The Blockade of Adenosine Deaminase Ameliorates Chronic Experimental Colitis through the Recruitment of Adenosine A$_{2A}$ and A$_{3}$ Receptors

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ABSTRACT

Adenosine modulates immune/inflammatory reactions. This study investigates the expression of adenosine deaminase in the inflamed colon, the effects of adenosine deaminase inhibitors on established colitis, and the recruitment of adenosine receptors by endogenous adenosine after adenosine deaminase blockade. Adenosine deaminase expression was determined by Western blot. The effects of 4-amino-2-(2-hydroxy-1-decyl)pyrazole[3,4-d]pyrimidine (APP; a novel adenosine deaminase inhibitor), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; a reference adenosine deaminase inhibitor), dexamethasone, and selective adenosine receptor antagonists were tested in rats with 2,4-dinitrobenzenesulfonic acid-induced colitis. Systemic (food intake, body and spleen weight) and colonic (macroscopic/microscopic damage, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and malondialdehyde (MDA)) inflammatory parameters were assessed. Test drugs were administered intraperitoneally for 6 days, starting at day 5 from colitis induction. Adenosine deaminase was detected in normal colon, and its expression was increased in inflamed tissues. Colitis was associated with decreased food intake and body weight, augmented spleen weight, and increased levels of colonic TNF-α, IL-6, and MDA. APP or EHNA, but not dexamethasone, improved food intake and body weight. APP, EHNA, and dexamethasone counteracted the increments of spleen weight, ameliorated macroscopic and microscopic indexes of inflammation, and reduced TNF-α, IL-6, and MDA levels. The beneficial effects of APP and EHNA on inflammatory parameters were prevented by the pharmacological blockade of A$_{2A}$ or A$_{3}$ receptors, but not A$_{1}$ or A$_{2B}$. The present results show that: 1) bowel inflammation is associated with an enhanced adenosine deaminase expression; and 2) the anti-inflammatory actions of adenosine deaminase inhibitors against chronic established colitis depend on the sparing of endogenous adenosine, leading to enhanced A$_{2A}$ and A$_{3}$ receptor activation.

Introduction

Inflammatory bowel diseases (IBDs) are chronic, idiopathic inflammations of the enteric wall associated with severe alterations of the enteric tissue architecture and functions (Neuman, 2007). These disorders are characterized by varying periods of active disease or flare-ups and remission, with great impact on well-being and a patient’s quality of life (Neuman, 2007). Although the precise etiological factors leading to the onset of these chronic inflammatory conditions remain unclear, increasing evidence supports the concept that IBDs arise from altered and complex interactions of environmental, genetic, and immunological factors (Torres and Rios, 2008). In the presence of inflammatory bowel disorders, the physiological balance of enteric immune system seems completely unsettled (Baumgart and Carding, 2007). In particular, a marked polarization of the enteric immune response toward an uncontrolled increase in the production of proinflammatory mediators, which overrides the endoge-
nous anti-inflammatory and/or healing pathways, has been observed (Torres and Rios, 2008). Such mediators can trigger the biosynthesis of further downstream factors, deeply involved in the propagation and maintenance of the inflammatory process, resulting in an excessive degree of collateral injuries to the intestinal mucosa (Camuesco et al., 2004).

As a consequence of massive bowel inflammation, the host attempts to counteract the uncontrolled immune reactions through the activation of various mechanisms. Several lines of evidence indicate that adenosine can play a significant part in the mitigation of abnormal inflammatory responses (Antonioli et al., 2008a). Indeed, under inflammatory conditions, adenosine, through dynamic changes in the expression and/or function of its ectoenzymes, participates actively in a fine-tuning of immune cellular activities, thus playing an important role in limiting the inflammatory insults to host tissues (Desrosiers et al., 2007; Antonioli et al., 2008b).

On the other hand, a close correlation has been found between the severity of inflammation and a local increase in both expression and activity of adenosine deaminase, the enzyme responsible for conversion of endogenous adenosine into inosine, which leads to a decreased availability of biologically active adenosine (Conlon and Law, 2004; Desrosiers et al., 2007). Based on this knowledge, the pharmacological inhibition of adenosine deaminase is regarded as a novel therapeutic approach to counteract inflammation in several pathological conditions (Adanin et al., 2002; Law et al., 2003; Kayhan et al., 2008). In line with this concept, we previously provided evidence on the protective effects exerted by drugs acting as adenosine deaminase inhibitors against the onset of experimental colitis.

In particular, we observed that, when started before the induction of colitis, the blockade of adenosine deaminase resulted in a significant amelioration of several inflammatory parameters (Antonioli et al., 2007).

