Spermine Inhibits Proinflammatory Cytokine Synthesis in Human Mononuclear Cells: A Counterregulatory Mechanism that Restrains the Immune Response

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Summary

The local production of proinflammatory cytokines mediates the host response to inflammation, infection, and injury, whereas an overexpression of these mediators can injure or kill the host. Recently, we identified a class of multivalent guanylhydrazone compounds that are effective inhibitors of proinflammatory cytokine synthesis in monocytes/macrophages. The structure of one such cationic molecule suggested a molecular mimicry with spermine, a ubiquitous endogenous biogenic amine that increases significantly at sites of inflammation and infection. Here, we addressed the hypothesis that spermine might counterregulate the innate immune response by downregulating the synthesis of potentially injurious cytokines. When spermine was added to cultures of human peripheral blood mononuclear cells stimulated with lipopolysaccharide (LPS), it effectively inhibited the synthesis of the proinflammatory cytokines tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, MIP-1α, and MIP-1β. The inhibition of cytokine synthesis was specific and reversible, with significant inhibition of TNF synthesis occurring even when spermine was added after LPS. The mechanism of spermine-mediated cytokine suppression was posttranscriptional and independent of polyamine oxidase activity. Local administration of spermine in vivo protected mice against the development of acute footpad inflammation induced by carrageenan. These results identify a distinct molecular counterregulatory role for spermine in downregulating the monocyte proinflammatory cytokine response.

During the early immune response to infection or injury, macrophages synthesize proinflammatory cytokines, which orchestrate the inflammatory reaction. Relatively small amounts of these cytokines produced locally in tissues benefit the host by activating antimicrobial pathways and stimulating tissue repair. Evidence of these protective mechanisms has been obtained in animal studies, where administration of anti-TNF antibodies worsens the severity and duration of Leishmania infection in mice (1), and mice rendered insensitive to TNF by knockout of TNF receptors are exquisitely sensitive to infection by intracellular pathogens (2). On the other hand, the uncontrolled release of larger amounts of cytokines, and the resultant mediator cascade, signals the onset of tissue injury and lethal shock (3–5). This potentially disastrous scenario is normally prevented by endogenous counterregulatory mechanisms that have evolved to inhibit cytokine overproduction. One class of endogenous cytokine synthesis inhibitors are the glucocorticoid hormones, which are produced during the stress response, and suppress immune activation and cytokine synthesis (6, 7). Another class is comprised of the anti-inflammatory cytokines (e.g., TGF-β and IL-10), which effectively inhibit macrophage activation and proinflammatory cytokine synthesis and prevent the injurious sequelae of cytokine excess (8–12). Lastly, prostaglandin E2, which accumulates at sites of inflammation, can also suppress TNF synthesis by increasing intracellular cAMP (13, 14). Together, these molecular mechanisms serve to counterregulate or dampen the inflammatory response, and to prevent the overabundant production of potentially injurious cytokines during the immune response to invasive stimuli.

The present study originated from our recent work focused on a class of low molecular weight multivalent guanylhydrazone compounds that suppress proinflammatory cytokine synthesis in activated monocytes/macrophages (15, 16). One of these, N,N′-bis[3,5-bis [1(amino-imi-

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nomethyl)hydrazono]-ethyl]phenyl]-decanediamide tetrahydrochloride (termed CNI-1493) effectively inhibits TNF translation and suppresses the production of the pro-inflammatory cytokines IL-1, IL-6, MIP-1α, and MIP-1β in human PBMCs (16, 17). Inhibition of proinflammatory cytokine synthesis by CNI-1493 is specific, because CNI-1493 does not inhibit synthesis of the anti-inflammatory cytokine TGF-β, nor does it prevent the upregulation of MHC class II induced by IFN-γ (16). By suppressing proinflammatory cytokine production in vivo, CNI-1493 protects mice against the lethal effects of endotoxin, and prevents the acute inflammatory response in carrageenan-induced footpad edema (15, 16). We considered it plausible that the cytokine inhibitory activities of this cationic anti-inflammatory molecule might be attributable to molecular mimicry with an endogenous molecule(s) that normally participates in counterregulating cytokine production.

