Inosine reduces inflammation and improves survival in a murine model of colitis

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Mabley, J. G., P. Pacher, L. Liaudet, F. G. Soriano, G. Haskó, A. Marton, C. Szabó, and A. L. Salzman. Inosine reduces inflammation and improves survival in a murine model of colitis. Am J Physiol Gastrointest Liver Physiol 284: G138–G144, 2003.—Inosine, a naturally occurring purine formed from the breakdown of adenosine, has recently been shown to exert powerful anti-inflammatory effects both in vivo and in vitro. This study evaluated inosine as a potential therapy for colitis. Colitis was induced in mice by the administration of dextran sulfate sodium (DSS). Oral treatment with inosine was begun either before the onset of colitis or as a posttreatment once colitis was established. Evaluation of colon damage and inflammation was determined grossly (body wt, rectal bleeding), histologically, and biochemically (colon levels of MPO, MDA, and cytokines). DSS-induced colitis significantly increased inflammatory cell infiltration into the colon. DSS-induced colitis also increased colon levels of lipid peroxidation, cytokines, and chemokines. Inosine protected the colon from DSS-induced inflammatory cell infiltration and lipid peroxidation. Inosine also partially reduced these parameters in an experimental model of established colitis. Thus inosine treatment may be a potential therapy in colitis.

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IT IS WELL RECOGNIZED THAT certain naturally occurring purines can exert powerful modulatory effects on the immune system. Nucleoside adenosine is the best characterized of these purines and has been shown to affect almost all aspects of an immune response (2, 8, 19). Adenosine and its analogs can effect the development of a variety of inflammatory diseases including endotoxin shock (18), rheumatoid arthritis (38), plural inflammation (35), or uveitis (27). Effects of adenosine are partly mediated by the inhibition of deleterious immune-mediated processes, including the release of proinflammatory cytokines and free radicals (16). Inosine is a naturally occurring purine, formed from the breakdown of adenosine by adenosine deaminase (3), and was widely believed to be without biological actions. However, our group has recently observed that inosine potently inhibits the release of proinflammato-
MATERIALS AND METHODS

Reagents were obtained from the following sources: DSS (MW 40,000) was from ICN Pharmaceuticals; inosine, human MPO, 1,1,3,3-tetramethoxypropane, thiobarbituric acid, sodium dodecyl sulfate, tetramethylbenzidine, hexadecyltrimethylammonium bromide, and hydrogen peroxide were from Sigma (St. Louis, MO); BALB/c mice were from Taconic Farms (Germantown, NY); and specific cytokine ELISA kits were from R&D Systems (Minneapolis, MN).

Induction of colitis and treatment. Male BALB/c mice, 8 wk of age, weighing 20–23 g were used for these studies. Animals were housed in rooms at a controlled temperature and light-dark cycle for 48 h before starting experimental protocols. All animal experiments were carried out in accordance with “Guide for the Care and Use of Laboratory Animals” [DHEW Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205] and with the approval of Inotek’s Institutional Animal Care and Use Committee.

Mice were fed 5% DSS, molecular mass 30–40 kDa dissolved in distilled water ad libitum throughout the experiment (31). Inosine was administered either orally by gavage or intraperitoneally twice a day. Control mice were treated with vehicle, which was either water for the oral inosine experimental protocol or saline for the intraperitoneal inosine experimental protocol. For the delayed inosine treatment experiments, mice were given vehicle up to the day inosine treatment was started. Inosine was given at doses ranging from 25 to 200 mg·kg⁻¹·day⁻¹ and was based on recent studies testing inosine in rodent models of inflammation (14, 18, 25). Intake of the DSS solution was monitored throughout the experiments and was found to be unchanged among experimental groups (data not shown).

Evaluation of colitis severity and drug effects. Parameters recorded in the experiments were body weight, colon length, mortality, and bleeding from the rectum as determined by ocular inspection. Mice were weighed on days 1 and 10 with the subsequent colitis-induced weight change expressed as a percentage of the original weight. Mice were killed by cervical dislocation, and the colon was resected between the ileocecal junction and the proximal rectum, close to its passage under the pelvis sternum. The colon was placed on a nonabsorbent surface and measured with a ruler. Colonic biopsies were taken for histological and biochemical analysis. One biopsy was fixed in 15% formaldehyde, embedded in paraffin, and sectioned (4-μm slices). The sections were then stained with hematoxylin and eosin and viewed by an investigator (blinded) and scored for inflammation severity (0 = none, 1 = mild, 2 = moderate, and 3 = severe) and extent (0 = none, 1 = mucosal, 2 = mucosal and submucosal, and 3 = transmural) as well as crypt damage (0 = none, 1 = basal 1/3, 2 = basal 2/3, 3 = crypts lost epithelium present, and 4 = crypts and surface epithelium lost), with representative sections being shown here.