Our previous experience has led to a number of issues that deserve further investigation. In particular, the following remain to be assessed: 1) the extent of adenosine deaminase expression in inflamed colonic tissue; 2) the anti-inflammatory efficacy of adenosine deaminase inhibitors in the setting of established colitis; and 3) the receptor pathways mediating the anti-inflammatory effects resulting from adenosine deaminase blockade.

To select appropriate doses of adenosine deaminase inhibitors, the potencies of erythro-9-(2-hydroxy-3-nonyl)adenine (EINA) and 4-amino-2-(2-hydroxy-1-decy)pyrazole[3,4-d]pyrimidine (APP) in blocking adenosine deaminase activity were assessed in vitro by an enzyme inhibition assay, performed as reported by Antonioli et al. (2007). The dose of dexamethasone was selected on the basis of a previous study performed on a rat model of colitis (Nakase et al., 2001).

The first part of the study was aimed at evaluating the efficacy of adenosine deaminase inhibitors to see whether they are effective against established colitis and the involvement of adenosine receptor subtypes in mediating the anti-inflammatory effects resulting from adenosine deaminase blockade. For this purpose, the effects of APP were tested, in either the absence or presence of selective A1, A2A, A2B, and A3 adenosine receptor antagonists, on systemic inflammatory parameters (food intake, body weight, spleen weight) and macroscopic score. Effective and selective doses of the receptor antagonists were chosen on the basis of previous reports (Liem et al., 2001; McLaughlin et al., 2006; Wakeno et al., 2006; Ilie et al., 2009), and they were then validated by means of preliminary experiments in the model of DNBs-induced colitis. In this setting, increasing doses of adenosine receptor antagonists were tested against effective doses of adenosine receptor agonists on colonic myeloperoxidase (MPO) levels, a reliable index of tissue inflammatory response, thus allowing the selection of effective and selective doses of antagonists to be used in subsequent experiments on adenosine deaminase inhibition. The mean findings on the effects of adenosine receptor antagonists versus the respective agonists are summarized in Table 1.

The second part of the study was designed to gain more detailed information on the role played by adenosine receptor subtypes involved significantly in the anti-inflammatory effects exerted by the adenosine deaminase inhibitors. For this purpose, the effects of test drugs were evaluated on microscopic colonic damage and tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and malondialdehyde (MDA) tissue levels.

The macroscopic score was evaluated for the whole colon, whereas microscopic and biochemical analyses were performed on tissue specimens taken from a region of inflamed colon immediately adjacent and distal to the gross necrotic damage. Therefore, at the time of sacrifice, portions of colonic tissues were immediately snap-frozen in liquid nitrogen and stored at −80°C for subsequent Western blot analysis, MPO, and cytokine and MDA assays or fixed in cold 4% paraformaldehyde for the assessment of microscopic damage score.

Assessment of Colitis. At the end of the treatments, colonic tissues were excised, rinsed with saline, and scored for macroscopic and histological damage, in accordance with the criteria reported previously by Antonioli et al. (2007). The criteria for macroscopic scoring of colonic damage were as follows: 1) presence of adhesions between colonic tissue and other organs (0, none; 1, minor; 2, major adhesions); 2) consistency of colonic fecal material (0, loose; 1, liquid stools); and 3) presence of ulceration (0, none; 1, hyperemia; 2, ulceration without hyperemia; 3, ulceration with inflammation at one side; 4, ≥2 sites of ulceration and inflammation; 5, major sites of damage; and 6, major sites of damage extending >2 cm). The score was then increased 1 unit for each millimeter of colonic wall thickness. Microscopic damage and inflammation were assessed by light microscopy on hematoxylin/eosin-stained histological sections obtained from whole gut specimens. The histological criteria included: mucosal architecture loss (0–3), cellular infiltrate (0–3), muscle thickening (0–3), crypt abscess (0, absent; 1, present),

### Materials and Methods

**Animals.** Albino male Sprague-Dawley rats, 250 to 300 g in body weight, were used throughout the study. The animals were fed standard laboratory chow and tap water ad libitum and were not subjected to experimental procedures for at least 1 week after their delivery to the laboratory. Their care and handling were in accordance with the provisions of the European Union Council Directive 86-609, which is recognized and adopted by the Italian Government.

**Induction of Colitis, Drug Treatments, and Experimental Design.** Colitis was induced in accordance with the method described previously by Antonioli et al. (2007). In brief, during a short anesthesia with isoflurane (Abbott Labs, Pomezia, Italy), 15 mg of 2,4-dinitrobenzenesulfonic acid (DNBS) in 0.25 ml of 50% ethanol was administered intrarectally via a polyethylene PE-60 catheter inserted 8 cm proximal to the anus. Control rats received 0.25 ml of 50% ethanol. Five and 11 days after DNBS or vehicle administration, rats were subjected to the evaluation of systemic and tissue inflammatory parameters and the expression of adenosine deaminase to assess the inflammatory status and expression pattern of the molecular target of adenosine deaminase inhibitors at the onset of drug treatments. All test drugs were administered intraperitoneally for 6 consecutive days, starting 5 days after the induction of colitis.

