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Suppression of macrophage inflammatory protein (MIP)-1α production and collagen-induced arthritis by adenosine receptor agonists

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1 Ligands of the various adenosine receptor subtypes modulate the production of pro-and anti-inflammatory cytokines. Here we evaluated the effect of adenosine and various ligands of the adenosine receptor subtypes (A_1, A_2, A_3) on the chemokine macrophage inflammatory protein (MIP) 1α production in immunostimulated RAW macrophages *in vitro*. Furthermore, we studied whether a selected A_3 adenosine receptor agonist inhibits MIP-1α production and affects the course of inflammation in collagen-induced arthritis.

2 In the cultured macrophages, the A_3 receptor agonist N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (IB-MECA), and, less potently, the A_2 receptor agonist 2-p-(2-carboxyethyl) phenethylamino-5′-N-ethyl-carboxamidoadenosine (CGS; 1–200 μM) dose-dependently suppressed the production of MIP-1α. The selective A_1 receptor agonist 2-chloro-N6-cyclopentyladenosine (CCPA, 1–200 μM) was ineffective, and adenosine was a weak inhibitor. The inhibition of MIP-1α production by the A_3 and A_2 agonist was associated with suppression of its steady-state mRNA levels.

3 Based on the *in vitro* data, we concluded that activation of A_3, and to a lesser extent A_2 adenosine receptors suppresses MIP-1α expression. Since IB-MECA was the most potent inhibitor of MIP-1α expression, we next investigated whether it affects the production of other pro-inflammatory mediators. We observed that IB-MECA (1–300 μM) inhibited, in a dose-dependent manner, the production of IL-12, IL-6, and, to a lesser extent, nitric oxide in the immunostimulated cultured macrophages.

4 Since MIP-α is a chemokine which enhances neutrophil recruitment into inflammatory sites, we investigated whether the A_3 agonist IB-MECA affects the course of inflammation, MIP-α production and the degree of neutrophil recruitment in arthritis. In a model of collagen-induced arthritis in mice, IB-MECA (0.5 mg/kg/day) reduced the severity of joint inflammation. IB-MECA inhibited the formation of MIP-1α, IL-12 and nitrotyrosine (an indicator of reactive nitrogen species) in the paws, and suppressed neutrophil infiltration.

5 We conclude that adenosine receptor agonists, most notably the A_3 agonist IB-MECA suppress the production of MIP-α, and exert anti-inflammatory effects. Therefore, stimulation of adenosine receptor subtypes A_3 and A_2 may be a strategy worthy of further evaluation for the abrogation of acute or chronic inflammatory disorders.

Keywords: Inflammation; cytokines; arthritis; xanthine; arthritis

Introduction

Adenosine is a purine nucleoside that is released from a variety of cells in response to metabolic stress or from the sympathetic nervous system, and occupies various adenosine receptor subtypes on target cells (Collis & Hourani, 1993; Cronstein, 1994). Ligands of the A_1, A_{2a}, A_{2b} and A_3 adenosine receptors have been shown to down-regulate the production of proinflammatory mediators, reduce polymorphonuclear leukocyte (PMN) recruitment and exert anti-inflammatory effects (Collis & Hourani, 1993; Cronstein, 1994). In addition, the anti-inflammatory properties of drugs such as methotrexate, sulphasalazine, and adenosine kinase inhibitors are related to their ability to release adenosine (Cronstein, 1994; Firestein *et al*., 1994; Gadangi *et al*., 1996).

In recent studies it was found that both A_1 and A_2 receptor agonists inhibited TNF-α production by RAW 264.7 macrophages or human monocytes, and the rank order of potency of agonists was characteristic of neither A_1 nor A_2 receptors (Le Vraux *et al*., 1993; Haskó *et al*., 1996), suggesting a possible involvement of the A_3 receptor. Moreover, adenosine, but not selective A_1 and A_2 receptor agonists enhanced IL-10 production in human monocytes (Le Moine *et al*., 1996). Using specific A_1, A_2, and A_3 receptor agonists and antagonists, it was subsequently demonstrated that inhibition of TNF-α production by LPS-stimulated U937 (human monocyte) cells was mainly an A_3 receptor-mediated process (Sajjadi *et al*., 1996). Similarly, ADO receptor agonists, in a dose-dependent manner characteristic of the A_3 receptor, blocked endotoxin induction of the TNF-α gene and protein expression in the murine J774.1 macrophage cell line (McWhinney *et al*., 1996).

Although the above studies demonstrated that various adenosine receptor agonists are able to modulate the production of pro- and anti-inflammatory cytokines (see for review: Haskó & Szabó, 1998), no information on the modulation by adenosine receptor ligands of the production of chemokines was previously available. Macrophage inflammatory protein (MIP)-1α is a low molecular weight protein, a CC chemokine, with potent inflammatory effects, which are mainly mediated by the promotion of neutrophil recruitment into inflammatory sites (Shanley *et al*., 1995; Standiford *et al*.,

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1995; Kunkel *et al*., 1996). Since MIP-1α is an important mediator of acute inflammation, and adenosine receptor ligands can exert potent anti-inflammatory effects (Cronstein, 1994; Gadangi *et al*., 1996; Haskó *et al*., 1996), here we investigated whether adenosine receptor ligands affect the production of MIP-1α protein and mRNA in macrophages. Since we found inhibition of MIP-1α expression by the adenosine receptor agonists, most notably by the A3 agonist, we went on to investigate whether the A_3 agonist IB-MECA affects MIP-1α production, the course of inflammation and neutrophil recruitment in a model of collagen-induced arthritis.

