate for at least three times. It should be noted that preliminary

experiments have shown that PMEA has no activity whatsoever upon the replication of the VSV.

IFN neutralization and characterization assays. The type of IFN produced was assayed by a neutralization assay previously described (Del Gobbo et al., 1991). Serum samples from control- and PMEA-treated mice were mixed with optimal dose of polyclonal rabbit anti-mouse IFN alpha/beta antibodies (Calbiochem, La Jolla, CA, USA) for 3-h at 4°C. After 2-h incubation at 4°C, the residual IFN activity in samples with/without anti-IFN antibodies was assayed as described above.

IFN characterization was carried out by adjusting the acidity of serum samples to pH 2 by 1 N HCL. Samples were then incubated overnight at 4°C, neutralized with 1 M NaOH, and tested for the residual IFN activity. All experiments were performed in quadruplicate at least three times.

Statistical analysis. Statistical analysis was performed by Student's *t*-test.

Results

In a first set of experiments we assessed the modulation of natural immune functions upon infrequent administration of PMEA. Fig. 1a shows that a single dose of 25 mg/kg PMEA results in a more than 3-fold increase of NK cell activity starting from 48 h after injection. PMEA-induced NK activity peaks at day 4, and returns to baseline at about day 8. Such enhancement is even more pronounced than that achieved with 5 consecutive daily injections of the same dose of PMEA (at most 2-fold enhancement of NK activity) (Fig. 1b). Toxicity of PMEA given as a single dose was not found in these experiments even at 50 and 100 mg/kg (measured in terms of death or decrease in body weight) (data not shown), thus suggesting that the immunostimulating effect of PMEA is achievable at concentrations far below those that are toxic.

Our previous experiments suggest that IFN production is somehow correlated to the NK enhancement mediated by PMEA. Thus, we measured the serum levels of IFN following PMEA administration. As shown in Fig. 2a, a substantial enhancement of IFN levels in sera was achieved from 24 h after administration of a single dose of PMEA (25 mg/kg). The peak of IFN production, achieved at day 4 after drug administration, was 825 units/ml, and was followed by a decline of such titer down to the baseline at day 7–8 after drug administration. As expected, no detectable IFN production was found in control mice. This suggests that the IFN production detected in PMEA-treated mice is linked to the administration of PMEA. Again, the amount of IFN produced in response to a single administration of PMEA was more pronounced than that obtained with 5 consecutive administrations of PMEA, which afforded levels of 500–600 U/ml (Fig. 2b). However, this difference was not statistically significant.

