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Antiretroviral agent (R)-9-(2-phosphonomethoxypropyl) adenine stimulates cytokine and nitric oxide production

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Abstract

The immunomodulatory properties of (R)-enantiomer of 9-(2-phoshonomethoxypropyl)adenine ((R)-PMPA), one of the most potent acyclic nucleotide analogs effective against human immunodeficiency virus (HIV), were investigated under in vitro conditions using murine peritoneal macrophages. Remaining without influence on interferon-γ and interleukin-2 expression, (R)-PMPA dramatically stimulated in a concentration- and time-dependent manner the secretion of tumor necrosis factor alpha (TNF-α) and interleukin-10. It also substantially augmented the production of nitric oxide (NO) induced by exogenous interferon-γ. Inhibitory experiments using neutralizing antibodies against TNF-α and/or interleukin-10 demonstrated that these two cytokines are major factors responsible for triggering the underlying mechanism(s) leading to enhanced NO production. The novel findings on the immunomodulatory potential of acyclic nucleotide analogs are discussed in the context of their possible implication in antiviral therapeutic efficacy. © 1997 Elsevier Science B.V.

Keywords: Acyclic nucleotide analog; (R)-PMPA ((R)-9-(2-phosphonomethoxypropyl)adenine); TNF-α (tumor necrosis factor α); IL-10 (interleukin-10); NO (nitric oxide)

1. Introduction

Acyclic nucleotide analogs, i.e., *N*-phosphonomethoxyalkyl-purines and -pyrimidines, possess both anti-DNA-viral and/or antiretroviral activities. Purine-based compounds, such as 9-(2-phosphonomethoxyethyl)adenine (PMEA) and 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine (PMEDAP), are also effective against human immunodeficiency virus (HIV-1, HIV-2), simian immunodeficiency virus (SIV) (Tsai et al., 1994) and feline immunodeficiency virus (FIV) (Hartmann et al., 1992). PMEA (Adefovir), a prototype compound, has shown antiviral activity in phase I/II clinical trials against acquired immunodeficiency disease (AIDS) (Collier et al., 1993; Walker et al., 1993; Cundy et al., 1995). Its oral prodrug bis(POM)-PMEA (Adefovir Dipivoxil) (Naesens et al., 1996) is currently being investigated in clinical studies (Barditch-Crovo et al., 1995). Outstanding effects against replication of immunodeficiency virus were recently observed with the R-enantiomer of 9-(2-phosphonomethoxypropyl)adenine [(R)-PMPA] (Balzarini et al., 1996), which completely inhibited the development of AIDS in a simian model of the immunodeficiency disease (Tsai et al., 1995; Bischofberger et al., 1996).

It is generally assumed that the antiviral activities of these compounds result mainly from the termination of DNA polymerase and subsequent inhibition of DNA synthesis (Votruba et al., 1990; Cherrington et al., 1995; Kramata et al., 1996). However, the explicit protective effect of (R)-PMPA against SIV infection prompted us to investigate possible immunomodulatory properties of this compound in its overall therapeutic efficacy. The study was further encouraged by findings of Calio et al. (1994) and Perno et al. (1996) that demonstrated stimulatory effects of PMEA and related compounds on the production of interferon-α/β and natural killer activity.

In this paper we present experimental data which demonstrate the potential of (R)-PMPA to considerably

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enhance the production of NO generated by interferon-γ and the intimate association of this effect with an increased secretion of TNF-α and interleukin-10.

2. Materials and methods

2.1. Animals

Female mice of the inbred strain C57BL/6, 7 to 9 weeks old, were purchased from Charles River Deutschland (Sultzfeld, Germany). They were kept in transparent plastic cages in groups of eight and maintained in an independent environmental air flow cabinet (ESI Flufrance, Wissous, France). The lighting was set on from 06.00 to 18.00 h and the temperature was kept at 22 ± 1°C.