MPO activity. Colon biopsies were homogenized (50 mg/ml) in 0.5% hexadecyltrimethylammonium bromide in 10 mM MOPS and centrifuged at 15,000 g for 40 min. Suspension was then sonicated three times for 30 s. An aliquot of supernatant (20 μl) was mixed with a solution of 1.6 mM tetramethylbenzidine 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 MPO activity per milligram of protein, which were determined with the Bradford assay.

Malondialdehyde assay. Malondialdehyde formation was used to quantify the lipid peroxidation in the colon and was measured as thiobarbituric acid-reactive material. Tissues were homogenized (100 mg/ml) in 1.15% KCl buffer. Two hundred microliters 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 H₂O. 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 was measured at 532 nm using 1,1,3,3-tetramethoxypropane as an external standard. The level of lipid peroxidation was expressed as nanomoles MDA per milligram of protein (Bradford assay).

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 was frozen at −80°C until assay. Cytokine levels were determined using ELISA.

RESULTS

Inosine treatment protects against DSS-induced colitis. Treatment of BALB/c mice with 5% DSS in their drinking water for 10 days resulted in clinical, gross, and histological signs of colitis. DSS-treated mice had a marked weight loss, shortened colon length, and gross rectal bleeding (Table 1) compared with mice receiving regular drinking water. Inosine treatment starting on day 1 dose-dependently reversed these effects (Table 1). Colon biopsies from DSS-treated mice had significantly increased levels of both MPO (Fig. 1A), indicative of inflammatory cell tissue infiltration, and MDA

Table 1. Inosine dose dependently attenuates the hallmarks of experimental colitis

<table>
<thead>
<tr>
<th>Groups</th>
<th>%Change in Body Weight</th>
<th>Colon Length, cm</th>
<th>Rectal Bleeding, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>−7.2 ± 3.5</td>
<td>6.0 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>DSS + vehicle</td>
<td>22.8 ± 1.1†</td>
<td>3.6 ± 0.1†</td>
<td>90‡</td>
</tr>
<tr>
<td>DSS + inosine, 25 mg·kg⁻¹·day⁻¹</td>
<td>17.8 ± 1.8†</td>
<td>5.0 ± 0.3§</td>
<td>80†</td>
</tr>
<tr>
<td>DSS + inosine, 50 mg·kg⁻¹·day⁻¹</td>
<td>16.6 ± 1.4‡</td>
<td>4.4 ± 0.2§</td>
<td>60‡</td>
</tr>
<tr>
<td>DSS + inosine, 100 mg·kg⁻¹·day⁻¹</td>
<td>14.9 ± 1.9‡</td>
<td>5.0 ± 0.3§</td>
<td>25§</td>
</tr>
<tr>
<td>DSS + inosine, 200 mg·kg⁻¹·day⁻¹</td>
<td>13.1 ± 0.8§</td>
<td>4.8 ± 0.1§</td>
<td>20§</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE from 10–20 animals. Male Balb/c mice were exposed to the dextran sulfate sodium (DSS) solution (5% wt/vol) ad libitum. Inosine (25, 50, 100, or 200 mg·kg⁻¹·day⁻¹) was administered orally twice daily starting on day 1 and on day 10 the experiment was terminated. The colon was dissected out and measured. Statistical analysis was conducted using one-way ANOVA followed by Student-Newman-Keuls multiple comparisons post hoc analysis. Fisher’s exact test, Mann-Whitney U-test, or Kaplan-Meier survival analysis as appropriate, with a P value of <0.05 considered significant.
and BSS-induced increases in these parameters (Fig. 1, sine again dose-dependently protected against the (Fig. 1), indicative of lipid peroxidation damage. Ino-

Fig. 1. Inosine dose-dependently reduces the levels of MPO (A) and malondialdehyde (MDA) (B) in the colons of mice with an acute colon inflammation induced by dextran sulfate sodium (DSS). Mice were exposed to DSS ad libitum for 10 days, treatment with inosine (25, 50, 100, or 200 mg·kg$^{-1}$·day$^{-1}$, twice a day) started on day 1. Results are expressed as means ± SE from 8–20 animals, statistical analysis was conducted by one-way ANOVA followed by Student-Newman-Keuls multiple comparisons post hoc analysis where $P < 0.05$ was considered significant. $^*P < 0.05$, $^{**}P < 0.01$ vs. untreated animals and $\dagger P < 0.05$, $\ddagger P < 0.01$ vs. DSS-treated animals.