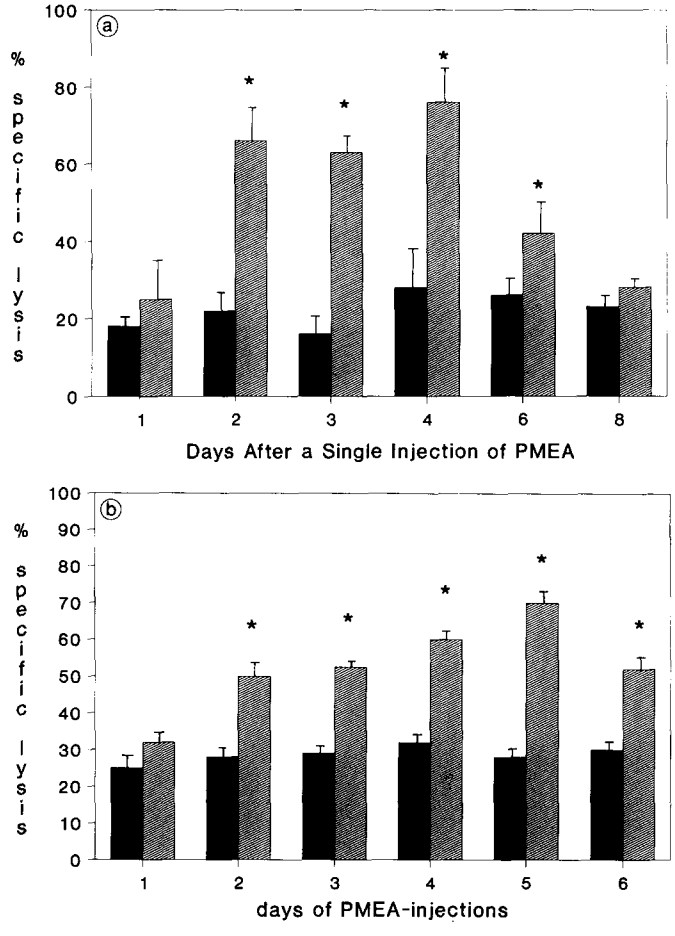

Fig. 1. NK cell activity in mice exposed to various schedules of i.p. administration of PMEA. Fig 1a: one single injection of 25 mg/kg PMEA; Fig. 1b: once daily injections of 25 mg/kg PMEA for 1 to 6 consecutive days. NK activity was evaluated at (1a) various days after the single injection or (1b) 24 h after the last injection. For this purpose, PMEA-treated and control mice were sacrificed and individual spleen NK cell activity was tested. The NK cell cytolytic activity is expressed as % specific ^{51}Cr-release at effector: target cell ratio of 100:1. The results represent the means of individual spleen NK cell activity of ten mice for each experimental group. Data were from a representative experiment out of three consecutive experiments. Black bars, control mice; slashed bars, PMEA-treated mice. Asterisks indicate the results that are significantly different from the corresponding control values, as calculated, using the Student's *t*-test, at $P < 0.001$.

Tests aimed at the characterization of the type of IFN induced by PMEA were then performed. Serum samples from PMEA-treated mice were incubated with polyclonal anti-IFNα/β antibody, and then tested for their biological activity as described in Materials and Methods. The antibody to IFNα/β significantly reduced the IFN activity of serum samples from PMEA-treated mice to about 20% of the original levels. Indeed, IFN titer of serum samples from PMEA-

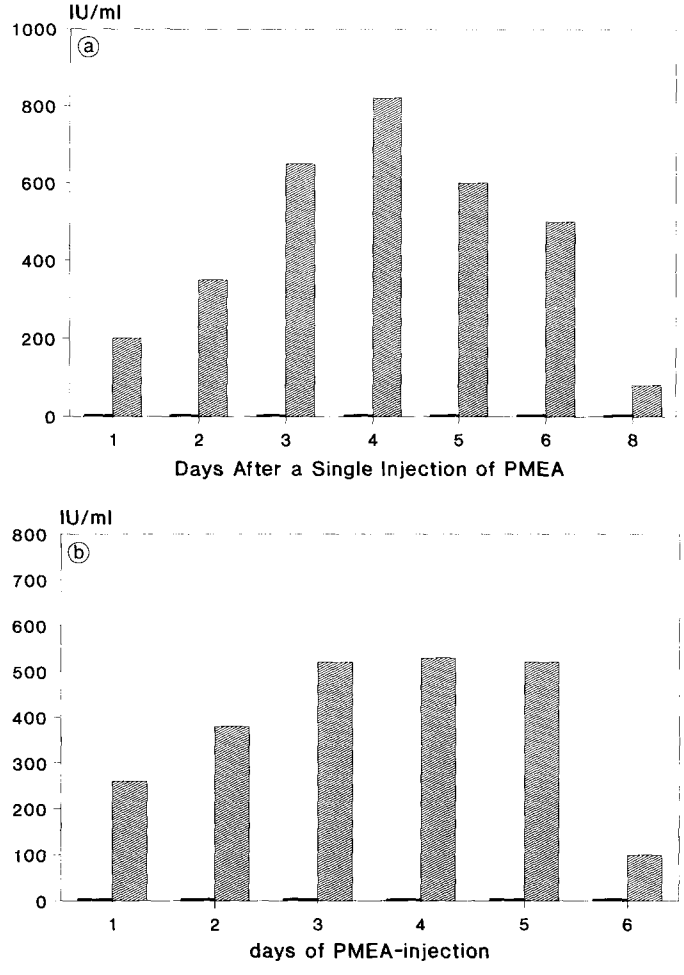

Fig. 2. Kinetics of interferon (IFN) production in mice treated with various schedules of PMEA. Fig. 2a: one single injection of 25 mg/kg PMEA; Fig. 2b: once daily injections of 25 mg/kg PMEA for 1 to 6 consecutive days. IFN production was evaluated at (2a) various days after the single injection or (2b) 24 h after the last injection. For this purpose, sera from control and PMEA-treated mice were assayed by reduction of cytopathic effect induced by vescicular stomatitis virus as described in the Materials and Methods. IFN titer is expressed in IU/ml referred to mouse IFN, standard preparation (see Materials and Methods). Each experiment was performed in duplicate at least three times. No IFN activity was detected in serum samples from control mice at any day tested. Black bars, control mice; slashed bars, PMEA-treated mice.

treated mice was about 750 IU/ml, while after incubation with rabbit anti-mouse IFN/immunoglobulins it declined to 150 IU/ml. In addition, pH-2 treatment completely destroyed the residual IFN-activity. These observations suggest that PMEA mainly induces IFNα/β, while the residual amount of IFN activity still present after neutralization with anti-α/β IFN antibodies (but destroyed by the pH-2-treatment) is conceivably due to a limited production of IFNγ.

