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.

colonic; dextran sodium sulfate; cytokines; purine

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 proinflammatory cytokines and chemokines by activated murine macrophages via a posttranscriptional mechanism and that this compound exerts powerful anti-inflammatory effects in murine endotoxic shock (14, 17), septic shock (25), and severe lung inflammation (24). Inosine also has anti-inflammatory effects in human monocytes, neutrophils, and epithelial cells in vitro (28), reducing both TNF-α production in response to LPS treatment as well as inhibiting the ability of human neutrophils activated with N-formyl-methionyl-leucyl-phenylalanine to induce cytochrome c reduction (28).

Several murine models of intestinal inflammation resemble human inflammatory bowel diseases such as Crohn’s disease and ulcerative colitis. Both of these diseases are characterized by chronically relapsing inflammation of the bowel of unknown origin. One of the murine models of inflammatory bowel disease can be induced by the oral administration of dextran sulfate sodium (DSS). Colitis induced by DSS exhibits lymphoid hyperplasia, inflammatory cell infiltration, and focal crypt damage (7, 10, 32). DSS-induced colitis also causes epithelial injury and ulceration (7, 10, 32). The underlying mechanism by which colitis is induced involves epithelial cell damage and phagocytosis of DSS, which leads to stimulation of lamina propria cells and increased production of proinflammatory cytokines (10). The cytokine profile of DSS-induced colitis was found to be similar to that found in human inflammatory bowel disease, with an increase in the levels of T helper (Th)1 cytokine mRNA transcripts including IL-1, IL-12, IFN-γ, and TNF-α (11). An increased colon IL-12 level appears to be pivotal in the development of colitis (15), with an anti-IL-12 antibody proving more effective in preventing experimental colitis in mice (13) than either anti-IFN-γ (13), anti-IL-1 (22), or anti-TNF-α (33). Although the murine DSS model of inflammatory bowel disease differs from the human disease, it has been recommended and is a widely used preclinical model for testing the efficacy of treatments for inflammatory bowel disease (5, 7, 12). In this study, we investigate the therapeutic efficacy of inosine in an animal model of inflammatory bowel disease.

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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, DRK/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 and recorded in the experiments were body weight, colon length, mortality, and bleeding from the rectum as determined by “Guide for the Care and Use of Laboratory Animals” [DHEW Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRK/NIH, Bethesda, MD 20205] and with the approval of Inotek’s Institutional Animal Care and Use Committee.

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 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.
and DSS-induced increases in these parameters (Fig. 1, sine again dose-dependently protected against the B), 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 †P < 0.05, ††P < 0.01 vs. DSS-treated animals.

(Fig. 1B), indicative of lipid peroxidation damage. Inosine again dose-dependently protected against the DSS-induced increases in these parameters (Fig. 1, A and B). Histological analysis of colon biopsies showed an accumulation of lymphocytes and marked crypt destruction with some surface mucosal layer disruption in the mice treated with DSS (Fig. 2B) compared with control mice (Fig. 2A and Table 2). Inosine protected against both the infiltration of neutrophils and against the mucosal damage (Fig. 2C and Table 2). Similar results were obtained when inosine was administered intraperitoneally. The colon length in vehicle-treated animals was reduced to 4.5 ± 0.2 cm, and this was significantly increased to 5.5 ± 0.2 and 5.7 ± 0.2 cm with 100 or 200 mg·kg⁻¹·day⁻¹ inosine intraperitoneally, 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 compared 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 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%, respectively, 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 inflammatory 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 production/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 either ×100 or ×400 magnification. Sections presented are representative of sections from 10 mice. Magnification of the first two columns is ×100 and the third column, ×400.

Fig. 2. Effect of inosine (200 mg·kg⁻¹·day⁻¹) on the morphological changes observed in the colon of mice treated with DSS for 10 days. Inosine was administered orally starting on day 1. On day 10, mice were killed 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 were viewed at either ×100 or ×400 magnification. Sections presented are representative of sections from 10 mice. Magnification of the first two columns is ×100 and the third column, ×400.
sine, 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).

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.
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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 animal 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 A2 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 κB, 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-1α 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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