0.2 cm with 100 or 200 mg·kg$^{-1}$·day$^{-1}$ inosine intra-

peritoneally, respectively. Similarly, rectal bleeding was significantly reduced from 60 to 10 and 0%. We also examined colon levels of MPO and MDA; in both cases, inosine significantly reduced the levels com-
pared with vehicle-treated animals. MPO levels were reduced from 337 ± 60 to 101 ± 21 and 69 ± 16 mU/mg protein with 100 or 200 mg·kg$^{-1}$·day$^{-1}$ inosine treatment. Similarly, MDA levels were reduced from 3.5 ± 0.5 to 1.9 ± 0.3 nmol/mg protein with 200 mg·kg$^{-1}$·day$^{-1}$ inosine. The level of MDA in the colon after 100 mg·kg$^{-1}$·day$^{-1}$ ip inosine was 2.5 ± 0.4 nmol/mg protein, which was not significantly different statistically from the vehicle-treated colon.

Inosine partially attenuates disease symptoms in es-
established colitis. Mice treated with 200 mg·kg$^{-1}$·day$^{-1}$ inosine starting on day 4 or 7 after commencement of DSS had an increased colon length but had no effect on either the colitis-mediated loss of body weight or the incidence of rectal bleeding (Table 3). Treatment of mice on day 4 with inosine also had lower colon levels of both MPO and MDA (Table 3). In long-term survival experiments, mice treated with 5% DSS for 30 days exhibited a 100% mortality rate by 20 days (Fig. 3). In contrast, mice treated with inosine (200 mg·kg$^{-1}$·day$^{-1}$) starting on day 1, 4, or 7 showed a marked increase in survival with 100, 70, and 30%, respecti-
vally, of mice alive on day 20, and even on day 30, 60, 20, and 10% of mice were still alive (Fig. 3).

Effect of inosine on the colon cytokine profile. DSS-
treated mice had greatly increased colon levels of in-
flammatory chemokines and cytokines (Fig. 4, A and B). Untreated mice had undetectable colon levels of chemokines or cytokines (data not shown). Inosine (200 mg·kg$^{-1}$·day$^{-1}$) significantly reduced the colon levels of chemokines (Fig. 4A) major intrinsic protein (MIP)-1 and -2 and proinflammatory cytokines (Fig. 4B) IL-1, IL-6, and IL-12. Inosine was also able to attenuate the colon levels of TNF (Fig. 4B).

DISCUSSION

We have demonstrated here that inosine effectively suppresses the development of experimental colitis in vivo. Inosine exerted anti-inflammatory effects when treatment began simultaneously with the application of DSS and was able to attenuate disease parameters in established colitis. Inosine also dramatically increased survival in a long-term disease model of colitis. Inosine markedly changed the colitis-induced cytokine profile of the colon. Inosine reduces colon levels of chemokines MIP-1α and -2, which are involved in the innate and adaptive immune response because of their ability to recruit, activate, and costimulate T cells and monocytes (42). Interestingly, the reduction of levels of MIP-1α by inosine appears to be a common observation seen not only in colitis but also in LPS-induced shock (14), septic shock (25), and lung inflammation (24), suggesting that increased chemokine levels are pivotal in the inflammatory process. Inhibiting their produc-
tion/expression may explain why inosine is protective.
in a wide variety of inflammatory conditions. Inosine-induced reduction of colon IL-12 levels, a cytokine pivotal in colitis (15), is also striking, mimicking the effectiveness of an anti-IL-12 antibody in protecting against colitis (13). These observations coupled with the reduction in colon levels of other Th1 cytokines such as IL-1, IL-6, and TNF-α may account for inosine’s mechanism of action in attenuating colitis.

Interestingly, we observed similar protection against colitis when inosine was administered intraperitoneally, suggesting a systemic effect of inosine as well as a possible local protective effect after oral treatment. It is conceivable that inosine may cause osmotic purging of the colonic lumen, thereby reducing the effective dose of DSS acting on the colon. Observation of inosine intraperitoneal treatment protecting against colitis demonstrates that this possible osmotic effect would not account for all of inosine’s mechanism of protection.

Indeed, we have given rats an oral dose of inosine and killed them at various time intervals so the inosine content of various sections of the digestive tract could be determined. After a single dose of 200 mg/kg inosine.

