Inosine Reduces Systemic Inflammation and Improves Survival in Septic Shock Induced by Cecal Ligation and Puncture

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Inosine is a naturally occurring purine formed from the breakdown of adenosine. Here we have evaluated the effects of inosine in a murine model of polymicrobial sepsis induced by cecal ligation and puncture (CLP). Mice subjected to CLP were treated with either inosine (100 mg/kg, intraperitoneally) or vehicle 1 h before and 6 h after CLP. After 12 h tumor necrosis factor α, interleukin 6 (IL-6), and IL-10 were measured in plasma. Biochemical markers of organ damage, liver NAD^+/NADH (indicator of the mitochondrial redox state), plasma nitrate, tissue myeloperoxidase (MPO, indicator of neutrophil accumulation) and malondialdehyde (MDA, indicator of lipid peroxidation), liver and lung chemokines (macrophage inflammatory protein 1α [MIP-1α] and MIP-2), and *ex vivo* vascular reactivity in aortic rings were also measured. Mice treated with inosine had significantly lower levels of circulating cytokines. Organ damage was significantly reduced by inosine treatment, which was associated at the tissue level with an increased hepatic NAD^+/NADH ratio, decreased MPO activity in the lung, reduced MDA formation in the gut and liver, and decreased MIP-1α and MIP-2 in the lung and liver. Furthermore, inosine significantly improved endothelium-dependent relaxant responses of aortic rings. These effects were associated with significant improvement of the survival of CLP mice treated with inosine, an effect that was still observed when inosine treatment was delayed 1 h after CLP, especially when it was associated with appropriate antibiotic treatment. Thus, inosine reduced systemic inflammation, organ damage, tissue dysoxia, and vascular dysfunction, resulting in improved survival in septic shock.

Keywords: endothelium; organ failure; purines; shock; vascular

Sepsis and septic shock are heterogeneous clinical syndromes complicating the course of severe infections. In spite of major advances in the understanding of the pathogenesis of septic shock, no satisfying therapy has emerged and treatment of this condition remains largely supportive. An overwhelming inflammatory and immune response, resulting in multiple organ damage, cardiovascular failure, and death, is a prominent feature of sepsis, and strategies able to limit this systemic inflammatory response might result in an improved outcome of septic shock patients. Because of the vast array of mediators involved, it is likely that treatments able to modulate several aspects of this inflammatory cascade will be more efficient than strategies targeting a single mediator (1).

Certain naturally occurring purines—the best characterized being the nucleoside adenosine—can exert potent antiinflammatory effects on the immune system. For instance, adenosine has been shown to reduce the production of proinflammatory cytokines by inflammatory (2, 3) and noninflammatory cells (4) stimulated by bacterial lipopolysaccharide (LPS). Inosine is another endogenous purine formed from the breakdown of adenosine by adenosine deaminase (5). It is released from cells on environmental stress such as hypoxia (6) and exposure to LPS (7). Inosine was widely believed to be inert and without biologic actions. However, our group has 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 potent antiinflammatory effects in murine endotoxic shock (8). In view of the major role of proinflammatory cytokines in the pathogenesis of septic shock, the present study was designed to investigate the potential antiinflammatory role of inosine in a clinically relevant model of septic shock represented by cecal ligation and puncture (CLP).

METHODS

In vivo studies were performed in accordance with National Institutes of Health guidelines and with the approval of the local IACUC committee.

Cecal Ligation Puncture Model

A total of 207 male BALB/c mice, 8 wk old, subjected to CLP, were used in this study. Mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), given intraperitoneally. Under aseptic conditions, a 2-cm midline laparotomy was performed to allow exposure of the cecum with adjoining intestine. The cecum was tightly ligated with a 3.0 silk suture at its base, below the ileocecal valve, and was perforated twice with an 18-gauge needle (top and bottom). The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites. The cecum was then returned to the peritoneal cavity and the laparotomy was closed with 4.0 silk sutures. In addition, n = 20 animals (10 treated with vehicle, 10 treated with inosine) were used for control purposes. All animals were then returned to their cages with free access to food and water.

Experimental Protocols

For the purpose of biochemical measurements (see below), 20 mice were made septic by CLP. Twelve hours later, the mice were reanesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), given intraperitoneally. Blood samples were obtained by cardiac puncture, followed by the harvesting of samples from the lung, liver, and gut (ileum), immediately frozen in liquid nitrogen, and then stored at $-70°$ C until assayed for myeloperoxidase activity, malondialdehyde formation, chemokine production (macrophage inflammatory protein 1α [MIP-1α] and MIP-2), and NAD^+/NADH, as detailed below. For the

(*Received in original form January 3, 2001; accepted in final form July 10, 2001*)

Supported, in part, by grants from the National Institutes of Health (R43GM59560 and R01GM60915).

Dr. Liaudet is on leave from the Critical Care Division, Department of Internal Medicine (University Hospital, Lausanne, Switzerland) and is supported by a grant from the ADUMED Foundation (Switzerland). Dr. Soriano is on leave from the Department of Critical Care Medicine (Hospital das Clinicas da Universidade de Sao Paulo, Brazil) and is supported by a fellowship from FAPESP (Brazil). Dr. Pacher is on leave from the Department of Pharmacology and Pharmacotherapy (Semmelweis University, Budapest, Hungary).