Methods

Effect of adenosine agonists on MIP-1α production in RAW 264.7 cells

The mouse macrophage cell line RAW 264.7 was cultured in Dulbecco's modified Eagle's medium. Cells were pretreated with $1-300\ \mu$M of adenosine, the selective A_1 receptor agonist 2-chloro-N6-cyclopentyladenosine (CCPA), the A_2 receptor agonist 2-p-(2-carboxyethyl) phenethylamino-5′-N-ethyl-carboxamidoadenosine (CGS-21680) and the A_3 receptor agonist N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (IB-MECA) (Collis & Hourani, 1993; Gallo-Rodriguez *et al*., 1994). Adenosine agonists were from Research Biochemicals Inc. (Natick, MA U.S.A.). After 30 min, cells were stimulated with LPS (10 ng/ml *Escherichia coli* 055 : B5 from Sigma, St. Louis, MO U.S.A.) and $1-3$ h later supernatants taken or cells harvested for MIP-1α protein or mRNA measurements. In a separate set of experiments, the effect of a 1 h pretreatment with the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 50 μM) (Wolberg & Zimmermann, 1985; Riches *et al*., 1985) was studied on the inhibitory effect of adenosine on MIP-1α production. MIP-1α was measured using murine ELISA kits purchased from Genzyme (Boston, MA U.S.A.) (detection limit: 1.5 pg/ml) as described previously (Hasko *et al*., 1998b). Test compounds did not interfere with the assay.

Effects of adenosine agonists on MIP-1α steady-state mRNA levels

Total RNA was extracted from each well using a guanidinium isothiocyanate/chloroform based technique (TRIZOL), followed by isopropanol precipitation. Cytoplasmic RNA (15 μg) was fractionated on a 1% formaldehyde gel and transferred to a nylon membrane. The cDNA for rat MIP-1α shares 92% homology with the mouse sequence and has demonstrated cross-hybridization between the two species (Shanley *et al*., 1995). This cDNA was radiolabeled with ^{32}P-dCTP (specific activity, 3,000 Ci/mM; NEN DuPont) by random priming (Pharmacia, Piscataway, NJ U.S.A.). Radioactivity of probes was determined by scintillation counting and 1.5×10^7 c.p.m. were applied to the Northern blot and hybridization was performed at 42°C for 16 h. The hybridized filters were serially washed at 53°C with $2\times$ sodium citrate, sodium chloride, 0.1% SDS ($2\times$SSC) solution. Autoradiography was done at −70°C on Kodak ×-OMAT-AR film. After probing for MIP-1α, membranes were stripped with boiling 5 mM EDTA and rehybridized with a ^{32}P-radiolabeled oligonucleotide probe for 18S ribosomal RNA.

Effect of IB-MECA on cytokine and NO production in RAW 264.7 cells

In the case of the A_3 agonist IB-MECA, we investigated whether the agonist also affects the production of IL-12, IL-6, and NO in RAW 264.7 macrophages. In these studies, the cells were pretreated with $1-300\ \mu$M of IB-MECA (Research Biochemicals Inc., Natick, MA U.S.A.). After 30 min, cells were stimulated with LPS (10 μg ml^{-1}), alone, or in combination with murine IFN-γ (200 u ml^{-1}; Genzyme, Boston, MA U.S.A.). This concentration of LPS is higher than the one used for the studies investigating the effect of adenosine receptor agonists on MIP-1α production. Our preliminary studies have demonstrated that higher concentration of LPS is required to achieve optimal levels of NO production in this cell type, and only the combination of this higher concentration of LPS in combination with IFN-γ resulted in detectable IL-12 production in these cells. The cultures were incubated at 37°C for 24 h, after which the culture supernatant fluids were collected and stored at −70°C. Cytokines were determined by ELISA kits that are specific against the murine cytokines. IL-12 (p40 and p70) and IL-6 were measured using ELISA kits purchased from Genzyme (Boston, MA U.S.A.). Plates were read at 450 nm on a Spectramax 250 microplate reader. Detection limits were 10 pg ml^{-1} for IL-12 (p40), 5 pg ml^{-1} for IL-12 (p70), and 5 pg ml^{-1} for IL-6. Assays were performed as described previously (Haskó *et al*., 1996; Szabó *et al*., 1997) and according to the manufacturer's instructions. IB-MECA did not interfere with any of the assays used. Nitrite (a stable breakdown product of NO) in culture supernatants was measured by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of medium. The OD_{550} was measured using the Spectramax 250 microplate reader (Haskó *et al*., 1998a,b).

Measurement of mitochondrial respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Haskó *et al*., 1996).

Collagen-induced arthritis

Male DBA/1J mice (9 wk) were obtained from the Jackson Laboratory (Bay Harbor, ME U.S.A.). Bovine type II collagen (CII) was dissolved in 0.01 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Dissolved CII was frozen at −70°C until use. Complete Freund's adjuvant (CFA) was prepared by the addition of *Mycobacterium tuberculosis* H37Ra at a concentration of 2 mg ml^{-1}. Before injection, CII was emulsified with an equal volume of CFA. Collagen-induced arthritis was induced as previously described (Szabó *et al*., 1998). On day 1, mice were injected intradermally at the base of the tail with 100 μl of the emulsion (containing 100 μg CII). On day 21, a second injection of CII in CFA was administered.

For stimulation of A_3 adenosine receptors, animals were treated with IB-MECA at a dose of 0.5 mg kg^{-1}. The selection of this dose was based on previous *in vivo* studies (Von Lubitz *et al*., 1994; Tracey *et al*., 1997; Auchampach *et al*., 1997). Animals were treated with either vehicle ($n=18$) or with IB-MECA ($n=18$; 0.5 mg kg^{-1}, i.p.) every 24 h, starting from day 18. Mice were evaluated daily for arthritis

by using a macroscopic scoring system ranging from 0–4 (0 = no signs of arthritis; 1 = swelling and/or redness of the paw or one digit; 2 = two joints involved; 3 = more than two joints involved; and 4 = severe arthritis of the entire paw and digits). The arthritic index for each mouse was calculated by adding the four scores of the individual paws. Severity indices were calculated for whole groups of mice (vehicle-treated or IB-MECA treated), and no animals were excluded from the calculations.