Table 2. Histological analysis of colonic sections from vehicle, DSS + vehicle, and DSS + inosine-treated mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Inflammation Severity</th>
<th>Inflammation Extent</th>
<th>Crypt Damage</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.5 ± 0.12</td>
<td>0.5 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>DSS + vehicle</td>
<td>2.5 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>2.6 ± 0.5</td>
<td>7.8 ± 0.8</td>
</tr>
<tr>
<td>DSS + inosine, 200 mg·kg⁻¹·day⁻¹</td>
<td>1.7 ± 0.3*</td>
<td>1.5 ± 0.2†</td>
<td>1.3 ± 0.3</td>
<td>4.5 ± 0.8‡</td>
</tr>
</tbody>
</table>

Scores are presented as means ± SE from 10 animals. Male Balb/c mice were exposed to the DSS solution (5% wt/vol) ad libitum. Inosine (200 mg·kg⁻¹·day⁻¹) was administered orally twice daily starting on day 1. On day 10, mice were sacrificed and colon biopsies were taken and fixed in 10% formalin solution. Samples were embedded in paraffin and sectioned (3-μm sections). Sections were stained with hematoxylin and eosin and viewed at ×400 magnification and scored blind for inflammation severity (0 = none, 1 = mild, 2 = moderate, and 3 = severe) and extent (0 = none, 1 = mucosal, 2 = mucosal and submucosal, and 3 = transmural) as well as crypt damage (0 = none, 1 = basal 1/3, 2 = basal 2/3, 3 = crypts lost epithelium present, and 4 = crypts and surface epithelium lost). Statistical analysis was conducted using the Mann-Whitney U-test, where * = a two-tailed P value of 0.0524, † = a two-tailed P value of 0.0068, and ‡ = a two-tailed P value of 0.036 vs. the DSS + vehicle treatment group.
Inosine attenuates disease hallmarks in established experimental colitis

Table 3. Inosine attenuates disease hallmarks in established experimental colitis

<table>
<thead>
<tr>
<th>Groups</th>
<th>%Change in Body Weight</th>
<th>Colon Length, cm</th>
<th>Rectal Bleeding, %</th>
<th>MDA Levels, nmol/mg protein</th>
<th>MPO Levels, mU/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-7.2 ± 3.5</td>
<td>6.0 ± 0.2</td>
<td>0</td>
<td>1.9 ± 0.1</td>
<td>55.8 ± 7.9</td>
</tr>
<tr>
<td>DSS + vehicle</td>
<td>22.8 ± 2.3†</td>
<td>3.6 ± 0.1†</td>
<td>90†</td>
<td>3.9 ± 0.3†</td>
<td>368.7 ± 51.4†</td>
</tr>
<tr>
<td>DSS + inosine (200 mg·kg⁻¹·day⁻¹)</td>
<td>12.9 ± 4.3‡</td>
<td>4.8 ± 0.3‡</td>
<td>20‡</td>
<td>2.4 ± 0.2‡</td>
<td>102.9 ± 10.1‡</td>
</tr>
<tr>
<td>DSS + inosine beginning on day 4</td>
<td>17.9 ± 1.8†</td>
<td>4.1 ± 0.2†</td>
<td>60†</td>
<td>2.7 ± 0.2‡</td>
<td>225.7 ± 25.5‡</td>
</tr>
<tr>
<td>DSS + inosine beginning on day 7</td>
<td>18.9 ± 1.3†</td>
<td>4.3 ± 0.1†</td>
<td>60†</td>
<td>3.0 ± 0.2‡</td>
<td>275.6 ± 25.8†</td>
</tr>
</tbody>
</table>

Data are means ± SE from 10–12 animals. Inosine (200 mg·kg⁻¹·day⁻¹) was administered orally twice daily starting on day 1, 4, or 7 following the start of DSS treatment. On day 10, the experiment was terminated and the animals sacrificed. The colon was dissected out and measured. Biopsies were taken for determination of colon levels of myeloperoxidase (MPO) and malondialdehyde (MDA). Statistical analysis was conducted using one-way ANOVA followed by Student-Newman-Keuls multiple comparisons post hoc analysis or Fisher’s exact test, where \( P < 0.05 \) was considered significant. *\( P < 0.05 \), †\( P < 0.01 \) vs. vehicle-treated animals and ‡\( P < 0.05 \), §\( P < 0.01 \) vs. DSS + vehicle-treated animals.

Fig. 3. Inosine treatment significantly improves survival of mice with an acute colon inflammation. Mice were exposed to DSS ad libitum for 30 days, treatment with inosine (200 mg·kg⁻¹·day⁻¹ twice a day) commenced on days 1, 4, and 7. The number of mice surviving each day was recorded. Results are expressed as %survival from 20 animals. Statistical analysis was conducted using a Kaplan-Meier survival analysis, where \( P < 0.05 \) was considered significant. Survival of the mice was improved by inosine, where \( P < 0.0001 \) for inosine treatment starting on day 1 or 4 and \( P = 0.0003 \) for inosine treatment starting on day 7.