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This article has an online data supplement, which is accessible from this issue's table of contents online at www.atsjournals.org

Am J Respir Crit Care Med Vol 164. pp 1213–1220, 2001
Internet address: www.atsjournals.org

measurement of vascular reactivity, 10 additional mice were made septic by CLP. After 12 h, the mice were killed by CO_2 inhalation, followed by a thoracotomy to allow excision of the aorta for vascular ring studies.

Treatment Groups

The animals were divided into two treatment groups, receiving either inosine at a dose of 100 mg/kg, intraperitoneally, or vehicle (isotonic saline, 0.5 ml) 1 h before CLP; the dose was repeated 6 h after. Additional doses of inosine or vehicle were given in the survival experiments (see specific protocols below).

Measurements

Circulating levels of tumor necrosis α, interleukin 6, and interleukin 10. The plasma concentrations of immunoreactive murine tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and IL-10 were determined by using commercially available enzyme-linked immunosorbent assays (ELISAs), according to the manufacturer protocol (R&D Systems, Minneapolis, MN).

Plasma nitrate and nitrite. Plasma nitrate and nitrite were measured as an index of nitric oxide production. First, nitrate in the plasma was reduced to nitrite by incubation with nitrate reductase (610 mU/ml) and NADPH (170 mM) at room temperature for 3 h. After 3 h, nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthalethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated H_3PO_4; 1:1, vol/vol). The optical density at 550 nm (OD_{550}, corrected for absorbance at 650 nm) was measured in a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentrations were calculated by comparison of OD_{550} values of standard solutions of sodium nitrite prepared in phosphate-buffered saline (PBS).

Quantitative bacterial cultures. For the purpose of quantitative bacterial cultures, 12 mice (5 mice receiving inosine pretreatment and 7 mice treated with vehicle) were challenged with CLP. After 24 h, the surviving animals (n = 4/group) were killed and blood was obtained, using sterile technique, by cardiac puncture. The liver and spleen were aseptically harvested, weighed, and homogenized in sterile PBS. Sequential dilutions (in PBS) of blood and tissue homogenates were plated on agar plates, and colony-forming units were counted after 24 h of incubation at 37° C (aerobic conditions). Quantitative cultures are expressed as colony-forming units per 100 mg of tissue or colony-forming units per milliliter of blood.

Plasma levels of alanine aminotransaminase, alkaline phosphatase, blood urea nitrogen, creatinine, and amylase. The plasma concentrations of alanine aminotransaminase (ALT), alkaline phosphates, blood urea nitrogen (BUN), creatinine, and amylase were determined enzymatically, using an automated VetScan chemistry analyzer (Abaxis, Union City, CA).

Measurement of NAD^+ and NADH by high-pressure liquid chromatography. Liver samples were homogenized in 400 μl of 0.2 M potassium cyanide, 0.06 M potassium hydroxide, and 1 mM bathophenanthrolinedisulfonic acid. Each sample was then extracted with chloroform and centrifuged in 4° C at 15,000 rpm for 5 min. A volume of 40 μl was injected on a high-performance liquid chromatography (HPLC) column. A Luna, 3 μm, 150 × 4.60 mm, C-18(2) HPLC column (Phenomenex, Torrance, CA) was used. The detectors used were an RF-10AXL fluorescence detector (wavelength: excitation, 330 nm; emission, 460 nm), and an SPD-10AV UV detector (wavelength, 254 nm). The mobile phase was 0.2 M ammonium acetate and HPLC-grade methanol with pH 5.88. The flow rate was 1 ml/min, with 96% ammonium acetate and 4% methanol initially and increasing up to 9% methanol in 25 min. NAD^+ and NADH levels were expressed as nanomoles per milligram of wet tissue.

Myeloperoxidase assay. Tissues were homogenized (50 mg/ml) in 0.5% hexadecyltrimethylammonium bromide in 10 mM 3-*N*-morpholinopropanesulfonic acid (MOPS) and centrifuged at 15,000 × *g* for 40 min. The suspension was then sonicated three times for 30 s. An aliquot of supernatant 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. Results are expressed as milliunits of myeloperoxidase (MPO) activity per milligram of protein, which were determined with the Bradford assay.

Malondialdehyde assay. Malondialdehyde (MDA) formation was utilized to quantify the lipid peroxidation in tissues and was measured as thiobarbituric acid-reactive material. Tissues were homogenized (100 mg/ml) in 1.15% KCl buffer. The homogenates (200 μl) were then added to a reaction mixture consisting of 1.5 ml of 0.8% thiobarbituric acid, 200 μl of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid (pH 3.5), and 600 μl of 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 was measured at 532 nm, using 1,1,3,3-tetramethoxypropane as an external standard. The level of lipid peroxides was expressed as nanomoles of MDA per milligram of protein (Bradford assay).

Measurement of MIP-1α and MIP-2 in lung and liver. The production of the chemokines MIP-1α and MIP-2 was determined in tissue homogenates from the lung and liver. Tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer. After centrifugation (14,000 rpm, 30 min), the concentrations of MIP-1α and MIP-2 were assayed in the clear supernatants, using a commercially available ELISA, according to the manufacturer protocol (R&D Systems). The levels of the chemokines were normalized to the tissue content in proteins, measured by the Bradford assay.