Histology and nitrotyrosine immunohistochemistry

At the end of the experiments (Day 35), animals were sacrificed under anaesthesia and paws and knees were removed and fixed for histological examination, which was done by an investigator blinded for the treatment regimen. For nitrotyrosine immunohistochemistry, joints were embedded in M1 medium and snap frozen in liquid nitrogen. Cryostat sections (6 μm) were cut with a microtome equipped with a carbide steel knife. Joint sections were analysed for the presence of nitrotyrosine, an indicator of peroxynitrite by immunohistochemistry (Szabó *et al*., 1998). Endogenous peroxidase was quenched with 0.3% H_2O_2 in PBS for 15 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate buffered saline for 60 min. The sections were then incubated overnight with 1 : 500 dilution of primary anti-nitrotyrosine antibody (Upstate Biotech, Saranac Lake, NY U.S.A.). Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories). In control experiments, sections were incubated with the anti-nitrotyrosine antibody in the presence of 10 mM nitrotyrosine. This intervention eliminated the nitrotyrosine staining presented in the figures.

Detection of cytokines, chemokines and nitrotyrosine in paw extracts

In another set of studies, aqueous joint extracts were prepared from control animals and from animals at 35 days of arthritis as described (Kasama *et al*., 1995), by homogenization in a lysis buffer in the presence of a mixture of protease inhibitors (10 μg ml^{-1} aprotinin, 20 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin A and 1 mM PMSF, pH 7.25). Extracts were analysed for the presence of IL-12, TNF-α MIP-1α using ELISA (Haskó *et al*., 1998a,b) and the presence of nitrated proteins, using Western blotting, as described (Szabó *et al*., 1998). Thirty μg of each sample was diluted in an equal volume of treatment buffer and heated to 95°C for 3 min. Samples were then loaded into 8–16% Tris-Glycine. Gels were run at 120 volts for 2 h, then transferred to 0.45 μm nitro-cellulose at 30 volts for 60 min using 1/2 × Towbin buffer system (1.45 g Tris, 7.2 g glycine, 800 ml di H_2O and 200 ml MeOH). The membrane was blocked in 1% BSA: 1% non-fat-milk in PBS-Tween (phosphate-buffered saline with 0.05% Tween 20) for 1 h then probed with rabbit anti-nitrotyrosine (Upstate Biotechnology, Saranac Lake, NY U.S.A.) 1 μg/ml in PBS-Tween overnight at 4°C. The blot was washed three times with PBS-T, once with H_2O, then incubated for 1.5 h with secondary antibody, goat anti-rabbit-HRP (1 : 3000). The blot was washed three times with PBS-T, once with di H_2O, then 1.5 ml mixed ECL chemiluminescence reagent (Amersham) was added for 1 min. The blot was then exposed to X-ray film for 60 s.

Myeloperoxidase measurements in paw homogenates

In addition, myeloperoxidase activity, an indicator of neutrophil infiltration, was also measured in the paws as described (Szabó *et al*., 1997). Paws were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 × *g* at 4°C. An aliquot of the supernatant was allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured by spectrophotometry at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of hydrogen peroxide/min at 37°C and expressed in milliunits per mg protein.

Data analysis and presentation

All values in the figures and text are expressed as mean ± s.e.mean of *n* observations, where *n* represents the number of wells (6–9 wells from two to three independent experiments) or the number of animals studied. Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with Dunnett's test. For the arthritis studies, Mann-Whitney U-test (2-tailed, independent) was used to test the statistical differences in the arthritic indices. A *P*-value less than 0.05 was considered statistically significant.

Results

Adenosine agonists suppress the expression of MIP-1α in immunostimulated cultured macrophages

LPS (10 ng/ml) induced a significant production of MIP-1α . The A_3 agonist IB-MECA, and, less potently, the A_2 agonist CGS-21680 suppressed the formation of MIP-1α , whereas the A_1 receptor agonist CCPA was without any inhibitory effect (Figure 1). Adenosine exhibited a marginal inhibitory effect, which amounted to a 21 ± 3% inhibition ($P < 0.01$) at 200 μM. The adenosine agonists tested did not affect mitochondrial respiration, as measured with the MTT assay (not shown).

Since adenosine is rapidly degraded by adenosine deaminase, we have tested whether inhibition of adenosine deaminase affected the degree of its inhibition. In the presence of the adenosine deaminase inhibitor EHNA (50 μM), no enhancement of the inhibitory effect of adenosine was observed (24 ± 5% inhibition at 200 μM; $n = 6$).

LPS induced a time-dependent increase in the steady-state mRNA levels for MIP-1α (Figure 2). The marked suppression of MIP-1α production by IB-MECA was associated with a marked, dose-dependent suppression of MIP-1α mRNA (Figure 3). Adenosine and the A_1 agonist failed to affect MIP-1α mRNA expression, while the A_2 agonist CGS-21680 inhibited steady state mRNA levels (Figure 3). Overall, there was a fairly good correlation between the degree of the inhibition of MIP-1α protein and MIP-1α mRNA expression by the adenosine agonists. However, the degree of inhibition of MIP-1α mRNA levels and the protein levels did not always correspond to each other. For instance, using the ELISA method, we found that CGS (at 200 μM) inhibited MIP-1α protein production by approximately 50% (Figure 1), while the inhibition of MIP-1α mRNA production was almost completely abolished (Figure 3). Differences in the time of the measurements (protein was measured at 3 h, mRNA at 2 h post-LPS), and/or variations in the sensitivity of the two assays

