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The adenosine A_3 receptor agonist, N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide, is protective in two murine models of colitis

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Abstract

This study evaluated the effects of the adenosine A_3 receptor agonist, N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (IB-MECA), in two murine models of colitis, the dextran sodium sulphate-induced colitis and the spontaneous colitis found in interleukin-10 gene deficient mice. IB-MECA was given orally twice a day at a dose of either 1 or 3 mg/kg/day. Evaluation of colon damage and inflammation was determined grossly (body weight, rectal bleeding) and biochemically (colon levels of myeloperoxidase, malondialdehyde, chemokines and cytokines). There was significantly increased inflammatory cell infiltration into the colon associated with an increase in colon levels of cytokines and chemokines; with subsequent free radical related damage in both dextran sodium sulphate-induced colitis and 10-week-old interleukin-$10^{-/-}$ mice. IB-MECA protected in both models against the colitis induced inflammatory cell infiltration and damage and attenuated the increases in colon inflammatory cytokine and chemokine levels. Thus activation of the adenosine A_3 receptor is effective in protecting against colitis.
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1. Introduction

Adenosine is a purine nucleoside released from cells in response to metabolic stress (Dubyak and el-Moatassim, 1993) or from the sympathetic nervous system (Hasko and Szabo, 1998), and occupies adenosine A_1, A_{2A}, A_{2B} and A_3 receptors on target cells. Adenosine has been shown to affect almost all aspects of an immune response (Apasov et al., 1995; Cronstein, 1994; Hasko and Szabo, 1998). Adenosine and its analogues can affect the development of a variety of inflammatory disease including endotoxin shock (Hasko et al., 1998), rheumatoid arthritis (Szabo et al., 1998), pleural inflammation (Schrier et al., 1990) and uveitis (Marak et al., 1988). The effects of adenosine are partly mediated by the inhibition of deleterious immune-mediated processes, including the release of pro-inflammatory cytokines and free radicals (Hasko et al., 2000). These anti-inflammatory effects of adenosine have been mimicked using a selective agonist of the adenosine A_3 receptor, N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (IB-MECA). IB-MECA has been shown to decrease interleukin-12 and interferon-γ production and to prevent lethality in endotoxemic mice (Hasko et al., 1998) as well as to suppress macrophage inflammatory protein-1α production and collagen-induced arthritis (Szabo et al., 1998).

There are several murine models of intestinal inflammation that resemble human inflammatory bowel diseases such as Crohn's disease and ulcerative colitis. Both these diseases are characterized by chronically relapsing inflammation of the bowel of unknown origin. In this study, we utilize two models of colitis, the first model of inflammatory bowel disease was induced by the oral administration of dextran sulphate sodium. Colitis induced by dextran sodium sulphate exhibits lymphoid hyperplasia, inflammatory cell infiltration, focal crypt damage, epithelial injury and ulceration (Cooper et al., 1993; Dieleman et al., 1998; Okayasu

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et al., 1990). Dextran sodium sulphate has toxic epithelial effects coupled with phagocytosis, which leads to stimulation of lamina propria cells and increased production of pro-inflammatory cytokines (Dieleman et al., 1998). The cytokine profile of dextran sodium sulphate-induced colitis was found to be similar to that found in human inflammatory bowel disease with an increase in the levels of Th1 cytokine mRNA transcripts including interleukin-1, interleukin-12, interferon-γ and tumor necrosis factor-α (Egger et al., 2000). The second model is a spontaneous model whereby the interleukin-10 gene deficient mouse develops colitis shortly after weaning at 4 weeks of age (Madsen et al., 1999a,b). By 8 weeks of age the colitis is well established with mucosal ulceration and extensive immune cell infiltration with production of inflammatory cytokines. We studied the effectiveness of IB-MECA on the colon inflammation of inetrleukin-$10^{-/-}$ mice treated from 8 to 10 weeks of age to determine its therapeutic potential, a model which has been used previously to demonstrate the effectiveness of poly (ADP-ribose) polymerase inhibitors as a therapy for colitis (Jijon et al., 2000). Although both murine models of inflammatory bowel disease differ from the human disease, they are widely used as pre-clinical models for testing the efficacy of treatments against inflammatory bowel disease (Bennett et al., 1997; Cooper et al., 1993; Elson et al., 1995).