2.2. Compounds

(R)-PMPA, i.e., the R-enantiomer of 9-(2-phosphonomethoxypropyl)adenine (Fig. 1), was synthesized as described elsewhere (Holý and Masojídková, 1995). A stock solution (10 mM) was prepared in incomplete phenol red-free RPMI-1640 culture medium containing $NaHCO_3$ (Sigma, St. Louis, MO, USA). Working concentrations were prepared by diluting the stock solution in complete RPMI-1640 (see below) and were used fresh. Adenosine, indomethacin and theophylline were purchased from Sigma and were prepared as given above (indomethacin in 0.01 M NaOH). All solutions were sterilized by filtration through non-pyrogenic 0.22 μm filters (Costar, Cambridge, MA, USA). The possible presence of lipopolysaccharide was tested using the chromogenic *Limulus amoebocyte* lysate assay (QCL-1000, BioWhittaker, Walkersville, MD, USA); test samples contained < 0.1 endotoxin units per ml.

2.3. Culture medium, isolation and cultivation of macrophages

Complete RPMI-1640 medium (SEVAC, Prague, Czech Republic) contained 10% heat-inactivated (56°C, 30 min)

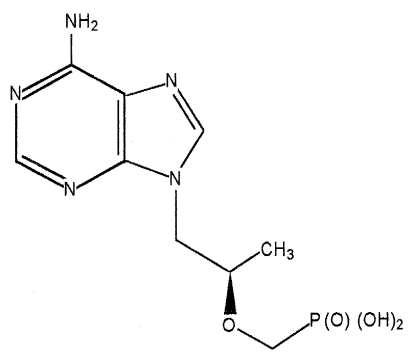

Fig. 1. Structural formula of (R)-PMPA, (R)-9-(2-phosphonomethoxypropyl)adenine.

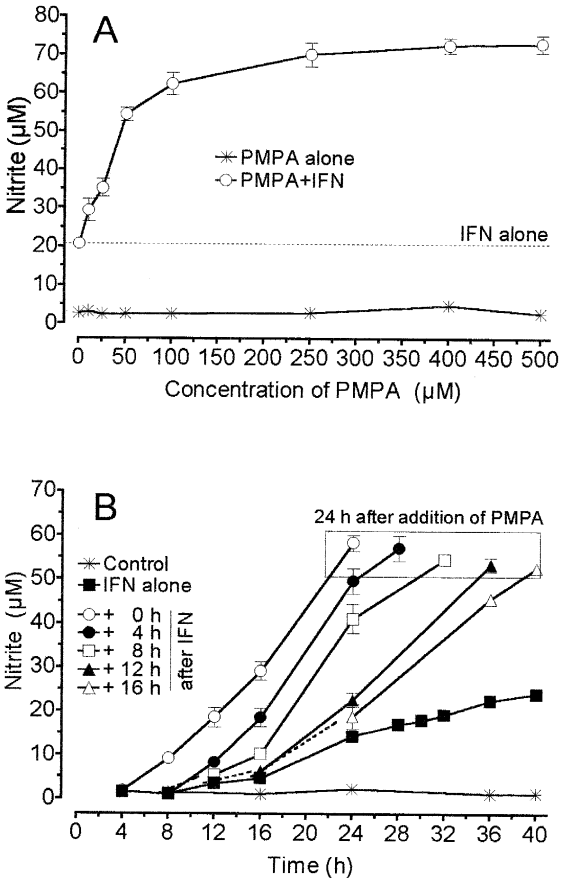

Fig. 2. Costimulatory effects of (R)-PMPA (PMPA) on the production of NO induced in murine peritoneal macrophages by interferon-γ (IFN-γ; 25 U/ml) in relation to: (A) concentration of PMPA and determined 24 h after their simultaneous administration, or (B) delayed addition of PMPA (250 μM) with respect to IFN-γ (= time zero) and determined 24 h after PMPA. The data are means ± S.E.M. of triplicate experiments variants and represent one of three separate experiments for part (A).

fetal bovine serum (ICN Biomedicals, High Wycombe, UK), 2 mM L-glutamine, 50 μg/ml gentamicin and 5×10^{-5} M 2-mercaptoethanol (all Sigma).