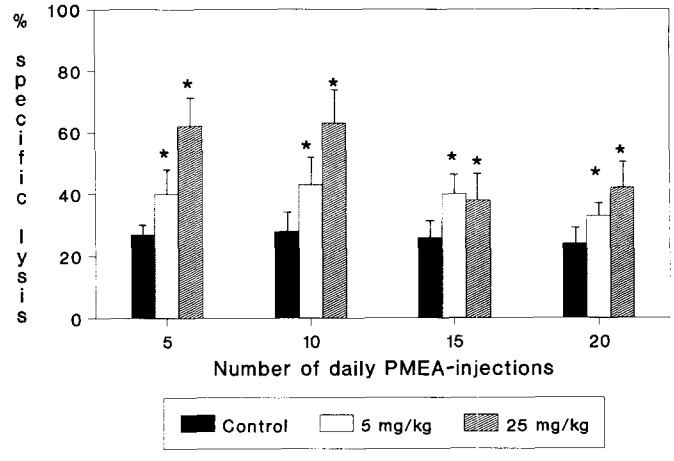

Fig. 3. Kinetics of NK cell cytolytic activity in mice inoculated i.p. once daily with PMEA for 20 consecutive days. Fig. 3a: treatment with 25 mg/kg PMEA. Fig. 3b: treatment with 5 mg/kg PMEA. 5, 10, 15 and 20 days after the beginning of treatment, PMEA-treated and control mice were sacrificed and individual spleen NK cell activity was determined as described in the legend to Fig. 1. Black bars, controls mice; slashed bars, PMEA-treated mice. Asterisks indicate the results that are significantly different from the corresponding control values, as calculated using the Student's t-test, at least at $P<0.05$.

We then assessed whether a prolonged administration of PMEA would induce a similar effect. Fig. 3a shows that the NK activity is quite well-enhanced by

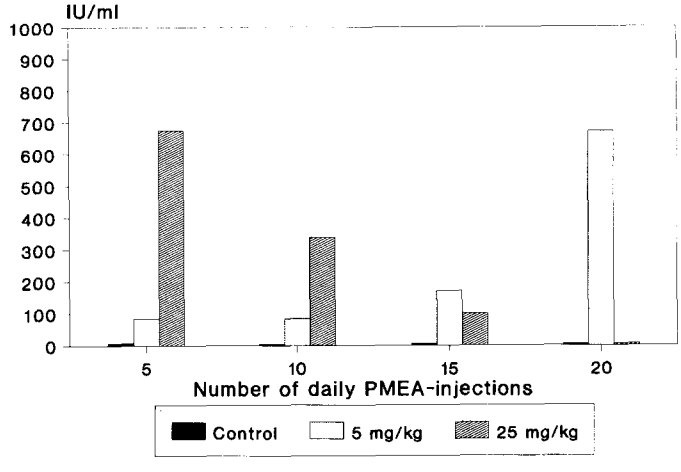

Fig. 4. Kinetics of interferon (IFN) induction in the serum of mice inoculated i.p. once daily with two different doses of PMEA for 20 consecutive days. 5, 10, 15 and 20 days after the beginning of treatment, sera from control and PMEA-treated mice were assayed by reduction of the VSV-induced cytopathic effect as described in the legend to Fig. 2. Black bars, control mice; dotted bars, mice treated with PMEA 5 mg/kg; slashed bars, mice treated with PMEA 25 mg/kg.

daily administration of 25 mg/kg PMEA up to day 20 after the beginning of the treatment, with the most marked enhancement noted at days 5–10. Thereafter, the increase of PMEA-mediated NK activity by long-term treatment was induced to a lesser exent, yet even at day 20 it was still more elevated in PMEA-treated mice than in control mice. The chronic stimulatory effect of PMEA appeared to be dose-dependent, since daily administration of PMEA at 5 mg/kg induced a less pronounced (but still clearly detectable) increase of NK activity (Fig. 3b).