Spermine, a ubiquitous biogenic amine that is positively charged at physiological pH, has been widely studied for its biological roles in the regulation of DNA synthesis, cellular proliferation, modulation of ion channel function, and as a second messenger in cellular signaling (18). A large body of evidence also implicates spermine as an inhibitor of immune responses. For example, spermine prevents nitric oxide (NO)[1] production in macrophages activated by bacterial endotoxin (19, 20), downregulates human neutrophil locomotion (21), and is immunosuppressive to T cells (22). Increased spermine levels have been measured in tissues following injury, inflammation, and infection, derived in part from the release of intracellular spermine from dying and injured cells, and in part by stimulated biosynthesis (23). It has been suggested that the accumulation of spermine, and the products of its oxidative metabolism via polyamine oxidase, mediate the anti-inflammatory activity found in inflammatory exudates, human pregnancy serum, plasma from arthritic rats, and human rheumatoid synovial fluid (21, 24–28). Although these and other studies implicate spermine in suppressing the innate immune response, it was unclear whether it might also counterregulate proinflammatory cytokine synthesis.

We show here that spermine effectively suppresses the synthesis of proinflammatory cytokines in human PBMCs. The mechanism of cytokine inhibition by spermine was post transcriptional, reversible, specific, and independent of polyamine oxidase activity. The in vivo application of spermine protected mice against the development of carrageenan-induced edema, giving evidence that spermine accumulation in tissues can counterregulate the acute inflammatory response.

Materials and Methods

Cell Isolation and Culture. Human PBMCs were obtained by elutriation from normal individual donors to the Long Island Blood Bank services (Melville, NY). PBMCs were isolated by density gradient centrifugation through Ficoll (Ficoll–Paque PLUS, Pharmacia, Piscataway, NJ) as previously described (16, 29) with a yield typically of 2×10^8 adherent cells per isolation. Cells were resuspended in RPMI-1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated human serum, 0.1% l-glutamine, and 0.01% gentamicin, seeded into either 24-well (at 5×10^6 cells/well, for RNA isolation) or 96-well plates (at 5×10^5 cells/well, for spermine uptake and cytokine assays), and cultured overnight at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Nonadherent cells were removed after overnight culture, and adherent cells (monocytes) were subjected to different experimental treatments as indicated. For studies of murine macrophage-like cells, RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), seeded into 24-well tissue culture plates (1×10^6 cells/ml RPMI 1640, 10% FBS, 2 mM glutamine) and subjected to experimental treatments as described for human PBMCs.

Cytokine Induction. To stimulate cytokine production from human PBMCs, freshly sonicated *Escherichia coli* endotoxin (LPS; Sigma Chem. Co., St. Louis, MO) was added to a final concentration of 100 ng/ml. In some experiments, IFN-γ (Boehringer Mannheim, Indianapolis, IN) was also added to a final concentration of 25 U/ml. To evaluate the effect of spermine on production of cytokines from the stimulated human PBMCs, freshly prepared spermine (Sigma) was added to human PBMCs at various concentrations 1 h before LPS/IFN-γ stimulation. After LPS/IFN-γ stimulation, supernatants were harvested and assayed for level of cytokines by ELISA assays. Lactate dehydrogenase (LDH) release, trypan blue exclusion, and metabolism of MTT were used to evaluate cytotoxicity.

Cytokine Assay. TNF levels in supernatants and cell lysates of human PBMCs were determined by ELISA using monoclonal and polyclonal antibodies raised against human TNF (Picower Institute for Medical Research, Manhasset, NY). Serial dilution of recombinant human TNF (0–10,000 pg/ml) was used in ELISA to generate standard curves. In brief, 96-well microtiter plates were coated with 60 μl of supernatants or standard hTNF solution at 4°C for 8–18 h (or at 37°C for 2 h). After washing with buffer containing 20 mM Tris–HCl (pH = 7.4), 150 mM NaCl, and 0.05% Tween 20, 60 μl of polyclonal TNF antibodies diluted (at 1:200) in buffer containing 10 mM Tris–HCl (pH = 7.4), 150 mM NaCl, 0.2% Tween 20, and 1% goat serum was added and incubated at room temperature for 2 h. After several washings to remove the unbound antibodies, the bound antibodies were then reacted for 30 min with 60 μl (1:2,500 dilution) alkaline phosphatase–conjugated goat anti–rabbit IgG (H+L) (Boehringer Mannheim, Indianapolis, IN). After washing, the amount of specifically bound alkaline phosphatase-conjugated antibodies was determined by assaying for alkaline phosphatase activity with freshly prepared p-nitrophenylphospate in diethanolamine buffer (10 mM diethanolamine and 0.5 mM $MgCl_2$, pH = 9.5). A substrate solution was added into each well and incubated for 30 min at room temperature. The absorption at 405 nm was determined with a automatic EIA analyzer. MIP-1α and MIP-1β levels were determined by specific sandwich ELISA as previously described (30), with the exception that coating (goat) and revealing (rabbit) antibodies specific for human MIP-1α and human MIP-1β were used. IL-1β, IL-6, and TGF-β levels were determined by commercially obtained ELISA kits according to the instructions of the manufacturer (Genzyme, Cambridge, MA). Cell lysates were prepared in lysis buffer (50 mM Tris–HCl, 0.5% NP-40, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 0.02% sodium azide, pH 7.4).