Mice ($n = 4$–8 in separate experiments), killed by cervical dislocation, were injected i.p. with 8 ml of sterile saline. Collected and pooled cells were washed, resuspended in culture medium and seeded into 96-well round-bottom microplates (Costar) in 100 μl volumes (2×10^5 cells/well). Adherent peritoneal cells (macrophages) were isolated by incubating the cells for 2 h at 37°C, 5% CO_2 and then thrice vigorously shaking the plate and washing the wells to remove nonadherent cells. Cultures were maintained at 37°C, 5% CO_2 in a humidified Heraeus incubator for 24 h. All experimental variants were set in triplicate.

2.4. Immunochemicals and cytokine assays

All the following items were purchased from Genzyme (Genzyme, Cambridge, MA, USA): recombinant mouse interferon-γ (specific activity: 1.1×10^7 U/mg); recombi-

nant mouse TNF-α (specific activity 1.33×10^8 U/mg); recombinant mouse interleukin-10 (specific activity 5.0×10^5 U/mg); polyclonal rabbit antibody against mouse TNF-α (bioactivity: ~ 10 μl neutralizes approximately 1000 units of mouse TNF-α in the standard L929 cell cytotoxicity assay); monoclonal rat antibody against mouse interleukin-10 (bioactivity 1–2 μg/ml neutralizes the interleukin-10 activity of an approximately 1:100 dilution of the monkey kidney COS cell line supernatant in the MC-9 cell costimulation assay).

The concentration of interleukin-2, interleukin-10, TNF-α and interferon-γ in macrophage supernatants was determined in accord with manufacturer's instructions, i.e., using the ELISA Kit reagents purchased from Genzyme.

2.5. Nitric oxide (NO) production by macrophages

The concentration of nitrites in supernatants was taken as a measure of NO production (Marletta et al., 1988). This was detected in individual cell-free samples (50 μl) incubated for 10 min at 37°C with an aliquot of a Griess reagent (1% sulfanilamide/0.1% naphtylethylendiamine/2.5% H_3PO_4). The absorbance at 540 nm was recorded using a Uniskan II microplate reader (Labsystems, Helsinki, Finland). A nitrite calibration curve was used to convert absorbance into μM nitrite.

2.6. Statistical analysis

The data were evaluated by means of analysis of variance and subsequent Dunnett's test, using the Prism program (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Influence of (R)-PMPA on production of nitric oxide (NO)

(R)-PMPA, though ineffective alone, significantly increased in a concentration-dependent manner the production of NO by murine peritoneal macrophages cultured 24 h in the presence of interferon-γ (25 U/ml) (Fig. 2A). With the nitrite level elicited by 500 μM (R)-PMPA being regarded as a maximum, the EC_{50} turned out to be 45 μM. The onset of the costimulatory effect occurred very soon after administration of (R)-PMPA and interestingly, its delayed supply (250 μM; 4, 8, 12, 16 h after interferon-γ)