IFN levels upon chronic administration of PMEA (5 or 25 mg/kg) are shown in Fig. 4. Interestingly, IFN production in mice treated with 25 mg/kg PMEA sharply decreased over time, was barely detectable at day 15, and was no longer present 20 days after the treatment had begun. By contrast, IFN production increased over time with the chronic administration of only 5 mg/kg of PMEA, and maximum production was detected at the latest day tested (i.e., 20th day), while NK enhancement was most pronounced following the first days of drug administration. Thus, the enhancement of NK activity was not strictly correlated with IFN production.

TABLE 1

Enhancement of NK cytotoxic activity induced by FPMPA

	% Specific Lysis			
FPMPA	Day 2	Day 3	Day 4	Day 5
No drug	28 ± 3.5	26.3 ± 2.5	25.2 ± 2.3	21.3 ± 2.5
12.5 mg/kg	27 ± 2.3	33.1 ± 3.4	23 ± 3	23 ± 3.4
25 mg/kg	35 ± 4.1	35 ± 3.8	32.2 ± 3.1	32.3 ± 2.9
50 mg/kg	45* ± 5.2	53.2* ± 4.3	55.2* ± 4.3	38.4* ± 3.3
100 mg/kg	58* ± 4.8	60.4* ± 5	75.2* ± 5.3	43.2* ± 3.5
200 mg/kg	56.5* ± 3.6	63* ± 4.8	70.1* ± 4.2	40.1* ± 3.9

2, 3, 4 and 5 days after the injection, FPMPA-treated and control mice were evaluated for NK activity as described in Materials and Methods section. Each experiment was run in quadruplicate and repeated three times.

*Statistically significant points (Student's t-test) compared to the respective controls ($P < 0.001$).

TABLE 2

IFN serum production induced by a single injection of FPMPA

	International Units/ml (IU/ml)				
FPMPA	Day 2	Day 3	Day 4	Day 5	Day 6
No drug	N.D.	N.D.	N.D.	N.D.	N.D.
12.5 mg/kg	100 ± 15	115 ± 13	150 ± 14	162 ± 27	25 ± 2
25 mg/kg	250 ± 22	300 ± 42	300 ± 20	210 ± 17	37 ± 5
50 mg/kg	350 ± 30	450 ± 33	465 ± 37	412 ± 38	36 ± 4
100 mg/kg	280 ± 22	400 ± 40	425 ± 22	353 ± 51	52 ± 5
200 mg/kg	130 ± 10	200 ± 22	205 ± 28	221 ± 23	48 ± 3

2, 3, 4, 5 and 6 days after the injection, FPMPA-treated and control mice were evaluated for IFN activity as described in the Materials and Methods section. Each experiment was repeated three times. N.D., not detectable.

In an additional set of experiments, we evaluated whether other drugs of the ANP family, such as PMEDAP and FPMPA, would modulate natural immune functions. As shown in Table 1, substantial and reproducible enhancement of NK activity was achieved with a single dose of FPMPA up to day 5 after drug administration, at all effector-target cell ratios tested. An average of 2-fold increase of NK activity (as compared to control) was achieved with single injections of 50, 100, and 200 mg per kg of FPMPA in a dose-dependent fashion, without any evident drug-mediated toxicity. This effect was paralleled by a concomitant enhancement of IFN production, with a dose-response curve that was similar to that shown for NK activity (Table 2).

Further experiments also showed that the highest doses of PMEDAP only induced a 1.7-fold maximum increase of NK activity compared to control (Table 3). This effect was less pronounced than that achieved with PMEA or FPMPA, being evident only at day 3 after drug administration, but still statistically significant. IFN production was also enhanced following PMEDAP administration (Table 4), albeit for a short period of time (i.e., 2–3 days after drug administration). Thus, both PMEDAP and FPMPA were

TABLE 3

Enhancement of NK cytotoxic activity induced by PMEDAP

	% Specific Lysis		
PMEDAP	Day 2	Day 3	Day 4
No drug	18.2 ± 3.1	19.8 ± 4.1	17.3 ± 2.5
12.5 mg/kg	14.1 ± 2.2	22.1 ± 3.2	18.2 ± 3.1
25 mg/kg	18.4 ± 3.5	23.5 ± 4.2	19.5 ± 2.3
50 mg/kg	17.5 ± 4.1	31.5* ± 3.4	21.2 ± 3.4
75 mg/kg	15.2 ± 3.1	33.5* ± 3.9	23.3 ± 4.2
100 mg/kg	14.3 ± 2.7	32.3* ± 2.8	20.6 ± 2.9

2, 3, and 4 days after the injection, PMEDAP-treated and control mice were evaluated for NK activity, as described in the Materials and Methods section. Each experiment was repeated three times.