In this study we investigate the potential therapeutic efficacy of the adenosine A_3 receptor agonist, IB-MECA, against inflammatory bowel disease.

2. Materials and methods

Reagents were obtained from the following sources: dextran sodium sulphate (MW 40,000) was from ICN (ICN Pharmaceuticals, California, USA); IB-MECA, human myeloperoxidase, 1,1,3,3-tetramethoxypropane, thiobarbituric acid, sodium dodecyl sulfate, tetra-methyl-benzidine, hexadecyltrimethylammonium bromide and hydrogen peroxide were from Sigma (St. Louis, MO, USA.); BALB/c mice were from Taconic farms (Germantown, NY, USA); interleukin-$10^{-/-}$ mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA) Specific cytokine assay kits were from R&D Systems (Minneapolis, MN, USA).

2.1. Induction of colitis and treatment

All animal experiments were carried out in accordance with the guidelines published by the NIH in "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) and with the approval of Inotek's Institutional Animal Care and Use Committee. The animals are housed in rooms at a controlled temperature and light/dark cycle.

Male BALB/c mice, 8 weeks of age were fed 5% dextran sulfate sodium, molecular weight 30–40 kDa dissolved in distilled water ad libitum throughout the experiment. IB-MECA was administered by gavage at either 1 or 3 mg/kg/day b.i.d. in distilled water with control mice being gavaged with vehicle. The dose of each compound was based on recent studies testing these compounds in rodent models of endotoxic shock and arthritis (Hasko et al., 1998; Szabo et al., 1998). Intake of the dextran sodium sulphate solution was monitored throughout the experiments was found to be unchanged between experimental groups (data not shown).

Male interleukin-$10^{-/-}$ mice were purchased at 6 weeks of age and allowed to acclimatize for 2 weeks until reaching 8 weeks of age. The mice were then treated with IB-MECA at 1 or 3 mg/kg/day in distilled water for 2 weeks. Once mice were 10 weeks of age they were sacrificed and the colon taken for analysis.

2.2. Evaluation of colitis severity and drug effects

The parameters recorded in the experiments were body weight, colon length, mortality, gross colon damage score and fresh rectal bleeding evaluated by ocular inspection. Mice were weighed on day 1 and day 10 with the subsequent colitis-induced weight loss expressed as a percentage of the mouse original weight. The mice were killed by cervical dislocation and the colon resected between the ileocecal junction and the proximal rectum, close to its passage under the pelvisternum. The colon was placed on a nonabsorbent surface and measured with a ruler. The feces was then removed and assessed and scored for damage and blood as described previously (Mabley et al., 2001) (0 = well-formed pellets no colon damage, 1 = colon with small amount of blood present mixed with feces, 2 = colon with large amount of blood present with feces, 3 = colon filled with blood no feces). The colonic biopsies were then taken for biochemical analysis.

2.3. Myeloperoxidase activity

Myeloperoxidase activity, an indicator of tissue neutrophil accumulation, was measured as previously described (Liaudet et al., 2002). Colon biopsies were homogenized (50 mg/ml) in 0.5% hexadecyltrimethylammonium bromide in 10 mM 3-*N*-morpholinopropanesulfonic acid and centrifuged at 15,000 × *g* for 40 min. The suspension was then sonicated three times for 30 s. An aliquot of supernatant (20 μl) was mixed with a solution of 1.6 mM tetra-methylbenzidine and 1 mM hydrogen peroxide. Activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37 °C, using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as milliunits of myeloperoxidase activity per mg protein, as determined with the Bradford assay.