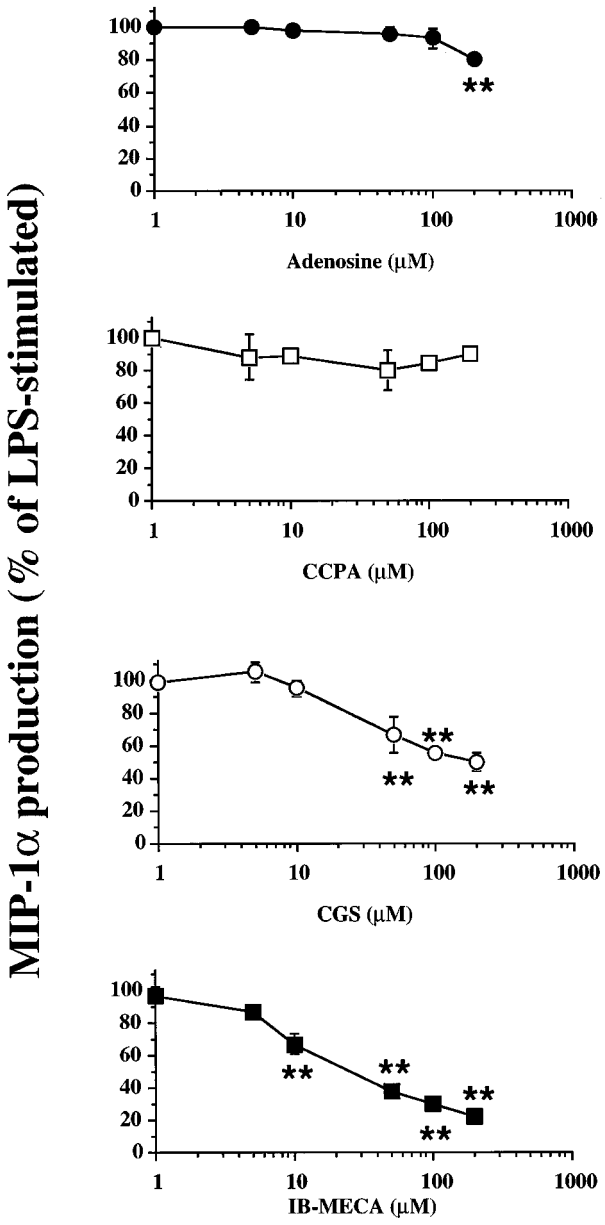

Figure 1 Effect of adenosine [ADO], the A_1 agonist CCPA, the A_2 agonist CGS, and the A_3 agonist IB-MECA on the production of MIP-1α at 3 h after stimulation with bacterial lipopolysaccharide LPS (10 ng ml^{-1}) in RAW macrophages. MIP-1α production in the absence of the inhibitors amounted to 6.6±1.3 ng ml^{-1}, and was considered 100%. $n=6-9$ wells from two to three independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ indicate significant inhibition of the production of MIP-1α.

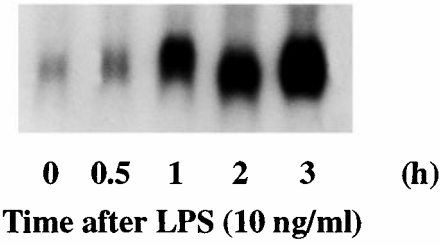

Figure 2 Effect of bacterial lipopolysaccharide LPS (10 ng/ml) on MIP-1α expression in RAW macrophages. Time-course of MIP-1α mRNA at 0–3 h after stimulation with bacterial lipopolysaccharide LPS (10 ng m^{-1}). Representative blots of $n=3-4$ blots are shown; 18S mRNA was unaffected by any of the treatments.

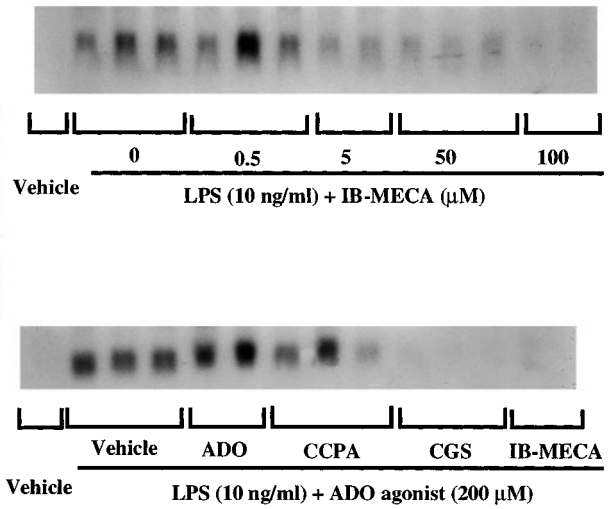

Figure 3 Effect of adenosine [ADO], the A_1 agonist CCPA, the A_2 agonist CGS, and the A_3 agonist IB-MECA on the production of MIP-1α mRNA in response to stimulation with bacterial lipopolysaccharide LPS (10 ng ml^{-1}) in RAW macrophages. (a) Dose-response showing the effect of 0.5, 5, 50 and 100 μM IB-MECA on the production of MIP-1α mRNA at 3 h after stimulation. (b) Effect of adenosine, CCPA, CGS and IB-MECA (200 μM each) on the production of MIP-1α mRNA at 3 h after stimulation. Representative blots of $n=3-4$ blots are shown; 18S mRNA was unaffected by any of the treatments.

used (ELISA *vs* Northern blotting) may be responsible for these discrepancies.