Measurement of vascular reactivity in isolated aortic rings. The thoracic aorta was cleared from periadventitial fat and cut into rings 0.5–1 mm in width, mounted in organ baths filled with warmed (37° C) and oxygenated (95% O_2, 5% CO_2) Kreb's solution (1.6 mM $CaCl_2$, 1.17 mM $MgSO_4$, 0.026 mM EDTA, 130 mM NaCl, 14.9 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 11 mM glucose). Isometric forces were measured with isometric transducers (Kent Scientific, Litchfield, CT), digitized with a MacLab A/D converter, and stored and displayed on a Macintosh computer. A tension of 1 g was applied and the rings were equilibrated for 60 min, followed by measurements of the concentration-dependent contraction to epinephrine (10^{-10} to 3×10^{-5} M) and relaxation to acetylcholine (10^{-9} to 10^{-5} M).

Survival Experiments

In a first protocol, 38 animals exposed to CLP received either inosine (100 mg/kg, intraperitoneally; n = 19) or vehicle (isotonic saline, 0.5 ml, intraperitoneally; n = 19), 1 h before CLP, repeated after 6 and 12 h. In a second set of experiments, 64 mice were exposed to CLP. Inosine (100 mg/kg, intraperitoneally; n = 28) or vehicle (isotonic saline, 0.5 ml, intraperitoneally; n = 36) was given after 1 h and additional doses were given after 12 and 24 h. In a last set of experiments, the effect of inosine or vehicle, coadministered with antibiotics, was analyzed in 53 mice. The animals were challenged with CLP, and after 1 h received an intraperitoneal treatment consisting of either inosine (100 mg/kg, n = 26) or saline (0.5 ml, n = 27), as well as a subcutaneous injection of gentamicin (10 mg/kg) and clindamycin (300 mg/kg), dissolved in 1 ml of saline. Additional doses of inosine and saline were given after 12 and 24 h, while the antibiotics were given every 12 h over a total of 3 d. The mortality of the animals was recorded over a 1-wk period.

Reagents

Inosine and all other chemicals were purchased from Sigma (St. Louis, MO).

Statistical Analysis

Data are expressed as means ± SEM in all figures. For the biochemical measurements in tissues (MPO, MDA, NAD^+, and NADH) and for the aortic ring studies, the means from the different treatment groups were compared by unpaired *t* test. The plasma variables were compared by a nonparametric test (Mann–Whitney). In the survival experiments, the survival curves of the different treatment groups were compared, using the log-rank test. Statistical significance was assigned to $p < 0.05$.

RESULTS

Plasma cytokines and nitrate concentrations are illustrated in Figure 1. As expected, CLP induced high levels of all mea-

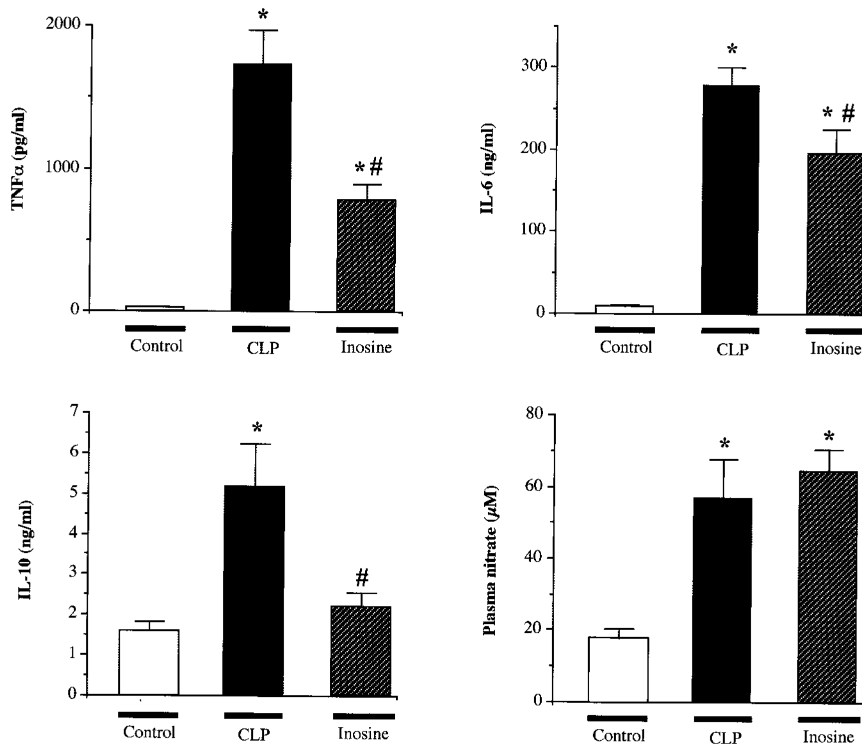

Figure 1. Circulating levels of TNF-α, IL-6, IL-10, and nitrate. Mice made septic with CLP were treated 1 h before and 6 h after CLP with either inosine (100 mg/kg, intraperitoneally; n = 10) or vehicle (isotonic saline, 0.5 ml, intraperitoneally). Plasma cytokines and nitrate were measured after 12 h of sepsis. Inosine-treated mice had significantly reduced circulating levels of all the measured cytokines; no influence was evident on the level of NO production. Values represent means ± SEM. *$p < 0.05$, indicated value versus control; #$p < 0.05$, CLP plus inosine versus CLP.