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**M**
Effects of increasing doses of adenosine receptor antagonists on colonic MPO levels in rats treated with adenosine receptor agonists

<table>
<thead>
<tr>
<th>Adenosine Receptor Antagonists</th>
<th>Control</th>
<th>DNBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4.8 ± 2.6</td>
<td>23.6 ± 4.5</td>
</tr>
<tr>
<td>CCPA (0.4 μmol/kg/day)</td>
<td>4.2 ± 3.8</td>
<td>15.7 ± 2.9</td>
</tr>
<tr>
<td>+ DPCPX (2 μmol/kg/day)</td>
<td>5.4 ± 2.4</td>
<td>16.4 ± 3.5</td>
</tr>
<tr>
<td>+ DPCPX (4 μmol/kg/day)</td>
<td>3.1 ± 2.2</td>
<td>21.6 ± 3.1</td>
</tr>
<tr>
<td>+ DPCPX (8 μmol/kg/day)</td>
<td>6.7 ± 2.3</td>
<td>22.8 ± 2.8</td>
</tr>
<tr>
<td>Vehicle</td>
<td>3.5 ± 1.9</td>
<td>25.8 ± 4.6</td>
</tr>
<tr>
<td>CGS 21680 (4 μmol/kg/day)</td>
<td>5.1 ± 2.8</td>
<td>8.8 ± 2.6</td>
</tr>
<tr>
<td>+ CSC (3 μmol/kg/day)</td>
<td>6.1 ± 3.1</td>
<td>23.4 ± 5.2</td>
</tr>
<tr>
<td>+ CSC (6 μmol/kg/day)</td>
<td>4.3 ± 1.6</td>
<td>21.7 ± 1.5</td>
</tr>
<tr>
<td>+ CSC (12 μmol/kg/day)</td>
<td>6.4 ± 4.5</td>
<td>22.8 ± 3.5</td>
</tr>
<tr>
<td>Vehicle</td>
<td>5.4 ± 2.0</td>
<td>21.9 ± 4.4</td>
</tr>
<tr>
<td>NECA (2 μmol/kg/day)</td>
<td>6.5 ± 2.8</td>
<td>30.4 ± 2.8</td>
</tr>
<tr>
<td>+ MRS 1754 (1 μmol/kg/day)</td>
<td>4.1 ± 3.6</td>
<td>31.5 ± 4.3</td>
</tr>
<tr>
<td>+ MRS 1754 (2 μmol/kg/day)</td>
<td>6.2 ± 4.4</td>
<td>17.3 ± 3.5</td>
</tr>
<tr>
<td>+ MRS 1754 (4 μmol/kg/day)</td>
<td>6.5 ± 2.7</td>
<td>16.2 ± 2.6</td>
</tr>
<tr>
<td>Vehicle</td>
<td>5.1 ± 3.4</td>
<td>24.9 ± 4.7</td>
</tr>
<tr>
<td>IB-MECA (1 μmol/kg/day)</td>
<td>4.8 ± 3.0</td>
<td>9.7 ± 2.9</td>
</tr>
<tr>
<td>+ MRS 1191 (3.5 μmol/kg/day)</td>
<td>5.0 ± 2.7</td>
<td>11.5 ± 2.5</td>
</tr>
<tr>
<td>+ MRS 1191 (7 μmol/kg/day)</td>
<td>6.4 ± 3.5</td>
<td>21.4 ± 3.0</td>
</tr>
<tr>
<td>+ MRS 1191 (14 μmol/kg/day)</td>
<td>3.8 ± 2.2</td>
<td>24.0 ± 4.1</td>
</tr>
</tbody>
</table>

CCP, 2-chloro-N-6-cyclopentyladenosine (A1 adenosine receptor agonist); DPCPX, 8-cyclopentyl-1,3-dipropylxanthine (A1 adenosine receptor antagonist); CGS 21680, 2-(2’-hydroxy-3’-methyl-4-phenylethynyl-6-phenyl-1,4-(piperazino)acetamide (A2 adenosine receptor agonist); NECA, 5-N-ethylcarboxamido adenosine (A2 adenosine receptor antagonist); CGS 21680 (2-p-chlorophenyl-2-pyridyl-1H-purine-5-yl)acetamide (A2B adenosine receptor antagonist); IB-MECA, 1-deoxy-1-(6-[3-(iodoophenyl)-methyl]-aminomethyl-9H-purin-9-yl)-N-methyl-β-sorbitoluracironudine (A3 adenosine receptor agonist); MRS 1191, 3-ethyl-5-benzyl-2-methyl-4-phenethyl-6-phenyl-1,4-([1,3]-dihydropyridine-3,5-dicarboxylate (A3 receptor antagonist).