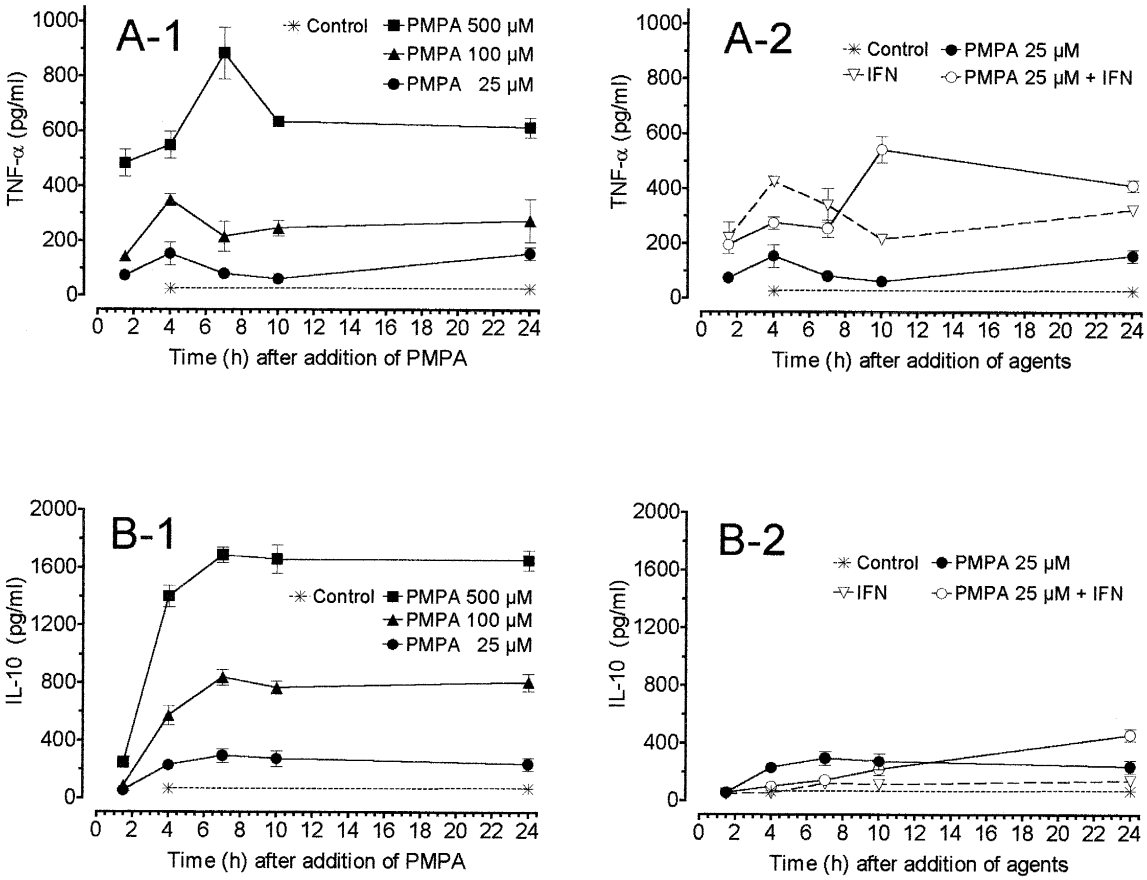

Fig. 3. Concentration of TNF-α (A1, A2) and interleukin-10 (IL-10) (B1, B2) in supernatants of murine macrophages cultured for indicated time intervals in the presence of (R)-PMPA (PMPA; 25, 100, 500 μM) added either alone (A1, B1, respectively) or jointly with interferon-γ (IFN-γ; 25 U/ml) (A2, B2, respectively). Data are means ± S.E.M. from a single experiment, each point being determined in triplicate.

had no influence upon the final (i.e., determined 24 h after addition of (R)-PMPA) NO-upregulatory effect (Fig. 2B).

Adenosine (tested at concentrations from 0.1 to 100 μM) remained without any influence on the production of NO induced by interferon-γ (data not shown).

3.2. Effects of (R)-PMPA on secretion of TNF-α, interleukin-2, interleukin-10 and interferon-γ

(R)-PMPA enhanced the secretion of TNF-α (Fig. 3A1) and interleukin-10 (Fig. 3B1) by murine peritoneal macrophages. The effects were closely related to the (R)-PMPA concentration and occurred somewhat earlier for TNF-α than for interleukin-10. While the interleukin-10 levels reached a plateau at approximately 7 h after addition of (R)-PMPA, the kinetics of TNF-α secretion differed. Its concentrations reached a maximum either at 7 h for the highest (R)-PMPA dose (500 μM) or at 3 h for 100 or 25 μM; then TNF-α concentrations decreased with a tendency to increase slightly again or remain constant.