sured cytokines. The time point chosen for this analysis (12 h after the onset of CLP) corresponds to the peak of circulating cytokines levels in this model (9). For both the proinflammatory cytokines TNF-α and IL-6 and the antiinflammatory cytokine IL-10, treatment with inosine induced a significant decrease in the plasma levels (TNF-α, from 1,727 ± 240 to 784 ± 112 pg/ml; IL-6, from 278 ± 23 to 198 ± 30 ng/ml; IL-10, from 5.3 ± 1.1 to 2.3 ± 0.4 ng/ml). In contrast, the plasma concentrations of nitrate were comparable and non-significantly different between noninosine-treated and inosine-treated mice (56 ± 11 and 64 ± 6 μM, respectively), indicating a lack of effect of inosine on NO production in this model.

Quantitative bacterial cultures demonstrated that all the animals subjected to CLP had positive cultures in blood and tissues after 24 h, with no statistically significant differences between the treatment groups. In mice treated with vehicle or inosine, the colony-forming units recovered were, respectively, 6.21 (± 2.95) × 10^8 versus 8.46 (± 5.54) × 10^8 CFUs/100 mg of tissue (liver, p = 0.73, *t* test), 3.03 (± 1.69) × 10^8 versus 1.97 (± 0.75) × 10^8 CFUs/100 mg of tissue (spleen, p = 0.58), and 1.38 (± 1.24) × 10^6 versus 0.39 (± 0.19) × 10^6 CFUs/ml (blood, p = 0.46).

The measurement of biochemical parameters of tissue injury revealed that inosine treatment significantly attenuated liver, kidney, and pancreas damage during CLP. Plasma ALT and alkaline phosphatase levels were reduced, respectively, from 382.5 ± 64.6 UI/L (CLP) to 127.5 ± 21.5 UI/L (CLP plus inosine), and from 104.6 ± 15.9 UI/L (CLP) to 34.9 ± 5.3 UI/L (CLP plus inosine; $p < 0.05$ for both variables). Regarding renal damage, inosine treatment reduced the plasma concentrations of urea nitrogen from 69.4 ± 2.4 to 23.1 ± 0.8 mg/dl ($p < 0.05$) and those of creatinine from 1.12 ± 0.05 to 0.38 ± 0.02 mg/dl ($p < 0.05$). In addition, the increase in plasma amylase noted after CLP in mice treated with vehicle (1,514 ± 146 UI/L) was also significantly suppressed by inosine treatment (504 ± 49 UI/L, $p < 0.05$).

Organ alterations were further investigated by the measurements of myeloperoxidase activity (MPO) and malondialdehyde (MDA) formation. With respect to MPO, we did not notice any significant increase in the gut and liver after CLP, when compared with control conditions, and there was no influence of inosine treatment. In contrast, CLP induced a significant increase in MPO in the lung, an effect that was significantly prevented by inosine treatment (control, 17.8 ± 2.7 mU/mg protein; CLP, 58.1 ± 8.6 mU/mg protein; CLP plus inosine, 32.7 ± 5.1 mU/mg protein, $p < 0.05$ versus CLP). In contrast to its influence on lung MPO, inosine did not influence the increase in lung MDA formation during CLP. The respective values of lung MDA in control, CLP, and CLP–inosine mice were 4.2 ± 0.7 nmol/mg protein, 6.2 ± 0.5 nmol/mg protein ($p < 0.05$ versus control), and 6.3 ± 0.3 nmol/mg protein ($p < 0.05$ versus control, p = NS versus CLP). Alternatively, inosine significantly attenuated MDA formation in the gut (CLP, 2.91 ± 0.16 nmol/mg protein; CLP plus inosine, 2.13 ± 0.11 nmol/mg protein, $p < 0.05$) and the liver (CLP, 9.57 ± 0.84 nmol/mg protein; CLP plus inosine, 5.37 ± 0.44 nmol/mg protein, $p < 0.05$).

Liver NAD^+ and NADH levels are shown in Table 1. Inosine administration was associated with a significantly maintained NAD^+ content. The ratio of NAD^+/NADH was thus significantly higher in inosine-treated animals during CLP.

To determine the potential influence of inosine on the expression of chemokines during CLP, the concentrations of MIP-1α (a CC chemokine) and MIP-2 (a C-X-C chemokine) were determined in homogenates from lung and liver. As illustrated in Figure 2, a massive increase in these two chemokines occurred in mice challenged with CLP, in both the lung (Figure 2A) and liver (Figure 2B), an effect significantly suppressed by inosine.

Vascular function was assessed by determining the *ex vivo* contractility and relaxation of thoracic aortic rings. Concentration–response curves to epinephrine and acetylcholine are

TABLE 1. NAD⁺ AND NADH CONTENT IN THE LIVER*

	NAD^+ (*nmol/mg*)	NADH (*nmol/mg*)	NAD^+/NADH
Control	19.01 ± 2.21	1.21 ± 0.04	14.9 ± 1.23
CLP	3.98 ± 1.24†	0.55 ± 0.16†	9.1 ± 2.83†
Inosine	15.23 ± 2.82	0.076 ± 0.014†‡	234.29 ± 66.72†‡

* Mice subjected to septic shock by CLP were treated with inosine (100 mg/kg, intraperitoneally) or vehicle (isotonic saline) 1 h before and 6 h after. NAD^+ and NADH were measured by HPLC in liver samples taken 12 h after the induction of CLP. Values represent means ± SEM.