2.4. Malondialdehyde assay

Malondialdehyde formation was utilized to quantify the lipid peroxidation in the colon and measured as thiobarbituric

acid-reactive material as described previously (Liaudet et al., 2002). Tissues were homogenized (100 mg/ml) in 1.15% KCl buffer. 200 μl of the homogenates were then added to a reaction mixture consisting of 1.5 ml 0.8% thiobarbituric acid, 200 μl 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (pH 3.5) and 600 μl distilled H_2O. The mixture was then heated at 90 °C for 45 min. After cooling to room temperature, the samples were cleared by centrifugation (10 000 × *g*, 10 min) and their absorbance measured at 532 nm, using 1,1,3,3-tetramethoxypropane as an external standard. The level of lipid peroxides was expressed as nmol malondialdehyde/mg protein (Bradford assay).

2.5. Colon cytokine levels

A third colon biopsy was removed and snap frozen in liquid nitrogen, the sample was then homogenized in 700 μl of a Tris–HCl buffer containing protease inhibitors. Samples were centrifuged for 30 min and the supernatant frozen at −80 °C until assay. Cytokine levels were determined using commercially available kits as described previously (Mabley et al., 2002b).

2.6. Statistical analyses

Data are presented as means ± S.E.M. Statistical analysis was performed using the χ^2 test, analysis of variance with Bonferroni's correction, or Student's *t*-test as appropriate; $P < 0.05$ was considered significant.

3. Results

IB-MECA treatment dose-dependently protected dextran sodium sulphate-treated mice from the clinical signs of colitis. IB-MECA treated mice had reduced weight loss and rectal bleeding, longer colon lengths and a marked attenuation of the gross colon damage signs of colitis (Table 1). Myeloperoxidase activity was found to be fourfold higher in colon biopsies from dextran sodium sulphate-treated mice as compared to control mice indicative of an invasion of the colon mucosa by polymorphonuclear leukocytes (Fig. 1A). Both doses of IB-MECA significantly reduced the colon myeloperoxidase levels (Fig. 1A). Malondialdehyde formation was used to quantify lipid peroxidation in the colon mucosa, a measure of oxidative stress. Colonic malondialdehyde levels were found to be elevated threefold by dextran sodium sulphate-treatment (Fig. 1B) and IB-MECA significantly reduced the increased malondialdehyde levels (Fig. 1B).

Dextran sodium sulphate-treated mice had greatly increased colonic levels of inflammatory cytokines (Fig. 2A) and chemokines (Fig. 2B). Untreated non-colitic con-