Of all the adenosine agonists tested, IB-MECA provided the most pronounced inhibition of MIP-1α production. Therefore, in subsequent studies we tested the effect of this agonist on the production of other inflammatory mediators (IL-12, IL-6, NO) by stimulated macrophages, and in an *in vivo* model of inflammation. A 24 h exposure to LPS (10 μg ml^{-1}) or IFN-γ (200 u ml^{-1}) alone did not induce detectable levels of IL-12 (p40 or p70) in RAW 264.7 macrophages (not shown). The combination of LPS (10 μg ml^{-1}) and IFN-γ (200 u ml^{-1}) induced the production of IL-12 p40 (Figure 4a), however, IL-12 p70 was not produced in detectable quantities even with this combination (not shown). Pretreatment of the cells 30 min before LPS + IFN-γ with IB-MECA caused a concentration-dependent suppression of IL-12 p40 production, as assessed at 24 h (Figure 4a). LPS (10 μg ml^{-1}) induced the release of IL-6 and nitrite (the breakdown product of NO) in the culture supernatants of the RAW 264.7 cells as measured 24 h after stimulation. IB-MECA, given 30 min before LPS, decreased IL-6 and nitrite production (Figure 4b and c). IB-MECA did not affect cell viability in any of these experiments as measured with the MTT assay (not shown). The more potent inhibition of IL-12 and MIP-1α and the less potent inhibition of NO and IL-6 production by IB-MECA may be consistent with two different A_3 receptor subtypes. However, the current studies were not designed to explore this possibility.

IB-MECA pretreatment protects mice against collagen-induced arthritis

Between days 26–35 after the first collagen immunization, the majority of the vehicle-treated animals progressively developed arthritis (Figure 5a–b). IB-MECA treatment (0.5 mg/kg once a day, i.p.) reduced the incidence of

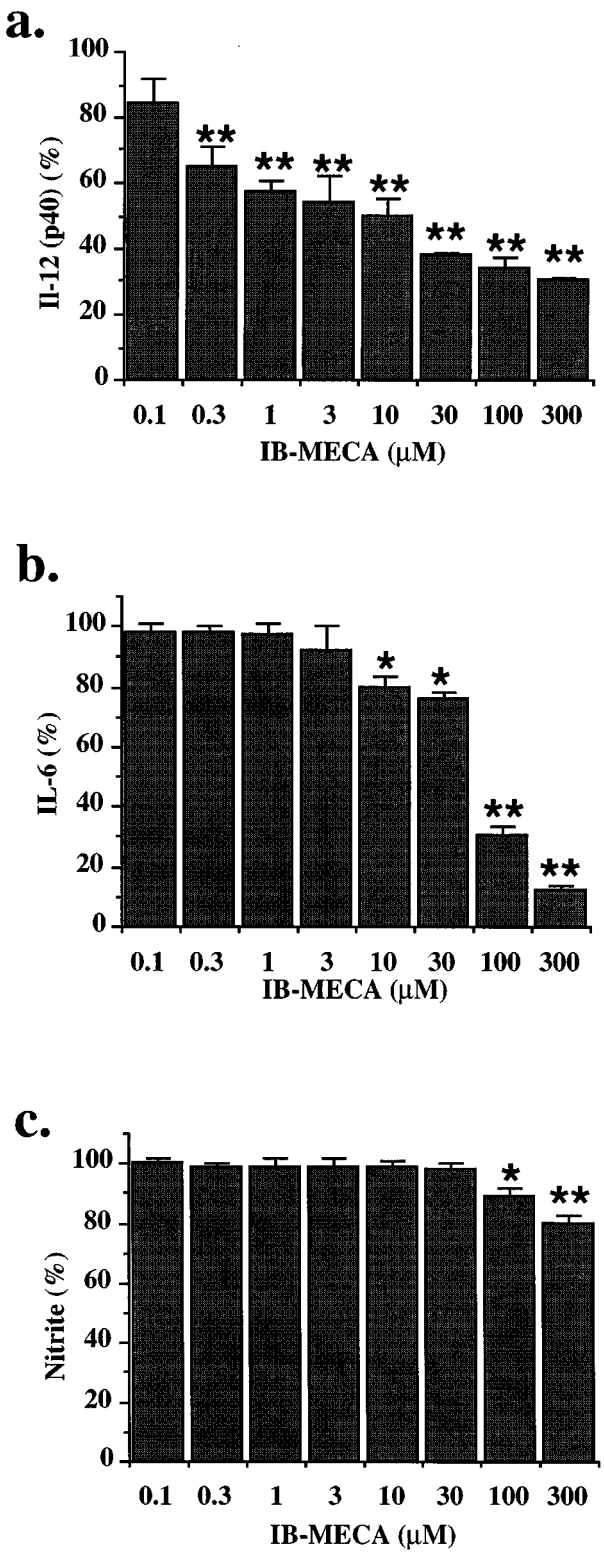

Figure 4 IB-MECA suppresses the production of inflammatory mediators in immunostimulated cultured RAW macrophages. Cells were stimulated with LPS (10 μg ml^{-1}) and IFN-γ (200 mU/ml) for 24 h for the measurement of IL-12 (p40), with LPS (10 μg ml^{-1}) for 24 h for IL-6 and nitrite measurements, or with LPS (10 ng/ml) and IFN-γ(200 mU ml^{-1}) for 3 h for the measurement of MIP-1α Absolute values of IL-12, IL-6 and nitrite in the absence of IB-MECA treatment amounted to 378 ± 23 pg ml^{-1}, 76 ± 4 ng ml^{-1} and 49 ± 5 μM respectively, and were considered 100%. $n=6-9$ wells from two to three independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ indicate significant inhibition by IB-MECA treatment.

arthritis (Figure 4a) and reduced the severity of the disease (Figure 5b). At day 35, histological evaluation of the paws in the vehicle-treated arthritic animals revealed signs of severe suppurative arthritis, with massive neutrophil, macrophage and lymphocyte infiltration. In addition, severe or moderate necrosis, hyperplasia and sloughing of the synovium was seen, with extension of the inflammation into the adjacent musculature (Figure 6a,b). In the IB-MECA treated animals, the degree of arthritis was significantly reduced: a moderate cell infiltration, coupled with mild to moderate necrosis and hyperplasia of the synovium was observed (Figure 6c). The reduced neutrophil infiltration was quantitated by measurement of paw myeloperoxidase. Arthritis caused an increase in myeloperoxidase levels by 89 ± 16 mU/mg protein ($n=12$). In the IB-MECA treated animals, there was a significantly lower degree of increase in the paw myeloperoxidase content at the end of arthritis (36 ± 6 mU/mg protein; $n=12$, $P<0.01$).