† $p < 0.05$, indicated value versus control.

‡ $p < 0.05$, CLP plus inosine versus CLP.

shown in Figure 3. CLP animals, but not the animals treated with inosine during CLP, exhibited suppressed contractions when compared with controls. We also noted a significant improvement of the relaxant response to acetylcholine in aortic rings harvested from inosine-treated mice, indicative of a reduction of sepsis-induced endothelial dysfunction by this purine.

The results of the survival experiments are shown in Figure 4. In the pretreatment protocol (Figure 4a), mice treated with vehicle started to die between 6 and 12 h after CLP, with a death rate reaching 60 and 80% at 24 and 36 h post-CLP, respectively. The overall mortality at the end of the observation period was 95% (1 of 19 animals alive). The onset of death was markedly delayed in mice receiving inosine treatment. Indeed, at 24 and 36 h after CLP, the mortality rate in this group was 11 and 58%, respectively. At the end of the study, the global survival in this group was 23% (5 of 19 mice alive). Comparison of the two survival curves, using the log-rank test, showed a significant ($p < 0.05$) survival advantage for mice treated with inosine. In the posttreatment protocol (Figure 4b), we also noted that administration of inosine significantly delayed the onset of death after CLP. Although the actual survival at the end of the observation period was similar between vehicle and inosine-treated mice, comparison of the global survival curves by the log-rank test yielded a significant difference between the two treatment groups. Figure 4c shows the result of the survival experiments using the coadministration of antibiotics and inosine or vehicle as a posttreatment strategy. Inosine-treated mice showed a significant improvement in survival, with a global survival rate of 24% at the end of the observation period, contrasting with a 7% survival rate in vehicle-treated animals.

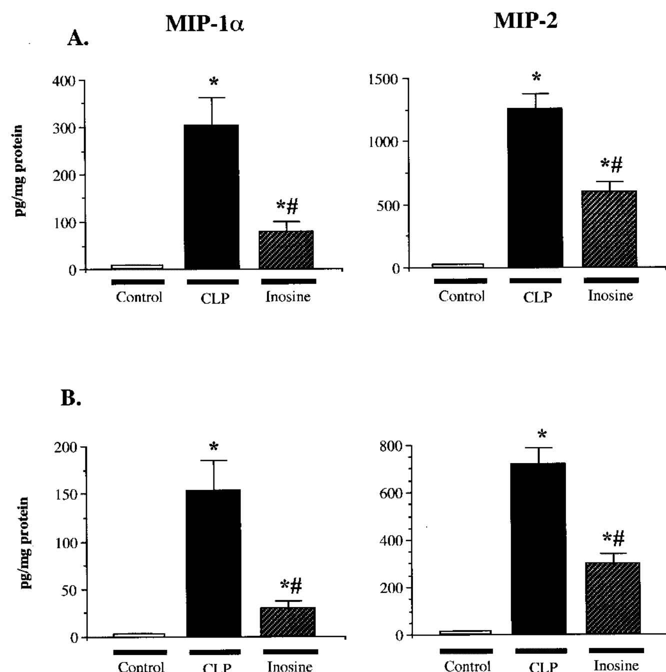

Figure 2. Expression of the chemokines MIP-1α and MIP-2 in liver and lung during CLP. Mice made septic with CLP were treated 1 h before and 6 h after CLP with either inosine (100 mg/kg, intraperitoneally; n = 10) or vehicle (isotonic saline, 0.5 ml, intraperitoneally; n = 10). Samples from the lung and liver were harvested after 12 h. There was no detectable level of MIP-1α and MIP-2 in control mice (not exposed to CLP). CLP induced a marked increase in both chemokines in the lung (*A*) and liver (*B*), an effect that was significantly reduced in animals receiving inosine treatment. *$p < 0.05$, indicated value versus control; #$p < 0.05$, CLP plus inosine versus CLP.

DISCUSSION

The main results of this study were that inosine, a naturally occurring purine, markedly reduced the systemic inflammation, multiple organ damage, and vascular endothelial dysfunction associated with sepsis induced by cecal ligation and perforation, leading to a significant improvement in survival.

Background and Previous Work

Previous data from our laboratory and other investigators indicated that inosine has marked antiinflammatory effects, both *in vitro* and *in vivo.* Inosine reduced the production of proinflammatory cytokines by murine macrophages stimulated by *Escherichia coli* lipopolysaccharide (LPS) (3, 8), and decreased the release of cytosolic enzymes from hypoxic rat lymphocytes (10). In a model of systemic inflammation induced by LPS, inosine limited the rise of cytokines TNF-α, IL-12, and interferon γ (IFN-γ) and the chemokine MIP-1α, and increased the production of the antiinflammatory cytokine IL-10. These effects were proposed to be mediated, at least in part, by an adenosine receptor mechanism, and it was suggested that inosine acted mostly through posttranscriptional mechanisms (8). The data presented in the current study confirm and extend these previous findings, by showing that inosine has major beneficial effects in a clinically relevant model of septic shock.