Interferon-γ per se enhanced TNF-α but had no effect upon interleukin-10 accumulation in macrophage supernatants (Fig. 3A2 and B2, respectively). The kinetic patterns of secretion of these cytokines were altered in the combined presence of (R)-PMPA (25 μM) + interferon-γ (25 U/ml), as compared with the effects of each of these agents alone. Concentrations of TNF-α were about intermediate up to 7 h after administration of (R)-PMPA + interferon-γ, but they were considerably higher (> sum of (R)-PMPA + interferon-γ) later, especially at 10 h (Fig. 3A2). Levels of interleukin-10 produced by (R)-PMPA alone (25 μM) were either suppressed (up to 7 h), unchanged (10 h interval), or increased (24 h interval) in the presence of interferon-γ (Fig. 3B2).

No changes were observed in the secretion of interleukin-2 or interferon-γ following administration of (R)-PMPA (data not shown).

Adenosine at 50 and 200 μM did not stimulate the secretion of TNF-α or interleukin-10 (data not shown).

3.3. Stimulation of nitric oxide (NO) production by interferon-γ in combination with TNF-α and/or interleukin-10. Inhibitory effects of antibodies against TNF-α and interleukin-10

The standard stimulatory effect of interferon-γ (25 U/ml) on NO production by murine peritoneal macrophages was significantly enhanced (determined after 24 h) by addition of TNF-α (300 pg/ml) and only marginally increased by interleukin-10 (750 pg/ml), both being ineffective in the absence of interferon-γ. The most prominent NO-stimulatory effect was achieved in the combined presence of interferon-γ + TNF-α + interleukin-10 (Fig. 4A). The concentration of the cytokines in this study was chosen so as to be close to their concentrations produced by 100 μM (R)-PMPA (viz., Fig. 3A1 and B1). There was no difference between their synergizing activity