A significant increase in MIP-1α and IL-12 p40 levels was detected in the aqueous extracts of the arthritic paws at 35 days (Figure 7), while no detectable TNF-α or IL-12 p70 levels were found in the extracts at this stage (not shown). In accordance with the results in LPS-stimulated cells or LPS-challenged animals, IB-MECA treatment prevented the

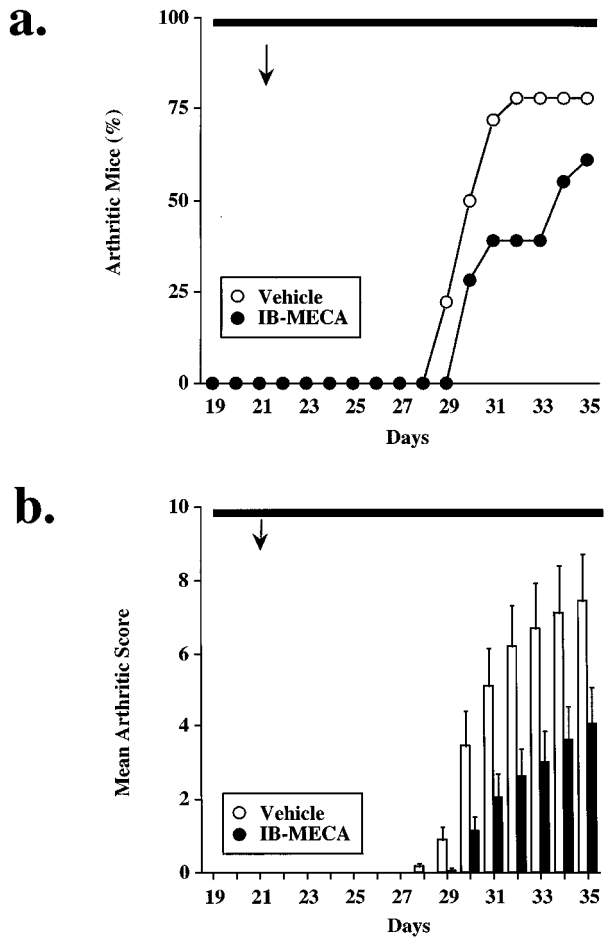

Figure 5 (a) IB-MECA suppresses collagen-induced arthritis in mice. The percentage of arthritic mice (mice showing clinical scores of arthritis >1) are represented. (b) Effect of IB-MECA on the severity of collagen-induced arthritis. Median arthritic score during collagen-induced arthritis. $n=10-12$. There was a significant increase in the arthritic score from day 28 ($^{*}P<0.01$), and there was a significant suppression of the arthritic score by IB-MECA from day 30 ($\#P<0.05$).

increase in IL-12 p40 and MIP-1α levels in the joints (Figure 7). Using immunohistochemistry (Figure 8a–c) and Western blotting of proteins in aqueous joint extracts (Figure 8d), we observed the appearance of nitrotyrosine-positive staining in the inflamed joints, but not in healthy control animals. IB-MECA treatment reduced the degree of nitrotyrosine staining (Figure 8).

Discussion

The signal transduction mechanisms triggered by various adenosine receptor subtypes are different in various cells. All adenosine receptors are G protein-coupled. Activation of A_2 receptors induces cyclic AMP (Collis & Hourani, 1993; Dubyak & El-Moatassim, 1993). An effect on cyclic AMP might have explained the inhibitory effect of the A_2 agonist on MIP-1α production, since dibutyril cyclic AMP can suppress the production of MIP-1α