[1] *Abbreviations used in this paper:* iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; NO, nitric oxide; PKC, protein kinase C.

Total RNA Isolation and Northern Blotting. Total RNA was isolated from human PBMCs by using the BRL TRIzol reagent kit as instructed by the manufacturer (GIBCO BRL, Gaithersburg, MD), separated on a 1% agarose gel with 2.2 M formaldehyde, and subsequently transferred to Biotrans nylon membrane (ICN, Aurora, Ohio). After UV cross-linking (at 150 mJ), the membrane was prehybridized at 45°C for 2 h in hybridization buffer containing 5× Denhardt's, 5× SSC, 50 mM NaH_2PO_4, 0.1% SDS, 50% formamide and 250 μg/ml salmon sperm DNA, and subsequently hybridized overnight at 45°C with [^{32}P]dCTP–labeled TNF or MIP-1α cDNA probes (using Radprime DNA Labeling System from BRL) in the same hybridization buffer. After two washings at room temperature for 20 min with 2× SSC, 0.1% SDS, two washings at room temperature for 20 min with 0.5× SSC, 0.1% SDS, and a final washing at 60°C for 30 min with 0.1× SSC, 0.1% SDS, the membrane was exposed to X-ray film at −70°C overnight and developed to visualize the signal. Level of TNF protein in the supernatants of human PBMCs were determined by ELISA for comparison to the corresponding TNF mRNA level.

Carrageenan-induced Footpad Inflammation. Paw edema was induced by injecting 50 μl λ-carrageenan (0.2% in phosphate buffer [1× PBS, pH 7.4]; Sigma, St. Louis, MO), into the plantar surface of the left hind footpad of female C3H/HeN mice (20–25 g body weight) either alone, or in combination with various concentrations of spermine. The right hind footpad was injected with 50 μl of PBS as control. 28 h after footpad injection, the thickness of the carrageenan- and saline-injected footpad was measured using calipers, and the data expressed as the difference between the diameters of the two footpads.

Results

Spermine Suppression of TNF Synthesis in LPS-activated Human PBMCs. To study the effect of spermine on TNF synthesis, human PBMCs purified by elutriation and adherence were stimulated with LPS (100 ng/ml). Whereas LPS is a potent inducer of TNF synthesis in these cells, pretreatment with spermine (60 min before LPS challenge) effectively suppressed LPS-induced TNF synthesis (Fig. 1). The 50% inhibitory concentration (IC_{50}) of spermine for TNF protein release was 20 ± 15 μM. Maximal TNF suppression was observed with spermine concentrations ⩾100 μM, which suppressed TNF production to levels that were 25–35% of the TNF levels produced by controls. Spermine did not interfere with ELISA detection of TNF as evidenced by the fact that TNF standard curves measured in the presence of spermine were comparable to TNF standard curves measured in its absence (data not shown). Moreover, TNF levels as assessed by the L929 cell cytotoxicity bioassay were also inhibited by addition of spermine (data not shown). Although the purified human PBMCs contained more than 95% monocytes, we wished to confirm that spermine inhibited TNF production in the murine macrophage-like cell line RAW 264.7. In these experiments, we observed that spermine inhibited LPS-induced TNF synthesis in RAW cells, and that the IC_{50} was 40 ± 20 μM, a value comparable ($P > 0.05$) to the IC_{50} determined in human PBMCs (see above).