Fig. 4. Effect of inosine on colon chemokine (A) and cytokine (B) levels after colitis. Cytokine levels were determined in colon biopsies from mice treated for 10 days with DSS ± inosine (200 mg·kg⁻¹·day⁻¹). Results are expressed as means ± SE from 10 animals. Statistical analysis was conducted by one-way ANOVA followed by Student-Newman-Keuls multiple comparisons post hoc analysis, where \( P < 0.05 \) was considered significant. ‡‡\( P < 0.01 \) vs. DSS-treated animals. MIP, major intrinsic protein.

Inosine, we were able to detect inosine down to the jejunum and a small amount in the ileum, but we were unable to detect inosine in either the contents of the cecum or colon (unpublished observations). It therefore appears that inosine is being absorbed and/or broken down before it reaches the colon and its protective effect in colitis is likely due to a systemic action rather than local. A systemic effect of inosine is supported by data we have obtained in other animal models of inflammation, where oral inosine treatment protected against diabetes (26) and arthritis (unpublished observations) and intraperitoneal administration protected against endotoxic shock (14), septic shock (25), and acute respiratory distress syndrome (24).

Inosine is considered an inactive metabolite in most biological systems, but recently, evidence from our...
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group and others has shown that this is not the case. For example, it prevents glial cell death during glucose deprivation (21), decreases the release of intracellular enzymes from hypoxic lymphocytes (6), improves renal function during ischemia (9), and removes the harmful effects of total hepatic ischemia. More recently, our group has demonstrated that inosine has anti-inflammatory effects in vitro (17) and in vivo in animals models of endotoxic shock, septic shock, and lung inflammation (14, 17, 24, 25). Inosine’s effects have been shown to be direct and not due to its degradation product, hypoxanthine (17). However, inosine’s anti-inflammatory effects are partially but not completely mediated by activation of adenosine receptors (17). It is possible that inosine produces its inhibitory effects on cytokine production via binding to A$_2$ receptors, a receptor shown to be present on monocytes and macrophages (30, 34). It has also been shown that the effect of inosine on cytokine release is posttranscriptional and does not involve interference with the activation of p38, p42/44, JNK, degradation of inhibitor kB, or elevation of intracellular cAMP levels (17). Inosine treatment was particularly effective in attenuating the rises in colon cytokine and chemokine levels observed in colitis. The marked reduction of both MIP-1$\alpha$ and MIP-2 may explain why there is less colon infiltration by inflammatory cells.

Inosine has also been shown to inhibit the enzyme poly(ADP-ribose) synthetase (PARS), albeit at high concentrations (41). Inhibition of PARS has been shown to be beneficial in many inflammatory diseases (37), including experimental colitis in the mouse (44) and rat (29). Inosine may also enhance endogenous antioxidant systems because the breakdown of inosine yields urate, a scavenger of oxyradicals and peroxynitrite (1, 4, 20, 39), both of which have been implicated in the pathogenesis of colitis (36, 43). We have also examined the effectiveness of a specific peroxynitrite decomposition catalyst in colitis and found it to be protective (23). However, the effects of a peroxynitrite scavenger on immune cell infiltration and cytokine/chemokine levels in the colon of colitic mice was minimal and do not compare with what we observed with inosine treatment, further evidence of a systemic anti-inflammatory mechanism of action of inosine in colitis.

The posttranscriptional nature of inosine’s action both on cytokine release, inhibition of PARS, and urate production may be considered preferable, because one would expect an increased window of therapeutic opportunity, i.e., inosine may remain effective in a post-treatment paradigm. Indeed, our data suggest that inosine is able to attenuate established colitis. Purines have also been shown to promote healing of small bowel ulcers in experimental enterocolitis (40), and this, too, may play a role in inosine’s posttreatment protective effects in colitis. Promotion of repair of damaged mucosa by inosine may explain the decrease in inflammatory cells infiltrating the colon.

In conclusion, we have demonstrated the effectiveness of inosine as a protective therapy in an experimental model of murine colitis. Inosine was not only able to prevent colitis development but also had a beneficial effect on the established disease. The current data, coupled with inosine’s excellent safety record, suggest that the concept of testing and developing inosine as a colitis therapy in humans may be justified.

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Drs. J. G. Mabley, P. Pacher, and A. Marton are employees of Inotek Pharmaceuticals; Drs. C. Szabó and A. L. Salzman are employees, owners, and stockholders of Inotek Pharmaceuticals.

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