Effects of Inosine on the Production of Cytokines during Sepsis

CLP induced a massive systemic inflammatory response, evidenced by the high levels of plasma TNF-α and IL-6. In agreement with our data about endotoxic shock (8), inosine reduced the production of these proinflammatory cytokines. Since both TNF-α and IL-6 are proximal mediators of septic shock, it is likely that their downregulation was a central mechanism underlying the overall beneficial effects of inosine. However, it is noteworthy that, in spite of their deleterious consequences on the host, proinflammatory cytokines, predominantly TNF-α, also play a major role in the defense against invading pathogens. Indeed, mice genetically deficient in TNF-α are extremely sensitive to bacterial peritonitis (9), and clinical trials evaluating anti-TNF-α strategies yielded disappointing results (11). Such concerns cannot be raised in our study because, although inosine depressed TNF-α production, this effect was not associated with a reduction in antibacterial defenses, as indicated by the results of the quantitative bacterial cultures performed in the liver, spleen, and blood. Therefore, the inosine-dependent reduction of TNF-α essentially

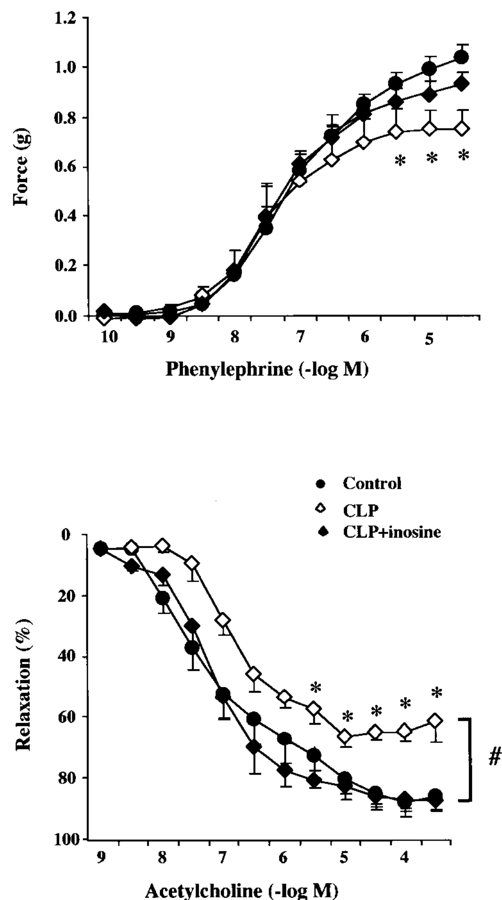

Figure 3. Vascular ring experiments. Mice made septic with CLP were treated 1 h before and 6 h after CLP with either inosine (100 mg/kg, intraperitoneally; n = 10) or vehicle (isotonic saline, 0.5 ml, intraperitoneally). Thoracic aortae (n = 5 per group) were harvested 12 h after CLP to measure their contraction to epinephrine (*top*) and relaxation to acetylcholine (*bottom*) *ex vivo.* With respect to control conditions, vascular contractility was significantly depressed by CLP in vehicle-treated, but not in inosine-treated, animals. However, the difference between the two groups of mice challenged with CLP was not statistically significant. In contrast, inosine significantly improved the relaxant response to acetylcholine during CLP. Values represent means ± SEM. $^{*}p < 0.05$, indicated value versus control; $^{\#}p < 0.05$ CLP plus inosine versus CLP.

blunted its proinflammatory effects without interfering with its antibacterial effects.

Another feature of CLP was the high plasma level of the antiinflammatory cytokine IL-10, which was reduced by inosine. This was an unexpected finding because inosine was previously reported to increase IL-10 in endotoxic shock (8), suggesting that the mechanisms regulating IL-10 production may be partially distinct during endotoxic and septic shock. In addition, IL-10 has been shown to be crucial in preventing overproduction of proinflammatory cytokines during systemic inflammation (12), pointing to potential harmful consequences of reducing IL-10 in such conditions. However, this was not the case in our study, in which inosine, although decreasing IL-10, consistently reduced inflammation after CLP. These findings are actually consistent with previous studies reporting deleterious effects of increased IL-10 production in animal models of septic shock. Indeed, IL-10 was reported to suppress lymphocyte proliferation and lymphokine production during sepsis (13), and the administration of anti-IL-10 monoclonal antibodies has been shown to improve the survival of mice after CLP (13), as well as in a two-hit model of CLP followed by the intratracheal instillation of *Pseudomonas* (14). Furthermore, administration of exogenous IL-10 failed to decrease morbidity or mortality in mice challenged with CLP (15). Overall, our data show that inosine led to a generalized downregulation of the activation state of murine macrophages, by mechanisms that remain to be characterized.