P < 0.05 significant difference vs. control.

P < 0.05 significant difference vs. DNBS alone.

P < 0.05 significant difference vs. the respective agonist alone.

and goblet cell depletion (0, absent; 1, present). All parameters of macroscopic and histological damages were recorded and scored for each rat by two observers blinded to the treatment. At the time of the experiment, the weight of spleen was also measured.

Western Blot Analysis

The colonic specimens were weighed and homogenized in radioimmunoprecipitation assay lysis buffer containing 10 mM HEPES, 30 mM NaCl, 0.2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 2% glyceraldehyde, 0.3 mM MgCl2, and 1% Triton X-100, using a polytron homogenizer (Cole Parmer, Vernon Hills, IL). The homogenates were spun by centrifugation at 20,000 revolutions/min for 15 min at 4°C, and the resulting supernatants were then separated from pellets and stored at −20°C. Protein concentration was determined in each sample by the Bradford method (Bradford, 1976) (Protein Assay Kit; Bio-Rad Laboratoires, Hercules, CA). To perform Western blot analysis of adenosine deaminase, aliquots of 30 μg of protein were separated by electrophoresis on 8% SDS-polyacrylamide gel electrophoresis and transferred onto a poly(vinylidene difluoride) membrane. The blots were then blocked for 2 h with 1% bovine serum albumin in Tween 20 phosphate-buffered saline (PBS-T) and incubated overnight at room temperature with a rabbit polyclonal primary antibody raised against adenosine deaminase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:1000). After repeated washings with 0.1% PBS-T, a peroxidase-conjugated donkey anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc.; dilution 1:10,000) was added for 1 h at room temperature. After repeated washings with PBS-T, immuno-reactive bands were visualized by incubation with chemiluminescent substrate (Immobilon reagent; Millipore Corporation, Billerica, MA) and exposed to Eastman Kodak (Rochester, NY) Image Station 440 for signal and densitometric image analysis. To ensure the equal loading and accuracy of changes in protein abundance, the protein levels were normalized to β-actin.

Determination of Tissue Myeloperoxidase. To validate the doses of adenosine receptor antagonists to be used against the adenosine deaminase inhibitors under study, preliminary experiments were performed to determine colonic MPO levels as reported previously by Antonioli et al. (2007). In brief, colonic samples (300 mg) were homogenized three times (30 s each) at 4°C with a polytron homogenizer (QIAGEN, Milan, Italy) in 1 ml of ice-cold 50 mM phosphate buffer, pH 6.0, containing 0.5% of hexadecyltrimethylammonium bromide to prevent the pseudoperoxidase activity of hemoglobin and solubilize membrane-bound MPO. The homogenate was sonicated for 10 s, frozen-thawed three times, and spun by centrifugation for 20 min at 18,000g. The supernatant was then recovered and used for determination of MPO by using a kit for enzyme-linked immunosorbent assay (Bioxtech; OXIS Research Inc., Portland, OR). All samples were assayed within 2 days from collection. The results were expressed as nanograms of MPO per 100 mg of tissue.

Evaluation of Tissue Cytokine Levels. Tissue TNF-α and IL-6 levels were measured with enzyme-linked immunosorbant assay kits (BioSource International, Camarillo, CA). For this purpose, tissue samples, stored previously at −80°C, were weighed, thawed, and homogenized in 0.3 ml of PBS, pH 7.2/100 mg of tissue at 4°C, and centrifuged at 13,400g for 20 min. Aliquots (100 μl) of the supernatants were then used for assay. Tissue TNF-α and IL-6 levels were expressed as picogram per milligram of tissue.

Evaluation of Tissue Malondialdehyde Levels. MDA concentration in colonic specimens was evaluated to obtain quantitative estimation of membrane lipid peroxidation. Colonic tissues were weighed, minced by forceps, homogenized in 2 ml of cold buffer (20 mM Tris-HCl, pH 7.4) by a polytron homogenizer (QIAGEN), and spun by centrifugation at 1500g for 10 min at 4°C. Colonic MDA concentrations were determined with a kit for colorimetric assay (Calbiochem, San Diego, CA), and the results were expressed as μmol of MDA per milligram of colonic tissue.

Drugs and Reagents. DNBS, dexamethasone, 3-ethyl-5-benzyl-2-methyl-4-phenethyl-6-phenyl-1,4-[1,3]-dihydropyridine-3,5-dicarboxylate (MRS 1191), chloroform, and TRIZol were purchased from Sigma-Aldrich (St Louis, MO). EHNA, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (A2B adenosine receptor antagonist); IB-MECA, 1-deoxy-1-(6-[3-(iodophenyl)-methyl]-aminomethyl-9H-purin-9-yl)-N-methyl-β-sorbitoluracironudine (A3 adenosine receptor agonist); MRS 1191, 3-ethyl-5-benzyl-2-methyl-4-phenethyl-6-phenyl-1,4-[1,3]-dihydropyridine-3,5-dicarboxylate (A3 receptor antagonist).