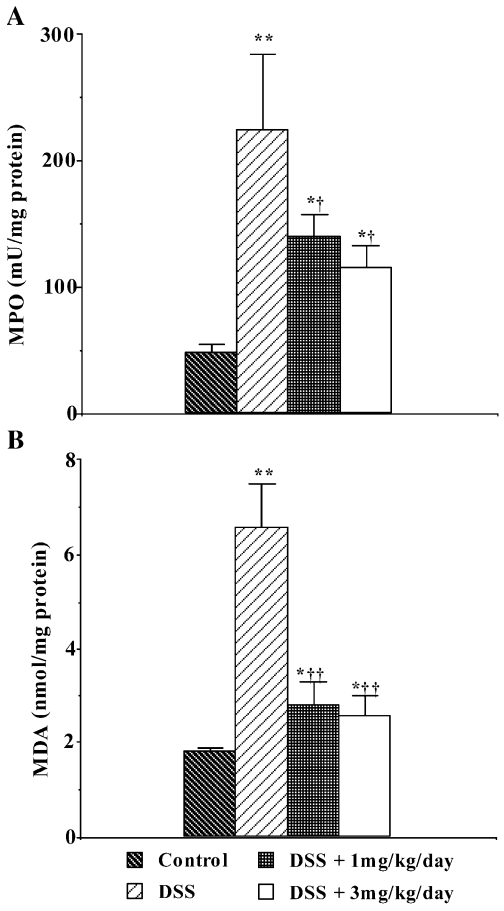

Fig. 1. IB-MECA reduces the levels of (A) MPO and (B) MDA in the colons of mice with an acute colon inflammation induced by dextran sodium sulphate (DSS). Mice were exposed to dextran sodium sulphate ad libitum for 10 days, treatment with IB-MECA (1 or 3 mg/kg/day) commenced on day 1. Results are expressed as mean ± S.E.M. from 8–15 animals. Statistical analysis was conducted using Student's unpaired *t*-test where $P < 0.05$ was considered significant. *$P < 0.05$, **$P < 0.01$ vs. untreated animals and †$P < 0.05$, ††$P < 0.01$ vs. dextran sodium sulphate treated animals.

Table 1
IB-MECA attenuates the symptoms of colitis

Groups	% Decrease in body weight	Colon length (cm)	Rectal bleeding (%)	Gross histological score (median)
Untreated	−3.5 ± 3.5	7.5 ± 0.2	0	0
DSS treated	24.9 ± 2[a]	4.3 ± 0.3[a]	80[a]	3
DSS + IBMECA (1 mg/kg/day)	23.5 ± 1.1[a]	4.9 ± 0.2[a]	60[a]	1
DSS + IBMECA (3 mg/kg/day)	15.3 ± 1.4[b,c]	5.6 ± 0.3[b,c]	20[b,c]	1

Mice were exposed to dextran sodium sulphate (DSS) ± IB-MECA (1 or 3 mg/kg/day) administered orally starting on day 1. On day 10, the colon was dissected out and measured and the scored (0 = well-formed pellets no colon damage, 1 = colon with small amount of blood present mixed with faeces, 2 = colon with large amount of blood present with faeces, 3 = colon filled with blood no faeces). The data is expressed as mean ± S.E.M. from 8 to 15 animals. Statistical analysis was conducted using Student's unpaired *t*-test or Fisher's exact test where $P < 0.05$ was considered significant.

[a] $P < 0.01$ vs. untreated animals.

[b] $P < 0.05$ vs. untreated animals.

[c] $P < 0.01$ vs. dextran sodium sulphate-treated animals.

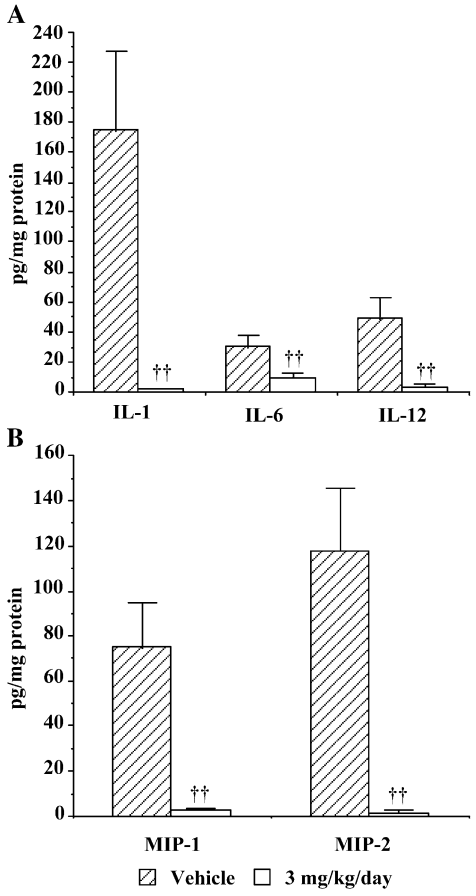

Fig. 2. Effect of IB-MECA on colon (A) cytokine and (B) chemokine levels following dextran sodium sulphate (DSS)-induced colon inflammation. Cytokine levels were determined in colon biopsies from mice treated for 10 days with dextran sodium sulphate ± IB-MECA (3 mg/kg/day). Results are expressed as mean ± S.E.M. from 10 animals. Statistical analysis was conducted using Student's unpaired *t*-test where $P<0.05$ was considered significant. ††$P<0.01$ vs. dextran sodium sulphate treated animals.

trol mice had undetectable colon levels of chemokines or cytokines (data not shown). IB-MECA (3 mg/kg/day) significantly reduced the colon levels of the pro-inflammatory cytokines, interleukin-1, interleukin-6, interleukin-12 and the chemokines, macrophage inflammatory protein-1α and macrophage inflammatory protein-2.