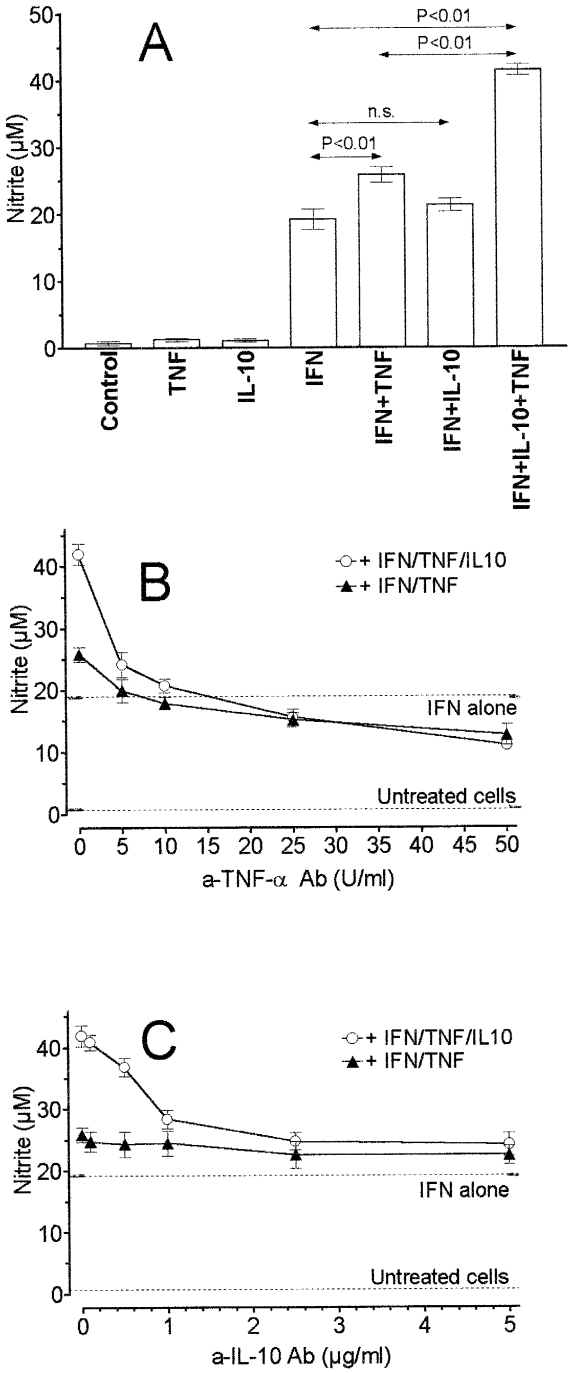

Fig. 4. Production of NO by murine peritoneal macrophages cultured in the presence of TNF-α (300 pg/ml), interleukin-10 (IL-10; 750 pg/ml), interferon-γ (IFN-γ; 25 U/ml), or in the combined presence of IFN-γ + TNF-α, IFN-γ + IL-10, or IFN-γ + TNF-α + IL-10 (A). Inhibition of NO production induced by IFN-γ + TNF-α, or IFN-γ + TNF-α + IL-10 (applied at concentrations given above) by antibodies against TNF-α (a-TNF-α Ab) (B), or IL-10 (a-IL-10 Ab) (C). All cytokines and antibodies were applied simultaneously, and NO production was determined 24 h afterwards. Each point or bar is a mean ± S.E.M. for quadruplicate culture wells.

when applied simultaneously with interferon-γ or after 2 h (not shown).

Antibodies against TNF-α (Fig. 4B) or interleukin-10 (Fig. 4C) in a concentration-dependent manner abolished the production of NO triggered by interferon-γ + TNF-α, or interferon-γ + TNF-α + interleukin-10. Anti-TNF-α antibody proved to be more potent than anti-interleukin-10 antibody.

3.4. Inhibition of enhancing effect of (R)-PMPA on interferon-γ-induced NO production by antibodies against TNF-α and / or interleukin-10

Based upon the findings obtained from the previous experiments, antibodies against TNF-α (50 U/ml) and/or interleukin-10 (2.5 μg/ml) were applied to macrophages cultured for 24 h in the presence of (R)-PMPA (50, 100 or 250 μM) + interferon-γ (25 U/ml). The production of NO was substantially suppressed by both anti-interleukin-10 and anti-TNF-α antibodies and even more so by their combined presence. The extent of inhibition was correlated with concentration of (R)-PMPA (Fig. 5); a combined administration of the two antibodies completely eliminated the NO-synergizing activity of (R)-PMPA at the highest dose tested (250 μM).

3.5. Effects of indomethacin or theophylline on (R)-PMPA-induced secretion of TNF-α and interleukin-10

Indomethacin, an inhibitor of prostaglandin synthesis, as well as theophylline, a nonspecific adenosine receptor antagonist, partially suppressed the secretion of both TNF-α and interleukin-10 induced by (R)-PMPA (200 μM) (Fig. 6A and B, respectively). In accord, they attenuated the costimulatory effect of (R)-PMPA on the production of NO induced by interferon-γ (Fig. 6C).

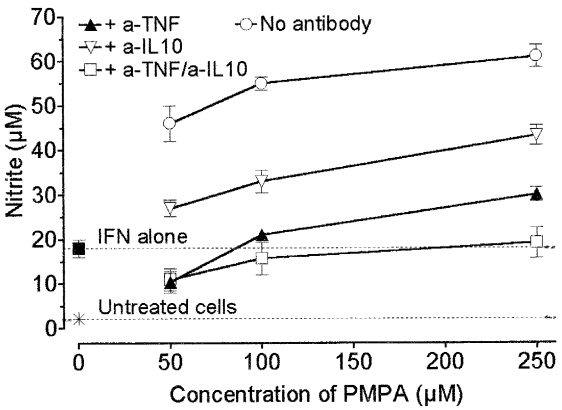

Fig. 5. Inhibition of costimulatory effects of (R)-PMPA (PMPA; applied at 50, 100, 250 μM) on interferon-γ (IFN-γ; 25 U/ml)-induced production of NO in murine macrophages by antibodies against TNF-α (a-TNF; 50 U/ml), or interleukin-10 (a-IL-10; 2.5 μg/ml), or both antibodies (a-TNF/a-IL-10). Concentration of nitrites was determined 24 h after simultaneous addition of PMPA + IFN-γ + antibodies. Individual points are means ± S.E.M. obtained from triplicate culture wells.