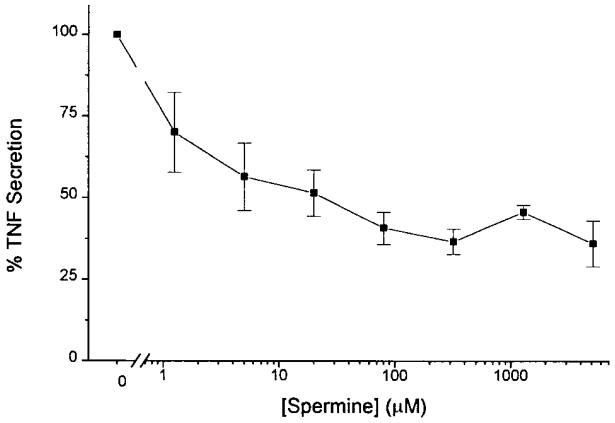

Figure 1. Spermine inhibits TNF synthesis from LPS-stimulated human PBMCs. Human PBMCs were exposed to the concentrations of spermine as indicated for 1 h, then stimulated by addition of LPS (100 ng/ml) and IFN-γ. TNF released into the conditioned supernatants collected after 4 h was measured by ELISA and results expressed as percentage of control (no spermine). Data shown are mean ± SEM of three independent experiments. (Control TNF = 7416 ± 763 pg/ml.)

We next studied the kinetics of spermine-mediated suppression of TNF synthesis in human PBMCs. In agreement with previous results showing that TNF is released within 2 h after stimulation of monocytes with LPS (31), pretreatment of monocytes with spermine before LPS, or coadministration of spermine simultaneously with LPS, was maximally effective in suppressing TNF synthesis (Table 1). However, significant inhibition was also observed, when spermine was added for up to 2 h after LPS (Table 1). To explore whether spermine-treated monocytes were capable of recovering the ability to synthesize TNF after spermine removal, we measured TNF production by human PBMCs that were incubated with spermine (35 μM) for 1 h, washed with PBS, and then incubated in fresh media without added spermine. TNF synthesis by washed PBMCs remained significantly decreased for up to 4 h after spermine removal from the cellular milieu (4-h washout, TNF = 1580 ± 376 pg/ml; control TNF = 3694 ± 214 pg/ml, $P < 0.05$). By 24 h after removing spermine, TNF synthesis was fully recovered to a level (TNF = 3211 ± 151 pg/ml) that was comparable to PBMCs that had never been exposed to spermine. Although recovery of TNF synthetic function by spermine-treated cells indicated that the mechanism of spermine inhibition of TNF synthesis could not be attributed to cytotoxicity, we also measured LDH release to assess cell viability. LDH activity was not increased in the media of spermine treated cells, even when spermine was added in concentrations exceeding 100-fold the IC_{50} for TNF suppression ([spermine, 3 mM] LDH = 18 ± 15 U/ml; versus controls [spermine, 0 mM] LDH = 21 ± 10 U/ml). Separate experiments using trypan blue exclusion and metabolism of MTT also confirmed that spermine concentrations ⩾3 mM were not toxic to human PBMCs during a 4 h treatment (data not shown).

Table 1. *Kinetics of Spermine-mediated Inhibition of TNF Synthesis from LPS and IFN-γ-stimulated Human PBMCs*

Time of spermine addition (min)*	TNF (percent of controls)
No spermine added	100
−60	26 ± 2
−30	26 ± 4
−5	33 ± 3
0	28 ± 3
+5	21 ± 3
+30	52 ± 7
+60	49 ± 8
+120	66 ± 7

*LPS and IFN-γ were added at time 0. Spermine (35 μM) was added at the relative times shown, and TNF in supernatants conditioned for 4 h after LPS was detected by ELISA. Data are mean ± SEM from six replicates of an experiment that was repeated twice.