Delaying the start of the IB-MECA treatment by 5 days also protected against dextran sodium sulphate-induced colon inflammation and oxidative damage but statistically significant protection was observed only at 3 mg/kg/day. The reduced colon length (4.6 ± 0.2 cm) was increased to 5.5 ± 0.4 ($P=0.09$) and 6.6 ± 0.4 ($P<0.001$) with 1 and 3 mg/kg/day IB-MECA, respectively. Colon myeloperoxidase (287 ± 86 mU/mg protein) and malondialdehyde (5.1 ± 0.5 nmol/mg protein) levels were reduced to 131 ± 29 ($P=0.2$) and 4.6 ± 0.5 ($P=0.5$) with 1 mg/kg and 66 ± 19 ($P=0.04$) and 3.6 ± 0.4 ($P=0.04$) with 3 mg/kg/day IB-MECA. As observed when 3 mg/kg/day IB-MECA treatment started on day 1; starting on day 5 IB-MECA treatment reduced colon levels of macrophage inflammatory protein-1α (22 ± 6 to 4 ± 0.6 pg/mg protein; $P<0.01$), macrophage inflammatory protein-2 (43 ± 10 to 5 ± 1 pg/mg protein; $P<0.001$), interleukin-1 (147 ± 31 to 29 ± 4 pg/mg protein; $P<0.001$) and interleukin-6 (32 ± 11 to 3 ± 0.5 pg/mg protein; $P<0.05$).

Interleukin-$10^{-/-}$ mice had significantly shorter colons and increased immune cell infiltration and oxidative damage at 10 weeks of age as compared to C57/BL6J control mice (Fig. 3). Treatment with IB-MECA for the 2 weeks prior to