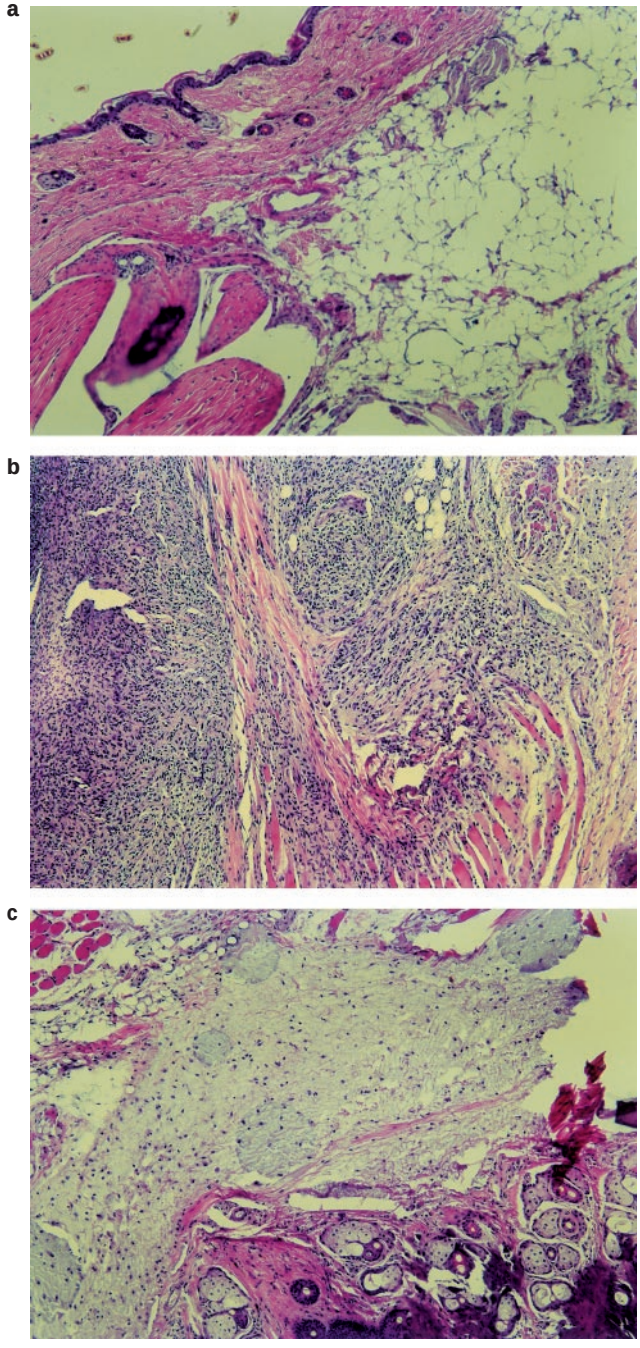

Figure 6 IB-MECA suppresses mononuclear cell infiltration in collagen-induced arthritis in mice. Representative histology of the paw (a) control; (b) arthritic; (c) IB-MECA treatment + arthritis. Sections were taken at 35 days. Note the reduction in the degree of mononuclear cell infiltration and myeloperoxidase content in the paws of the IB-MECA treated arthritic animals. Magnification: × 20.

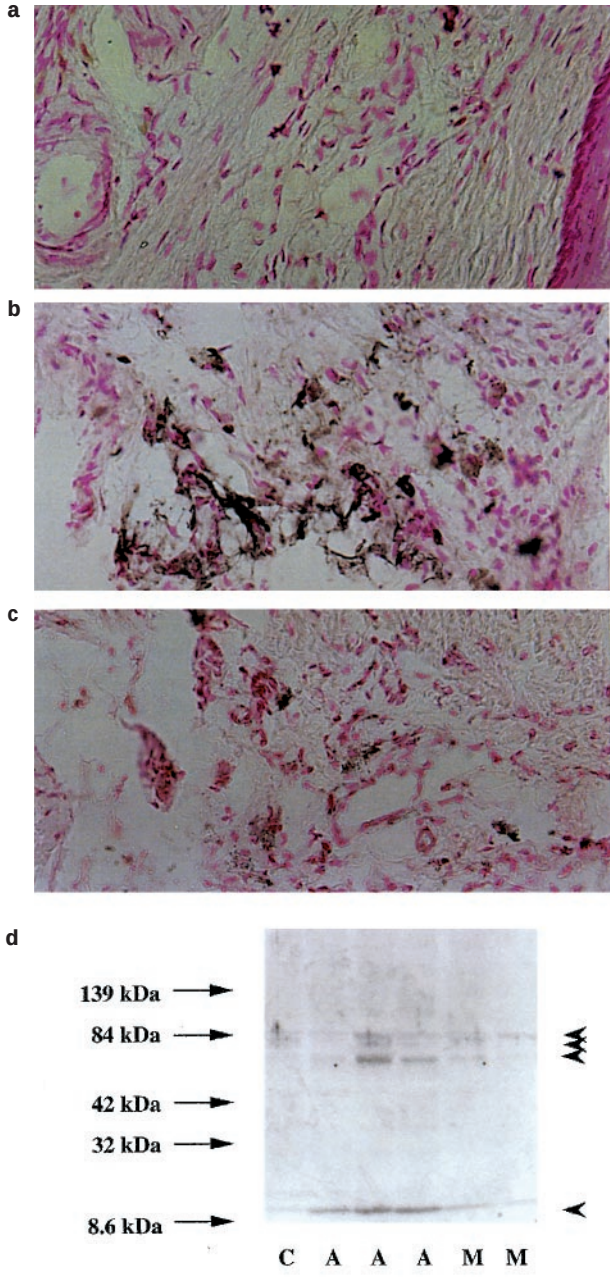

Figure 7 IB-MECA suppresses nitrotyrosine formation in the joints of mice subjected to collagen-induced arthritis. Nitrotyrosine immunostaining in (a) the paw of a control mouse, (b) paw of a mouse at 35 days of collagen-induced arthritis (c) and paw of an IB-MECA treated animal subjected to collagen-induced arthritis. In part (d), nitrotyrosine Western blots from paw extracts of control mice (C), mice after 35 days of collagen-induced arthritis (A), and IB-MECA treated mice subjected to collagen-induced arthritis (M) are shown. Note the marked increase in nitrotyrosine staining in the paws in arthritis, and the suppression of the staining with IB-MECA treatment. Also, note the increased tyrosine nitration of several proteins (indicated with arrowheads): three proteins (Mw: approximately 60–80 kDa), and a low molecular weight protein or protein fragment (Mw: approximately 10 kDa). Representative pictures or gels of $n = 3$ experiments are shown. For parts (a–c), magnification: × 40.

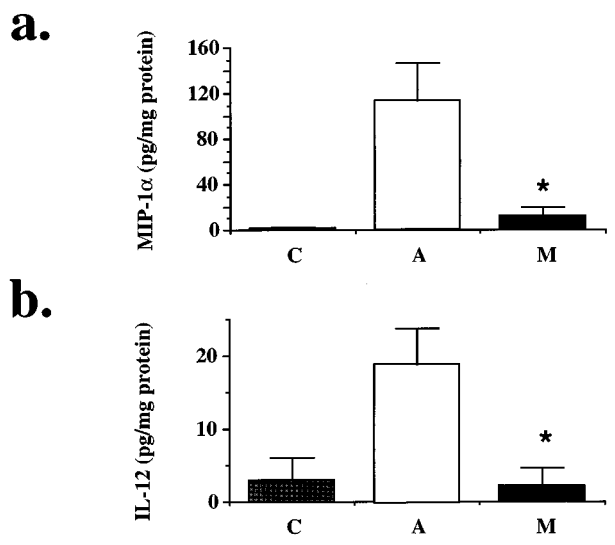

Figure 8 IB-MECA suppresses the production of inflammatory mediators in aqueous paw extracts of mice subjected to collagen induced arthritis. Levels of MIP-1α and IL-12 are shown in samples from control animals (C), from collagen-induced arthritis at 35 days (A), and IB-MECA treated mice subjected to collagen-induced arthritis (M) at 35 days. $n=10-12$. $^{*}P<0.05$ indicates suppression of IL-12 and MIP-1α production in arthritis by IB-MECA.