P < 0.05 significant difference vs. control.

P < 0.05 significant difference vs. DNBS alone.

P < 0.05 significant difference vs. the respective agonist alone.

Results

Adenosine Deaminase Expression

Western blot analysis showed a detectable expression of adenosine deaminase in colonic tissues from vehicle-treated rats (Fig. 1). In tissues from animals with colitis, the expression of adenosine deaminase was increased significantly at day 11 after the administration of DNBS (Fig. 1).
Evaluation of Colonic Inflammation under Control Conditions

Five days after intracolonic DNBS administration, there was a significant decrease in food intake and body weight, with concomitant increments of spleen weight and bowel inflammatory parameters (macroscopic and microscopic damage, TNF-α, IL-6, and MDA), compared with vehicletreated animals (Table 2). Likewise, at day 11 after the induction of colitis, food intake and body weight gain were lower, whereas other systemic and colonic indexes of inflammation were significantly increased in comparison with vehicle-treated animals, and these parameters were similar to those recorded in inflamed animals at day 5 (Table 2).

Effects of Test Drugs on Colonic Inflammation

Food Intake, Body Weight, and Spleen Weight. After a course of 6-day administration to animals with established colitis, APP (15 μmol/kg/day) or EHNA (90 μmol/kg/day) ameliorated food consumption, whereas no significant effects on food intake were observed in rats receiving dexamethasone (0.25 μmol/kg/day) (Table 3). Under the same conditions, the delay in body weight gain associated with colitis was counteracted after treatment with APP or EHNA (Table 3), whereas dexamethasone was without effect on this parameter (Table 3). Spleen weight was significantly increased in DNBS-treated rats, and such an increment was reduced by administration of APP, EHNA, or dexamethasone (Table 3).

The beneficial effects exerted by APP on food intake and body weight in rats with established colitis were counteracted by administration of CSC (6 μmol/kg/day) and, to a lower extent, MRS 1191 (7 μmol/kg/day), whereas no significant effects were obtained with the A1 or A2B receptors antagonists DPCPX (4 μmol/kg/day) and MRS 1754 (2 μmol/kg/day), respectively (Fig. 2). In addition, the effects of APP administration on spleen weight were blunted by the A2A receptor antagonist, whereas no significant effects were observed in the presence of the other receptor antagonists (Fig. 2). Moreover, the adenosine receptor antagonists did not exert any significant influence on food intake, body weight, or spleen weight when tested alone (Fig. 2).

Macroscopic Damage. Animals with colitis treated with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day), or dexamethasone (0.25 μmol/kg/day) for 6 days displayed a significant reduction in the macroscopic damage score (Fig. 3A). The adenosine receptor antagonists did not modify the macroscopic damage associated with chronic colitis when tested alone (Fig. 3B). However, CSC (6 μmol/kg/day) prevented the ameliorative effects exerted by APP, whereas no appreciable effects were observed with the administration of DPCPX (4 μmol/kg/day), MRS 1754 (2 μmol/kg/day), or MRS 1191 (7 μmol/kg/day) (Fig. 3B).

Microscopic Damage. Colonic specimens obtained from inflamed rats treated with APP (15 μmol/kg/day) showed a significant improvement in microscopic scores (Fig. 4A). Likewise, EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day) induced a significant amelioration of histological damage (Fig. 4A). The A2A or A3 receptor antagonist did not affect the microscopic damage of inflamed colon when tested alone (Fig. 4B). The beneficial effects exerted by APP

![Image](http://jpet.aspetjournals.org/)

**Fig. 1.** Western blot analysis of adenosine deaminase (ADA) in colonic tissues obtained from vehicle-treated animals (V) or rats with DNBS-induced colitis at days 5 and 11. Each column represents the mean value ± S.E.M. obtained from six animals. *P < 0.05, significant difference versus vehicle.

**Table 2**

Systemic and tissue inflammatory parameters in animals treated with DNBS or vehicle at day 5 or 11.