Effects of Inosine on Organ Damage and Vascular Function during Sepsis

CLP was associated with signs of multiple organ damage, involving the liver, kidney, and pancreas, that were all significantly attenuated by inosine. At the tissue level, inosine reduced the formation of malondialdehyde in the gut and liver, indicating a partial suppression of oxidative stress in these organs. This is of particular relevance because oxidant stress is a likely mechanism of gut mucosal barrier dysfunction in sepsis, a condition that is able to amplify and perpetuate the initial systemic inflammatory response (16). Indeed, we reported that inosine suppresses gut mucosal barrier failure induced by LPS in mice (17). An additional finding of our study was the significantly higher hepatic NAD^+/NADH ratio, which reflects the redox state in liver mitochondria (18) in inosine-treated mice (Table 1). A reduced NAD^+/NADH ratio has been shown to be an early and sensitive indicator of the onset of tissue dysoxia (18, 19), notably in sepsis, in which dysoxia may develop as a consequence of either perfusion abnormalities (hypoxic hypoxia), altered cellular handling of oxygen (cytopathic hypoxia), or both (20). Our results then indicate that inosine reduced sepsis-induced tissue dysoxia, at least in the liver, although the underlying mechanisms cannot be directly inferred from our data. In all likelihood, this influence of inosine on hepatic metabolism played a determinant role in decreasing organ damage in our septic animals.

Finally, we also found that inosine reduced neutrophil sequestration in the lung, a well-known consequence of sepsis eventually leading to the acute respiratory distress syndrome (21). Data have indicated that the C-X-C chemokine MIP-2 represents a major mediator regulating neutrophil trafficking in the lungs under septic conditions (22). In agreement with these data, we found a massive increase in the pulmonary levels of MIP-2 in mice challenged with CLP. In contrast, mice treated with inosine exhibited a marked reduction of MIP-2 production, which probably accounted in large part for the reduced lung neutrophil infiltration noted in these animals. It is noticeable that a similar downregulation of MIP-2 production by inosine was measured in the liver, but this effect did not translate into a reduction of MPO activity in this organ. In fact, there was no accumulation of neutrophils in the liver of septic mice, indicating that MIP-2 did not play a major role as a neutrophil chemoattractant in this organ, which is consistent with previous data from the literature (22). In addition to reducing MIP-2 production, inosine also massively decreased the expression of MIP-1α, a CC chemokine acting mainly as a monocyte chemoattractant (23), in the lung and the liver. Thus, beyond its effects on the production of proinflammatory cytokines, inosine also appears to be an important modulator of leukocyte trafficking under septic conditions by downregulating the expression of various chemokines. Because strategies aimed at inhibiting various chemokines have been shown to decrease tissue injury and mortality in experimental sepsis (22, 24, 25), it is likely that these effects of inosine largely contributed to improve the outcome of CLP in our study.

Another effect of inosine was the suppression of the endothelial dysfunction induced by CLP, indicated by a significant improvement in the vascular relaxation to acetylcholine. Al-

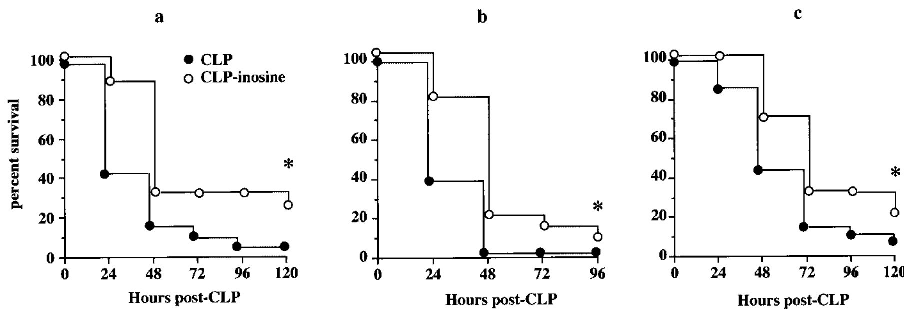

Figure 4. Survival experiments. Mice were made septic by CLP and were treated 1 h before and 6 and 12 h after CLP with either inosine (100 mg/kg, intraperitoneally; n = 19) or vehicle (isotonic saline, 0.5 ml, intraperitoneally; n = 19). In this pretreatment protocol (*a*), inosine-treated animals had a significantly improved survival during the 7-d observation period (no change in mortality was observed after Day 5 in both experimental groups). In a second study (posttreatment, *b*), inosine (100 mg/kg, intraperitoneally; n = 28) or vehicle (isotonic saline, 0.5 ml, intraperitoneally; n = 36) was administered 1 h after CLP, with additional doses given after 12 and 24 h. Although mortality at the end of the observation period was similar between treatment groups, analysis of the global survival profile showed a significant difference, inosine delaying the onset of death after CLP (no change in mortality was observed after Day 4 in both experimental groups). In a third study (*c*), animals were made septic by CLP and were treated after 1 h with antibiotics (gentamicin, 10 mg/kg and clindamycin, 300 mg/kg) given subcutaneously in 1 ml of saline, in association with either inosine (100 mg/kg, intraperitoneally; n = 26) or vehicle (isotonic saline, 0.5 ml, intraperitoneally; n = 27). Additional doses of antibiotics were given every 12 h over 3 d, while inosine or vehicle was repeated after 12 and 24 h. Inosine significantly delayed the onset of death in CLP animals and was associated with an improved survival rate at the end of the observation period (24 versus 7% survival) (no change in mortality was observed after Day 5 in both experimental groups). The survival curves represent the summary of two (*a*), 3 (*b*), and 3 (*c*) independent experiments. *$p < 0.05$, log-rank test.

though the mechanisms of endothelial dysfunction in sepsis remain largely unresolved, a depressed activity of endothelial NO synthase (eNOS) has been consistently reported in experimental models (26, 27), and might be related in part to a negative effect of TNF-α on eNOS mRNA stability (28). Thus, we can speculate that inosine reversed endothelial dysfunction by reducing TNF-α levels. In contrast to its effect on vascular relaxation, we did not find any effect of inosine on vascular contractility. This was indeed consistent with the lack of influence of inosine on NO production, the major mediator of vascular hypocontractility in sepsis.