*Statistically significant points (Student's t-test) at day 3 compared to the corresponding controls ($P<0.02$).

TABLE 4

IFN serum production induced by a single injection of PMEDAP

	International Units/ml (IU/ml)		
PMEDAP	Day 2	Day 3	Day 4
No drug	N.D.	N.D.	N.D.
12.5 mg/kg	N.D.	N.D.	N.D.
25 mg/kg	N.D.	N.D.	N.D.
50 mg/kg	42 ± 12	43 ± 18	N.D.
75 mg/kg	150 ± 23	110 ± 24	N.D.
100 mg/kg	255 ± 53	132 ± 43	N.D.

2, 3, and 4 days after the injection, PMEDAP-treated and control mice were evaluated for IFN activity, as described in the Materials and Methods section. N.D., not detectable.

able to enhance the host's natural immunity in a fashion similar to that of PMEA, suggesting that this effect is shared by at least three members of the ANP family.

Discussion

Acyclic nucleoside phosphonate (ANP) analogues represent a class of compounds of great interest in view of their potential use for the treatment of retrovirus and/or herpes virus infections. The acyclic side chain of the molecules markedly influences their antiviral activity spectrum, the FPMP derivatives being more active against retroviruses, and the HPMP derivatives more effective against DNA viruses. PMEA and its diaminopurine-derivative PMEDAP occupy an intermediate position in that they are active against both DNA viruses and retroviruses.

The results shown in this paper strongly suggest that ANP analogues have a substantial activity upon natural immunity. This effect is mainly characterized by a significative enhancement of natural killer activity and IFN production in mouse models. Since IFN is a potent inducer of NK function, it is conceivable that the immunomodulating effect of PMEA and its analogues is primarily mediated by the induction of IFN production, that then in turn stimulates NK function. Our results support this hypothesis, since we found a clear and reproducible correlation between the induction of IFN and the enhancement of NK cell activity. Nevertheless, the discrepancy between IFN production and NK enhancement during the chronic administration of PMEA suggests that the ANP-mediated immunomodulation may be also related to factors other than the direct induction of IFN. It is then conceivable that PMEA activates a number of cellular systems that interact through a cytokine network, thus resulting in the enhancement of natural immunity.

It should be noted that a single administration of PMEA or its analogues PMEDAP and FPMPA induced the greatest enhancement of NK and IFN activity, such effect being maintained for several days after treatment. This is in agreement with recently published data, showing that infrequent dosing of PMEA is less toxic and more effective in inhibiting the formation of tumors induced by Moloney murine sarcoma virus (Balzarini et al., 1990b; Naesens et al., 1991). Furthermore, data of Balzarini et al. indicated that PMEA-diphosphate (the active form of PMEA) has an extraordinarily long intracellular half-life, i.e., about 16 to 18 h (Balzarini et al., 1991a). Thus, it is conceivable that the infrequent administration of PMEA or its derivatives (even as single doses), may suffice to turn on both IFN production and NK activity, and to maintain them for a considerable period of time.

It should also be noted that the enhancement of NK activity induced by daily administration of PMEA is maintained for at least 20 days after the beginning of the treatment. This suggests that prolonged stimulation of NK activity can be achieved during a long-term treatment with this drug. On the other hand,

hyporesponsiveness develops with regard to IFN induction by high doses of PMEA, which is in agreement with what is generally observed with IFN inducers such as poly I:C (Stewart, 1979). However, this hyporeactivity does not occur with low doses of PMEA.

These results may be relevant for the therapy of HIV infection (as well as for cytomegalovirus and other herpesvirus infections) for at least two reasons. First, a regimen of continuous treatment with such compounds should be achievable without any evident toxicity upon the immune system. This is particularly relevant for HIV infection, since a number of authors have recently described an impaired NK cell and antibody-dependent cellular cytotoxicity in HIV-infected patients (Bonavida et al., 1986; Tyler et al., 1990; Scott-Algara et al., 1992). Second, the enhancement of the immune system, together with the direct drug-mediated inhibition of virus replication, may operate in an additive or even synergistic fashion, thus potentially achieving greater antiviral efficacy. Preliminary data obtained with ANP in mice infected with an RNA virus (not sensitive to the direct antiviral effect of ANP), support the hypothesis that enhancement of the immune system contributes to the host resistance to the virus infection.

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