<table>
<thead>
<tr>
<th>Food Intake</th>
<th>Body Weight Variation</th>
<th>Spleen Weight</th>
<th>Macroscopic Damage Score</th>
<th>Microscopic Damage Score</th>
<th>TNF-α pg/ml</th>
<th>IL-6 pg/ml</th>
<th>MDA μmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (day 5)</td>
<td>34.4 ± 3.7</td>
<td>15.2 ± 1.9</td>
<td>100.0 ± 5.0</td>
<td>1.4 ± 0.4</td>
<td>1.5 ± 0.3</td>
<td>4.1 ± 2.7</td>
<td>31.4 ± 3.9</td>
</tr>
<tr>
<td>DNBS (day 5)</td>
<td>16.8 ± 4.1*</td>
<td>-5.2 ± 1.8*</td>
<td>130.0 ± 3.5*</td>
<td>8.1 ± 1.4*</td>
<td>4.5 ± 0.9*</td>
<td>15.7 ± 4.2*</td>
<td>125 ± 6.8*</td>
</tr>
<tr>
<td>Vehicle (day 11)</td>
<td>38.0 ± 2.9</td>
<td>22.0 ± 3.5</td>
<td>100.0 ± 6.5</td>
<td>1.2 ± 0.5</td>
<td>1.3 ± 0.5</td>
<td>5.5 ± 3.0</td>
<td>27.0 ± 4.1</td>
</tr>
<tr>
<td>DNBS (day 11)</td>
<td>22.0 ± 3.5*</td>
<td>6.5 ± 2.9*</td>
<td>141.7 ± 5.8*</td>
<td>6.9 ± 1.2*</td>
<td>3.8 ± 0.7*</td>
<td>24.0 ± 4.8*</td>
<td>136 ± 3.8*</td>
</tr>
</tbody>
</table>

*P < 0.05 significant difference vs. the respective control value.

**Table 3**

Effects of APP, EHNA, or dexamethasone (DEX) on food intake, body weight, and spleen weight.

<table>
<thead>
<tr>
<th>Inflammatory Parameters</th>
<th>DNBS (day 11)</th>
<th>APP (15 μmol/kg/day)</th>
<th>EHNA (90 μmol/kg/day)</th>
<th>DEX (0.25 μmol/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake (g)</td>
<td>20.0 ± 4.0</td>
<td>23.5 ± 4.5*</td>
<td>28.0 ± 3.5*</td>
<td>20.5 ± 5.0</td>
</tr>
<tr>
<td>Body weight variation (%)</td>
<td>± 4.9 ± 2.5</td>
<td>± 18.0 ± 3.0*</td>
<td>± 16.8 ± 3.2*</td>
<td>± 1.2 ± 1.7</td>
</tr>
<tr>
<td>Spleen weight (%)</td>
<td>137.4 ± 6.2</td>
<td>108.7 ± 3.5*</td>
<td>115.0 ± 4.8*</td>
<td>105.4 ± 3.7*</td>
</tr>
</tbody>
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*P < 0.05 significant difference vs. DNBS.
Systemic inflammatory parameters estimated in rats with colitis treated with APP alone or upon combined administration with DPCPX (4 μmol/kg/day i.p.), CSC (6 μmol/kg/day i.p.), MRS 1754 (2 μmol/kg/day i.p.), or MRS 1191 (7 μmol/kg/day i.p.). A, food intake. B, body weight variation. C, spleen weight variation. Treatments with test drugs were started at day 5 from the induction of colitis with DNBS. Each column represents the mean value ± S.E.M. obtained from eight animals. *, P < 0.05, significant difference versus control; a, P < 0.05, significant difference versus DNBS alone; §, P < 0.05, significant difference versus DNBS plus APP.

Fig. 3. A, macroscopic damage score estimated in rats with DNBS-induced colitis in either the absence or presence of treatment with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day), or dexamethasone (0.25 μmol/kg/day i.p.). B, effects of DPCPX (4 μmol/kg/day i.p.), CSC (6 μmol/kg/day i.p.), MRS 1754 (2 μmol/kg/day i.p.), or MRS 1191 (7 μmol/kg/day, i.p.) on macroscopic damage in either the absence or presence of concomitant APP administration. Treatments with test drugs were started at day 5 from the induction of colitis with DNBS. Macroscopic and microscopic scores were estimated at day 11. Each column represents the mean value ± S.E.M. obtained from eight animals. *, P < 0.05, significant difference versus control; a, P < 0.05, significant difference versus DNBS alone; §, P < 0.05, significant difference versus DNBS plus APP.

or EHNA on the microscopic patterns of colonic inflammation were markedly counteracted by the concomitant administration of CSC and, to a lower extent, MRS 1191 (Fig. 4B). The A₂A or A₃ receptor antagonist did not show significant effects in dexamethasone-treated rats (Fig. 4B).

**TNF-α and IL-6 Levels in Colonic Tissues.** Treatments with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day), or dexamethasone (0.25 μmol/kg/day) significantly decreased the concentrations of TNF-α and IL-6 in the inflamed colonic tissues (Figs. 5A and 6A). CSC or MRS 1191 were without effect on TNF-α and IL-6 when administered alone to inflamed rats. However, the blockade of A₂A or A₃ receptors counteracted the decrease in TNF-α and IL-6 tissue levels induced by APP or EHNA in animals with colitis (Figs. 5B and 6B). The ameliorative effects of dexamethasone were
unaffected by treatment with CSC or MRS 1191 (Figs. 5B and 6B).