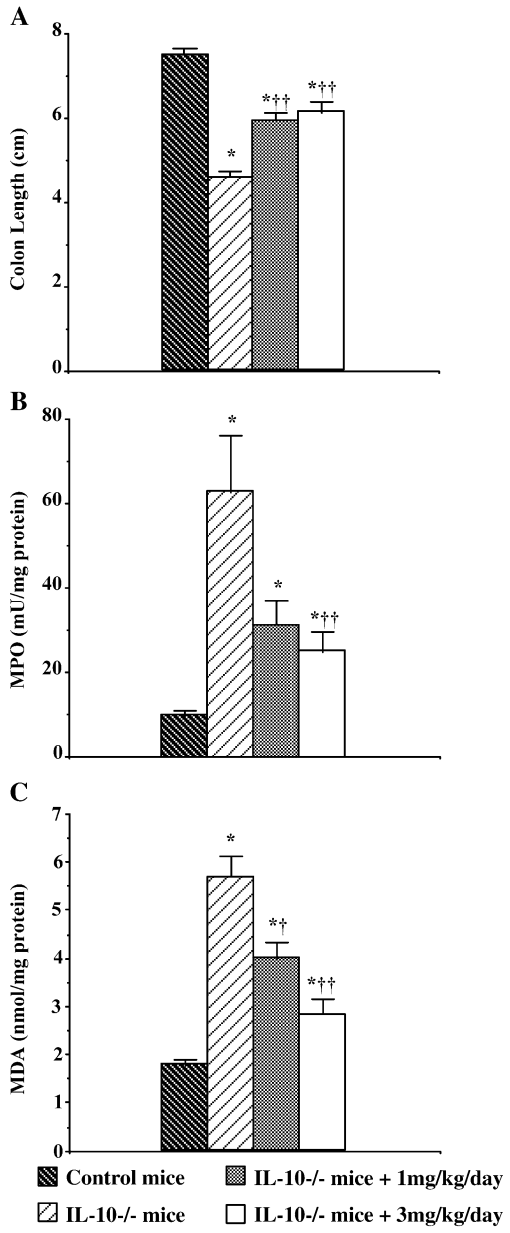

Fig. 3. Effect of IB-MECA on (A) colon length and colon levels of (B) MPO and (C) MDA in interleukin-$10^{-/-}$ mice. The 8-week-old interleukin-$10^{-/-}$ were treated with IB-MECA (1 or 3 mg/kg/day) for 2 weeks, at 10 weeks of age the mice were sacrificed and the colons removed and analyzed. Results are expressed as mean ± S.E.M. from 10 animals. Statistical analysis was conducted using Student's unpaired *t*-test where $P<0.05$ was considered significant. *$P<0.05$, vs. control C57/BL6J mice and †$P<0.05$, ††$P<0.01$ vs. interleukin-$10^{-/-}$ mice.

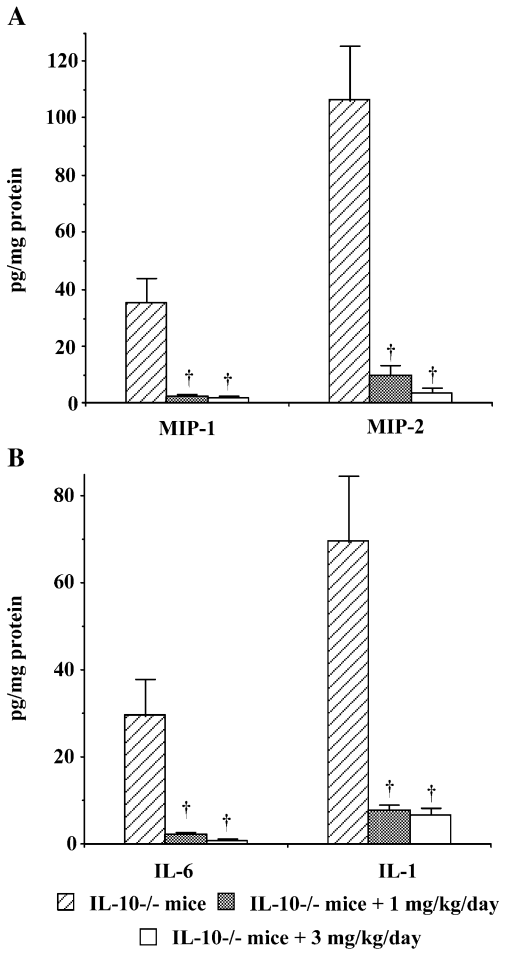

Fig. 4. Effect of IB-MECA on colon (A) chemokine and (B) cytokine levels from interleukin-$10^{-/-}$ mice. Chemokine/cytokine levels were determined from colon biopsies from 10-week-old interleukin-$10^{-/-}$ mice treated for 2 weeks ± IB-MECA (1 or 3 mg/kg/day). Results are expressed as mean ± S.E.M. from 10 animals. Statistical analysis was conducted using Student's unpaired *t*-test where $P<0.05$ was considered significant. †$P<0.01$ vs. vehicle treated interleukin-$10^{-/-}$ mice.

sacrifice dose dependently increased the colon length (Fig. 3A) and decreased immune cell infiltration (Fig. 3B) and oxidative damage (Fig. 3C). This protective effect of IB-MECA was associated with a marked decrease in colon levels of the chemokines (Fig. 4A), macrophage inflammatory protein-1α and macrophage inflammatory protein-2, along with the inflammatory cytokines interleukin-6 and interleukin-1 (Fig. 4B).