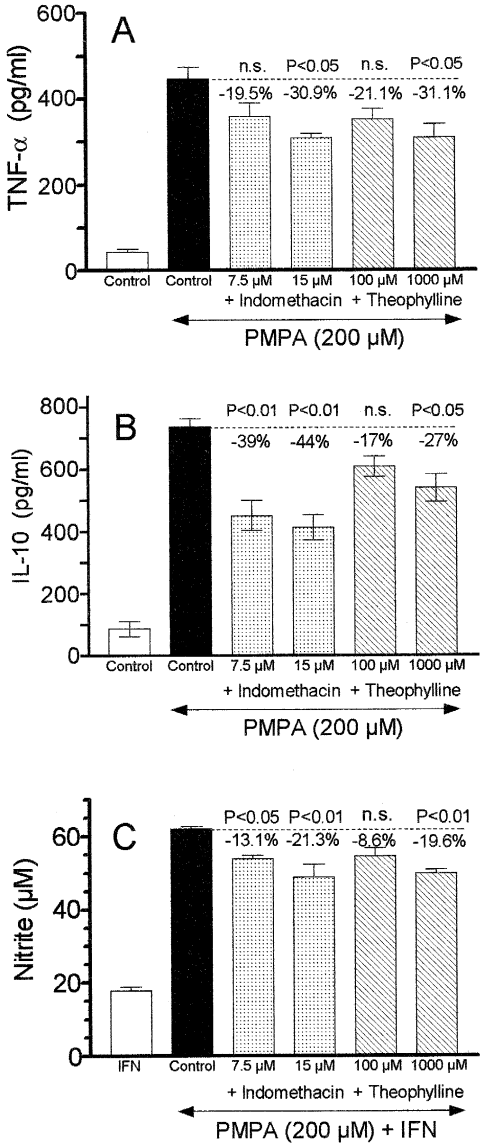

Fig. 6. Suppressive effects of indomethacin and theophylline on secretion of TNF-α, or interleukin-10 (IL-10) by murine peritoneal macrophages cultured for 4 h in the presence of (R)-PMPA (PMPA) alone (A and B, respectively), or on production of NO in murine peritoneal macrophages cultured for 24 h in the presence of interferon-γ (IFN-γ) + PMPA (C). The bars represent means ± S.E.M. obtained from triplicate determinations.

4. Discussion

The interference of an antiretroviral-active acyclic nucleotide analog (R)-9-(2-phosphonomethoxypropyl)adenine, (R)-PMPA, with factors that are known to have vital functions within the system of immune defence mechanisms was investigated. We found that (R)-PMPA is a powerful stimulator of

TNF-α and interleukin-10 secretion by murine macrophages, while it is inactive in altering the expression of interleukin-2 and interferon-γ. (R)-PMPA has also remarkably augmented the production of NO induced in macrophages by exogenous interferon-γ, but not if it was administered alone. Concentration-dependent effects upon cytokine and NO production were found to run in parallel and suggested thus an intimate, presumably causal relationship between the two parameters of macrophage activation. This possibility was substantiated by the outcomes of experiments in which the effects of exogenous interferon-γ plus TNF-α and/or interleukin-10 on formation of NO were analyzed. These two cytokines, though being ineffective by themselves, acted in a synergistic manner and profoundly enhanced NO production triggered by interferon-γ. The NO-enhancing effects were differentially counteracted by specific antibodies against TNF-α or interleukin-10, the former being more effective. The same attenuating or virtually inhibitory effects of these antibodies on NO production were observed in macrophages cultured in the presence of interferon-γ + (R)-PMPA. Joint addition of anti-TNF-α and anti-interleukin-10 antibodies completely abolished NO production, even under conditions in which each of them alone was only partially inhibitory, e.g., when (R)-PMPA was applied at relatively high concentration (250 μM). The findings support the view (Corradin et al., 1993) that interleukin-10 may upregulate macrophage activity.