**MDA Levels in Colonic Tissues.** Treatments with APP (15 µmol/kg/day), EHNA (90 µmol/kg/day), or dexamethasone (0.25 µmol/kg/day) significantly attenuated the increase in colonic MDA levels associated with established colitis (Fig. 7A). The blockade of A₂A receptors with CSC or treatment with the A₂ receptor antagonist MRS 1191 did not influence the oxidative injury of inflamed colon when tested alone, but CSC significantly reversed the antioxidant actions promoted by both adenosine deaminase inhibitors (Fig. 7B). CSC or MRS 1191 did not exert significant effects in rats treated with dexamethasone (Fig. 7B).

**Discussion**

The present study was performed to investigate the efficacy of adenosine deaminase blockade against established colitis and characterize the adenosine receptors that are recruited by endogenous adenosine after its preservation from catabolic inactivation. To pursue these aims, the effects of the novel adenosine deaminase inhibitor APP and its standard comparator EHNA were assayed during the chronic phase of colitis elicited by DNBS, and dexamethasone was used as a comparator with known anti-inflammatory activity to assess the relative anti-inflammatory potency of both adenosine deaminase inhibitors. In this curative dosing protocol, the administration of test drugs was started 5 days after DNBS administration, when chronic colonic inflammation was fully established (Tran et al., 1999). At this stage, and up to day 11, the inflammation was associated with reduced weight gain, diarrhea, mucosal ulceration, and bowel wall thickening. Significant increments of inflammatory cytokines (TNF-α, IL-6) and oxidation products (MDA) were also recorded in the inflamed colon. All of these observations support the evidence that from days 5 to 11 after DNBS administration there was a steady active inflammatory response of colon resulting from an abnormal activity of inflammatory cells infiltrating the intestinal tissue.

Our molecular analysis provided the first demonstration that a significant increase in adenosine deaminase expression occurs at the level of the inflamed colonic tissue. In keeping with our observation, previous studies have reported...
increments of adenosine deaminase expression and activity in different models of inflammation, including sepsis, small bowel enteritis, and hypoxia (Cohen et al., 2002; Eltzschig et al., 2006) and in the serum of patients with rheumatoid arthritis (Sari et al., 2003) or pancreatitis (Ibiş et al., 2007). However, the cellular localization of adenosine deaminase in normal or inflamed colonic tissues was not investigated in the present study, and it remains to be established whether the induction of adenosine deaminase in the setting of colitis occurs at intracellular or extracellular sites and whether such an increment depends on its increased expression in resident cells and/or accumulating immune cells. Based on previous studies, both of these circumstances seem to be conceivable, because it has been shown that adenosine deaminase was expressed in mucosal cells of normal colon (Dinjens et al., 1989), and the expression of this enzyme was found to be increased in different populations of immune cells, including macrophages and lymphocyte T, during inflammatory responses (Martín et al., 1995; Conlon and Law, 2004). It is noteworthy that the increase in adenosine deaminase expression, occurring in concomitance with the inflammatory bowel reaction, might enhance the catabolic degradation of adenosine, thus resulting in an impairment of immune modulation by endogenous adenosine pathways and a consequent worsening of inflammation and tissue injury.

Previous reports suggest that adenosine deaminase may act as a critical regulatory factor, endowed with the ability of immune modulation by endogenous adenosine pathways and a consequent worsening of inflammation and tissue injury. When considering bowel inflammation, our present experimental data demonstrated that the pharmacological blockade of adenosine deaminase promotes a significant attenuation of inflammatory responses (Dong et al., 1997; Desrosiers et al., 2007). It is noteworthy that the increase in adenosine deaminase expression, occurring in concomitance with the inflammatory bowel reaction, might enhance the catabolic degradation of adenosine, thus resulting in an impairment of immune modulation by endogenous adenosine pathways and a consequent worsening of inflammation and tissue injury.
EHNA were testified by improvements of all inflammatory parameters, including systemic indexes (food intake, body weight, spleen weight), macroscopic and histological scores, tissue cytokines (TNF-α and IL-6), and tissue oxidation (MDA). In this study, another relevant finding was that the improvement of colitis and related inflammatory indexes, associated with adenosine deaminase inhibition, were prevented by the blockade of A2A, and to a minor extent of A3 receptors, whereas A1 and A2B receptors did not seem to be significantly involved. Taken together these data support the concept that: 1) inhibitors of adenosine deaminase are effective not only in protecting bowel tissues from the onset of inflammation, but also in blunting the inflammatory response in the setting of established colitis; and 2) the anti-inflammatory actions resulting from inhibition of the catalytic breakdown of endogenous adenosine are driven by the recruitment of A2A and A3 receptors.