4. Discussion

We have demonstrated that activation of the adenosine A_3 receptor exerted anti-inflammatory effects in colitis. IB-MECA treatment reduced colon infiltration by immune cells and the oxidative damage associated with such an infiltration. Physical symptoms of colitis including weight loss and rectal bleeding were reduced in mice treated with IB-MECA. IB-MECA protective effects were not just prophylactic as IB-MECA protected against colitis in a post treatment paradigm. Delaying treatment with IB-MECA for 5 days in the dextran sodium sulphate-induced colitis model reduced both the immune cell infiltration and the oxidative damage of the colon. Similarly in the interleukin-$10^{-/-}$ mice, which at 8 weeks of age have established colitis, 2 weeks treatment with IB-MECA is able to partially reduce the infiltration of immune cells into and oxidative damage of the colon. The marked effects of IB-MECA on colon cytokine levels in both colitis models may provide an explanation for the protective effects of this adenosine A_3 receptor agonist.

Adenosine A_3 receptors are present on monocytes and macrophages (McWhinney et al., 1996; Sajjadi et al., 1996) and the adenosine-mediated reduction in tumor necrosis factor-α production following lipopolysaccharide stimulation of a human monocytes cell line or a mouse macrophage cell line was found to be mainly an A_3 receptor mediated process (Sajjadi et al., 1996). Adenosine receptor agonists also differentially regulated macrophage production of interleukin-10, tumor necrosis factor-α and nitric oxide production in vitro, effects that were also seen in endotoxemic mice in vivo (Hasko et al., 1996). Adenosine also inhibits interleukin-12, macrophage inflammatory protein-1α and interferon-γ production from macrophages (Hasko et al., 1998, 2000; Szabo et al., 1998) with IB-MECA treatment closely mimicked these anti-inflammatory effects (Broussas et al., 1999; Hasko et al., 1996, 1998; Szabo et al., 1998).

Inflammatory cytokines play a central role in inflammatory bowel disease (Hans et al., 2000). Anti-interleukin-12 antibodies have proved very effective in preventing experimental colitis in mice (Fuss et al., 1999) with anti-interferon-γ (Fuss et al., 1999), anti-interleukin-1 (Kojouharoff et al., 1997) or anti-tumor necrosis factor-α (Olson et al., 1995) also proving effective. IB-MECA markedly reduced the increased colon levels of the inflammatory cytokines interleukin-12, interleukin-1 and interleukin-6 in both dextran sodium sulphate-induced colitis and in the interleukin-$10^{-/-}$ mice and may indicate a potential mechanism of action by which IB-MECA protects against colitis. In both murine models of colitis there are markedly increased colon levels of the chemokines macrophage inflammatory protein-1α and macrophage inflammatory protein-2, which play a role in the innate and adaptive immune response because of their ability to recruit, activate and co-stimulate T cells and monocytes (Ward et al., 1998). Levels of both macrophage inflammatory protein-1α and macrophage inflammatory protein-2 were significantly reduced with IB-MECA treatment indicating that IB-MECA may be able to alter the development of chemotactic gradients, thereby acting as a powerful down-regulator of leukocyte trafficking in inflammatory conditions. However, the protective effects of IB-MECA against colitis in interleukin-$10^{-/-}$ mice indicate that IB-MECA's protection is not mediated through changes

in interleukin-10 levels. Adenosine and IB-MECA have also been shown to reduce macrophage nitric oxide formation (Hasko et al., 1996), an effect that may contribute to its effectiveness in colitis as inhibition of inducible nitric oxide synthase activity (Zingarelli et al., 1998, 1999) or scavenging peroxynitrite (Mabley et al., 2002a) reduces colitis susceptibility.

There has been indirect evidence to suggest that adenosine may be protective in colitis. Several drugs used in the treatment of colitis, including adenosine kinase inhibitors (Siegmund et al., 2001), methotrexate (Egan et al., 1999), and sulfasalazine (Axelsson et al., 1998) all have been proposed to release adenosine and thereby exert their beneficial effects. Our data with the specific adenosine A_3 receptor agonist, IB-MECA, suggest that adenosine protective effects, at least in part, are mediated through activation of the A_3 receptor subtype. The development of a specific a human A_3 receptor agonist may therefore be a potential therapy in the treatment of inflammatory bowel disease in humans.

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