Signalling pathways for the (R)-PMPA-induced production of cytokines are not yet clear. In as much as (R)-PMPA is an adenine phosphonate analog, we compared its effects with the effects of adenosine. Striking differences between them exist. Adenosine did not have an effect on the production of TNF-α and interleukin-10, or on the production of NO induced by interferon-γ. At concentrations comparable to those chosen for (R)-PMPA, adenosine has been found to augment the production of interleukin-10 by human monocytes stimulated with TNF-α (by 116% at 100 μM), hydrogen peroxide, or lipopolysaccharide (Le Moine et al., 1996). It has been speculated that the costimulatory activity could be due to mobilization of intracellular cyclic AMP (cAMP). However, in sharp contrast to (R)-PMPA, neither adenosine nor other cyclic AMP-elevating agents, such as prostaglandins (Mauël et al., 1995), are able, by themselves, to stimulate the expression of the interleukin-10 protein (Kambayashi et al., 1995). Moreover, not only is adenosine unable to increase the production of TNF-α, but it even inhibits it (Sajjadi et al., 1996). The two effects of adenosine are supposed to result from the activation of adenosine receptors (Haskó et al., 1996). Our preliminary findings indicate that adenosine receptors and prostaglandins are also shared, albeit only partially, by obviously distinct signalling pathways that determine the cytokine and NO stimulatory effects of (R)-PMPA. More sophisticated experiments are now needed to elucidate possible mechanism(s) for this action.

A number of cytokines have been shown to influence the replication of retroviruses. Thus, expression of HIV can be suppressed by interferon-γ (Dhawan et al., 1995), interferon-α (Gendelman et al., 1990), interferon-τ (Dereuddre-Bosquet et al., 1996), transforming growth factor-β (Poli et al., 1991), interleukin-10 (Kootstra et al., 1994; Akridge et al., 1994; Saville et al., 1994), interleukin-13 (Montaner et al., 1993) and interleukin-16 (Fauci, 1995). Interleukin-1 (Lepe-Zuniga et al., 1987), interleukin-6 (Breen et al., 1990), TNF-α (Poli et al., 1990), granulocyte/macrophage colony-stimulating factor and macrophage colony-stimulating factor (Perno et al., 1996) can augment HIV replication. It is highly probable that the integrated action of these factors may be quite different. For example, interleukin-10 upregulates cytosolic TNF-α receptors that are natural antagonists of the biological activities of TNF-α (Joyce et al., 1994) and it also downregulates the expression of mRNA for interleukin-1, interleukin-6, or TNF-α (Fiorentino et al., 1991; Bogdan et al., 1991; Cassatella et al., 1993).

NO has proved to be effective against various viruses (Karupiah et al., 1993) including retroviruses (Akarid et al., 1995). The main source of the inducible form of NO synthase, which upon appropriate stimulation produces large amounts of NO, is the macrophage (Förstermann et al., 1995). Macrophages, monocytes, and dendritic cells are known also to be a reservoir of HIV and may act as a major vector of its dissemination (Crowe and Kornbluth, 1994). Several acyclic nucleotide analogs have profound in vitro activity against HIV in monocyte/macrophages and peripheral blood lymphocytes (Balzarini et al., 1991). Although enhanced NO production in HIV-infected patients has been observed, its significance in the disease pathogenesis is largely unclear (Torre and Ferrario, 1996).

With respect to these facts, the therapeutic value of the ability of (R)-PMPA to stimulate the secretion of cytokines and to costimulate the production of NO remains to be firmly established. Further investigations should elucidate whether and how these effects are manifested in humans and virus-infected cells.

Acknowledgements

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