There is increasing evidence in the literature indicating a marked involvement of A2A and A3 receptors in the modulation of immune cell activity and inflammation (Antonioli et al., 2008a; Haskó et al., 2008). In particular, previous works have shown that A3 is the primary receptor responsible for the inhibitory effect of adenosine on the production of proinflammatory cytokines and reactive oxygen species at sites of inflammation in different experimental models, including asthma, chronic obstructive pulmonary disease, and arthritis (Haskó et al., 2000, 2008; Haskó and Cronstein, 2004). Furthermore, studies from other groups have shown that the direct stimulation of A2A receptors, via selective agonists, produced beneficial effects in different models of bowel inflammation (Odashima et al., 2005). In addition, a study by Kreckler et al. (2006) showed that cytokine release from murine peritoneal macrophages was suppressed predominantly via A2A receptor activation.

In recent years, special attention has been paid to the role played by A3 receptors in the regulation of inflammatory responses. Pharmacological agents targeting this receptor pathway are being tested in preclinical models of inflammation or hypoxic damage with encouraging results (Haskó et al., 1998; Szabó et al., 1998; van der Hoeven et al., 2008), and some A3 receptor agonists have already entered the phase of clinical development for the treatment of rheumatoid arthritis (Silverman et al., 2008). Several lines of evidence suggest that the activation of A3 receptors can favorably affect the outcome of intestinal inflammation, thus representing a promising target for the development of a novel therapeutic strategy against IBDs (Mabley et al., 2003). However, in the present study the relevance of A3 receptors in mediating the beneficial effects resulting from adenosine deaminase inhibition seems to be less consistent in comparison with A2A receptors. In particular, the involvement of A3 receptors in mediating the anti-inflammatory effects resulting from adenosine deaminase blockade seem to be appreciable for few phlogistic parameters. One explanation for this differential recruitment of receptor subtypes could be ascribed to an increase in the activity of nucleoside transporters arising from the activation of A2A receptors (Pinto-Duarte et al., 2005; Ye and Rajendran, 2009). In this regard, the greater stimulation of A2A receptors by the pharmacological blockade of adenosine deaminase could be related to an increased activity of nucleoside transporters to avoid a spread of extra-cellular adenosine, sufficient to activate A3 receptors and the low-affinity A2B receptor.

In the present study the A2A or A3 receptor antagonists did not exacerbate the severity of bowel inflammation when tested alone. This finding might be explained by the reduced bioavailability of endogenous adenosine resulting from the enhanced catalytic activity of adenosine deaminase at the level of the inflamed colon. Consistent with this view, Lee et al. (2006) showed that in murine microglial cells stimulated by exposure to lipopolysaccharide A2A or A3 receptor antagonists did not worsen the inflammatory response in comparison with vehicle. Moreover, Van der Hoeven et al. (2008) demonstrated that two proinflammatory actions of murine neutrophils (i.e., superoxide production and chemotaxis) did not differ when comparing wild-type with A2A or A3 knockout animals. However, McColl et al. (2006) observed that in leukocytes migrated into air pouches of mice lacking A2A receptors the expression of TNF-α was significantly higher in comparison with wild-type animals, thus suggesting that different models of inflammation can be associated with different degrees of adenosine-dependent anti-inflammatory activity mediated by A2A or A3 receptors stimulation. As far as A2B receptors are concerned, previous reports showed conflicting evidence about their antiinflammatory/proinflammatory role during intestinal inflammation (Kolachala et al., 2008; Frick et al., 2009). In our study, A2B receptors did not seem to be significantly involved in the modulation of colonic inflammatory response. This discrepancy could depend on a number of differences between the present work and those mentioned above. In particular, there are differences in species, experimental model of bowel inflammation, and drug administration protocol. Moreover, in our preliminary experiments the administration of the A3 receptor agonist 5-N-ethylcarboxamidoadenosine was associated with an increase in colonic myeloperoxidase levels and this effect was counteracted by the A2B receptor antagonist MRS 1754, thus suggesting that the exogenously induced activation of A2B receptors is likely to promote proinflammatory effects (L. Antonioli et al., unpublished data). For these reasons, it is conceivable that in the setting of the present study A2B receptors were not able to take a significant part to the anti-inflammatory response resulting in adenosine deaminase blockade.

Overall, the present results suggest that the pharmacological modulation of adenosine deaminase represents a novel and promising therapeutic strategy for the management of bowel inflammation. Indeed, the specific and selective inhibition of this enzyme, markedly expressed in inflamed bowel tissues, produced ameliorative effects against an established inflammatory environment, acting on several aspects of the inflammatory reaction. Most importantly, the enhanced levels of endogenous adenosine, resulting from adenosine deaminase inhibition, down-regulated the production of key proinflammatory cytokines, counteracted tissue oxidative injury, and attenuated the severity of colonic injury via recruitment of the A2A and A3 receptor pathways.

References


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