(Martin & Dorf, 1991). On the other hand, A_3 receptor stimulation in macrophages does not involve cyclic AMP, protein kinase A, nor the transcription factor NF-kappa B, while the composition of the AP-1 transcription complex can be altered by stimulation of this receptor subtype (Sajjadi *et al*., 1996). The delineation of the cellular signalling mechanism by which A_3 stimulation suppresses cytokine or MIP-1α production requires further studies.

The A_1 agonist was without significant inhibitory effect on the production of MIP-1α in our conditions, and adenosine was a weak inhibitor. Adenosine is mainly an A_1 receptor agonist, and a modest A_2 and A_3 agonist (Collis & Hourani, 1993). Our studies with the adenosine deaminase inhibitor demonstrated that rapid degradation of adenosine, by adenosine deaminase present in foetal calf serum are not responsible for the low level of the inhibition. We propose that adenosine's weaker potency on A_2 and A_3 receptors, may be responsible for the low efficacy of adenosine on MIP-1α production.

In addition to the inhibition of MIP-1α production, the current study also demonstrated that IB-MECA suppressed IL-12, IL-6 and NO production in immunostimulated cultured macrophages. Recently, adenosine receptor agonists, in a dose-dependent manner characteristic of the A_3 receptor, have been shown to block the endotoxin-mediated induction of the TNF-α gene and protein expression in the murine J774.1 macrophage cell line (McWhinney *et al*., 1996). In a recent study, we reported that stimulation of A_3 receptors by the selective A_3 receptor agonist IB-MECA decreased plasma TNF-α and increased IL-10 in LPS-treated mice (Haskó *et al*., 1996). The results of the present study extend these previous observations.

The *in vitro* studies in RAW 264.7 cells only represent an approximation of the characteristics of the primary or resident macrophages, or the *in vivo* situation in arthritis, where resident macrophages play a role in the production of chemokines, cytokines and NO. Nevertheless, the modulation of chemokine, cytokine and NO production by adrenergic, purinergic receptors shows remarkable similarities in monocytic/macrophage cells and in primary monocytes/macrophages (see: Haskó & Szabó, 1998). In the current study, the *in vivo* measurements of NO, IL-12 and MIP-1α production in the joint extracts also showed a good correlation with our *in vitro* findings in the RAW cells. It is conceivable that in the arthritis model, suppression of IL-12, MIP-1α and NO production by IB-MECA represent important, interrelated modes of anti-inflammatory action. It is well established that IL-12 plays a central role in the early events of the induction phase of arthritis. This was best demonstrated by the experiments of Germann *et al*. (1995), who showed that IL-12 could replace mycobacteria and trigger autoimmune arthritis in DBA/1 mice immunized with chicken CII in incomplete Freund's adjuvant. Furthermore, these authors showed that IL-12 was able to potentiate the arthritis induced with chicken CII in CFA. Further evidence for the importance of IL-12 in adjuvant-induced arthritis is that the course of the disease was suppressed in IL-12 deficient mice, or in mice treated with anti-mIL-12 antibodies (McIntyre *et al*., 1996; Joosten *et al*., 1997). Once the inflammatory process has been initiated by IL-12, macrophages and other cell-types become activated and release a variety of mediators such as TNF-α, IL-6, IL-1, MIP-1α, and NO, which maintain the active inflammatory state. From these mediators, the chemokine MIP-1α, released either from fibroblasts or macrophages, appears to be an important pathogenic factor in the development of arthritis, due mainly to its chemotactic effect on inflammatory cells (Al-Mughales *et al*., 1996; Kunkel *et al*., 1996). The reduction of neutrophil recruitment (demonstrated by the suppression of paw myeloperoxidase levels in the current study), may be, at least in part, due to suppression of paw MIP-1α levels (Kasama *et al*., 1995; Kunkel *et al*., 1996). The inhibitory effect of the A_3 receptor agonist on NO release may also be considered beneficial, as the overproduction of this free radical, and its reactive reaction product, peroxynitrite, have been shown to contribute to the pathophysiology of inflammatory joint disease (Kaur *et al*., 1994; McCartney-Francis *et al*., 1994; Connor *et al*., 1995). Nitrotyrosine formation is generally accepted as a specific 'footprint' of peroxynitrite (Beckman *et al*., 1996), although recent studies proposed additional pathways of tyrosine nitration, related to myeloperoxidase-dependent conversion of nitrite to NO_2Cl and NO_2 (Halliwell, 1997; Eiserich *et al*., 1998). Nitrotyrosine may rather serve as a collective indicator for the production of reactive nitrogen species (Halliwell *et al*., 1997) in the joints.

The present study provides evidence that stimulation of A_3 and A_2 receptors inhibits MIP-1α production, by suppressing of its mRNA expression. Furthermore, the present study provides evidence that an agonist of A_3 receptors prevents inflammation in collagen-induced arthritis. The mechanisms of this anti-inflammatory effect include the down-regulation of the production of the pro-inflammatory mediators MIP-1α, IL-12, and NO. Anti-inflammatory properties of drugs such as methotrexate (Cronstein *et al*., 1991; 1993; Cronstein, 1994), sulphasalazine (Gadangi *et al*., 1996), and adenosine kinase inhibitors (Firestein *et al*., 1994; Cronstein *et al*., 1995) are related to their ability to release adenosine at the sites of inflammation. We hypothesize that these anti-inflammatory effects may be, at least in part, related to A_3 and A_2

receptor stimulation. We propose that stimulation of adenosine receptor subtypes A_3 and A_2 may be a strategy worthy of further evaluation for the suppression of acute and chronic inflammatory disorders.

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