Effects of Inosine on Survival of CLP Mice

We found that inosine significantly improved the survival of CLP. We have previously reported that inosine improved survival in endotoxic shock (8), but the present data are of greater clinical relevance, being obtained in a model of polymicrobial sepsis. The effect of inosine was observed not only in a pretreatment strategy, but also when treatment was started 1 h after the onset of sepsis, especially when inosine was associated with antibiotics. Antibiotics alone only slightly delayed mortality, whereas the coadministration of inosine provided significant protection up to 7 d after CLP. Although the main effect of inosine was to delay the onset of death, it was still associated with a significant survival advantage 7 d after CLP (24% survival with inosine plus antibiotics, compared with 7% with antibiotics alone). It is noticeable that the model of CLP chosen in the present study was particularly severe because cecal perforation was achieved with a large-bore needle (18-gauge). Therefore, although not tested in this study, it is possible that inosine would provide a greater survival advantage in a less severe model of sepsis. Taken together, these data indicate that inosine may represent a valuable approach to reduce systemic inflammation and to improve outcome in clinical septic shock.

Several distinct strategies have been previously reported to improve survival in mice challenged with cecal ligation and puncture. The steroid hormone dehydroepiandrosterone reduced short-term mortality, an effect associated with a reduction of TNF-α release and an improvement of the activity of T cellular immunity (29). Recombinant heparin-binding protein, which enhances monocyte phagocytic activity, was also found to improve short-term survival when administered before CLP (30). Recombinant human tissue factor pathway inhibitor improved the survival of CLP, although this beneficial effect was observed only in CLP performed with a small-bore (23-gauge), not a large-bore (21-gauge), needle (31). In another study, a reduction in CLP mortality was reported after the administration for six consecutive days of recombinant granulocyte-macrophage colony-stimulating factor, which improved the killing of bacteria in various tissues (32). Finally, neutralization of the macrophage migration inhibitory factor (MIF) by anti-MIF antibodies was shown to produce a marked increase in survival, even when treatment was delayed up to 8 h after CLP, defining a critical part for MIF in the pathogenesis of septic shock (9). Although it is difficult to compare the results of the aforementioned studies with our data, it is worth mentioning that inosine was effective in a severe model of CLP, performed with a large-bore (18-gauge) needle, and that its protective effects were still present when inosine was administered after the septic challenge. Also, our study provides evidence that inosine, beyond its immunomodulatory effects, also affords protection from several important pathophysiologic alterations associated with sepsis, such as oxidant stress, tissue neutrophil infiltration, end-organ damage, and vascular dysfunction.

Potential Mechanisms of the Inosine Mode of Action

Our studies suggest that the effect of inosine is not sensitive to nucleoside uptake into cells and is partially mediated via adenosine receptors (8). In the presence of adenosine receptor (A1 and A2) antagonists, the inhibition by inosine of TNF-α release by stimulated macrophages was significantly abrogated (8). The effect of inosine was suppressed, but not abolished, in peritoneal macrophages lacking A2a receptors, indicating the partial involvement of this receptor subtype (33). Subsequent studies demonstrated that inosine failed to alter the activation of nuclear factor κB and various stress kinases (p42/44 MAPK, p38 MAPK, and JNK), and did not modify the expression of a host of cytokine mRNAs in LPS-stimulated macrophages (8), pointing to a posttranscriptional effect of inosine on cytokine production. Another potential mechanism of action of inosine may involve interference with the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) in response to DNA injury and oxidant-induced cell death (34). Activated PARP cleaves NAD^+ and depletes the cellular ATP pools, causing necrotic cell death. PARP has also been implicated in the transcriptional regulation of inflammatory mediators (34). Purines, including inosine, share structural similarities with NAD, the substrate of PARP. We have

demonstrated (35) that purines inhibit PARP activation *in vitro*. It is therefore conceivable that PARP inhibition contributed to the antishock effects of inosine *in vivo*. A PARP inhibitory action may also explain the maintenance of cellular NAD levels in the presence of inosine, as noted in the current study (Table 1). Finally, inosine may also enhance endogenous antioxidant systems because the breakdown of inosine yields urate, a scavenger of oxyradicals and peroxynitrite (36–39). Uric acid administration has been shown to exert protective effects in animal models of circulatory shock (40).

Conclusion and Implications

In summary, our data provide the first experimental demonstration that inosine reduces the consequences of septic shock induced by cecal ligation and perforation in the mouse, by decreasing the production of cytokines, limiting tissue inflammation and end-organ damage, and improving vascular function, thereby leading to an improvement in survival. The current data, coupled with the known excellent safety profile of the purine and the lack of inosine's hemodynamic effects may suggest that the concept of testing and developing inosine as an antishock agent may be justified.

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