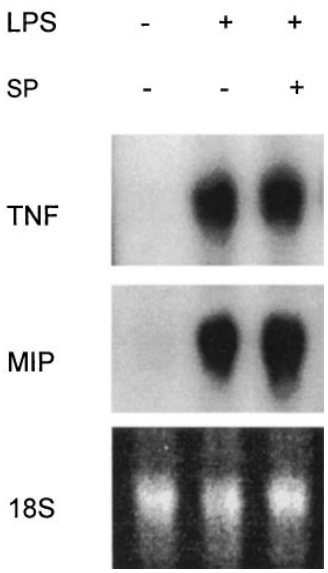

Figure 2. Spermine suppression of TNF and MIP-1α is posttranscriptional in LPS-stimulated human PBMCs. Northern blots for TNF and MIP-1α in human PBMCs performed 4 h after stimulation by LPS (100 ng/ml). Where indicated, spermine (35 μM) was added 1 h before LPS. Results are representative of two separate experiments using 6 μg of total RNA applied per lane.

Spermine Suppression of TNF Is Posttranscriptional and Specific. The mechanism of spermine action on cytokine synthesis was initially investigated by Northern blot analysis of total RNA extracted from LPS-activated human PBMCs using [^{32}P]dCTP–labeled TNF and MIP-1α cDNA as probes. In agreement with previous results (17), TNF and MIP-1α mRNA were not detected in human PBMCs in the absence of LPS, but were significantly increased 2 h after addition of LPS (100 ng/ml) (Fig. 2). Steady state levels of TNF and MIP-1α mRNA were not decreased by addition of spermine at a concentration (35 μM), although TNF and MIP-1α protein synthesis in the same cell cultures was suppressed to a level that was just 25 and 8% of controls, respectively ($P < 0.05$) (Fig. 3, *a* and *c*). We obtained similar results in separate experiments using quantitative PCR methodology in which the steady state level of TNF mRNA was found to be comparable between spermine-treated (35 μM) and untreated LPS-stimulated PBMCs, whereas spermine inhibited TNF protein synthesis in the same cells (data not shown).

To define further the posttranscriptional action of spermine on the release of TNF and MIP-1α, we assayed the cellular levels of these cytokines in lysates of LPS-stimulated cells. Exposure of human PBMCs to spermine significantly inhibited the levels of cell-associated TNF and MIP-1α (Fig. 3, *b* and *d*). We observed a similar IC_{50} for spermine inhibition of cell-associated and secreted cytokine levels. Because the levels of cell-associated cytokines were diminished and not increased, these results provide direct evidence that the mechanism of spermine action is by inhibiting cytokine synthesis, and not through suppressing cytokine release.

The specificity of spermine inhibition of proinflammatory cytokine synthesis was determined by measuring the levels of other macrophage-derived cytokines in the supernatants of LPS-activated human PBMCs. We observed spermine dose-dependent suppression of the proinflammatory cytokines MIP-1β, IL-1β, and IL-6, from LPS-activated human PBMCs (Fig. 4). The IC_{50} for spermine inhibition for each of these four proinflammatory cytokines was ~2 μM, with maximal suppression of MIP-1α, MIP-1β, and IL-6 exceeding 90%. However, the effects of spermine on IL-1β synthesis differed somewhat from these other cytokines, because at saturating spermine concentrations, IL-1β synthesis was only decreased by 65% as compared with controls. The residual amount of IL-1β synthesis (35%) that could not be inhibited by spermine treatment was similar to the residual amount of TNF synthesis that persisted in the presence of spermine (see above). Additional evidence for the specificity of spermine inhibition of proinflammatory cytokine synthesis was given by the observation that spermine failed to suppress the constitutive production of TGF-β from human PBMCs (Fig. 4 *d*), even when spermine was added in concentrations more than 100-fold higher than the IC_{50} for the proinflammatory cytokines. When considered together, these results indicate that spermine specifically and reversibly inhibits proinflammatory cytokine synthesis in human PBMCs.

Spermine Inhibits TNF Synthesis in Serum-free Media and in the Presence of Polyamine Oxidase Inhibition. Szabo and coworkers (19, 20, 32) recently reported that spermine suppressed the induction of inducible nitric oxide synthase (iNOS) in the cell line J774.2, and found that the molecular basis for this suppression was dependent upon oxidation of spermine by polyamine oxidase present in bovine serum. To address the possible role of polyamine oxidase-mediated spermine oxidation in the mechanism of spermine action on inhibiting proinflammatory cytokine synthesis in primary human monocytes, we measured TNF production in human PBMCs that had been cultured and stimulated with LPS under serum-free conditions. We observed a spermine dose-dependent suppression of TNF in the absence of added serum (Fig. 5 *a*), and determined that the IC_{50} under

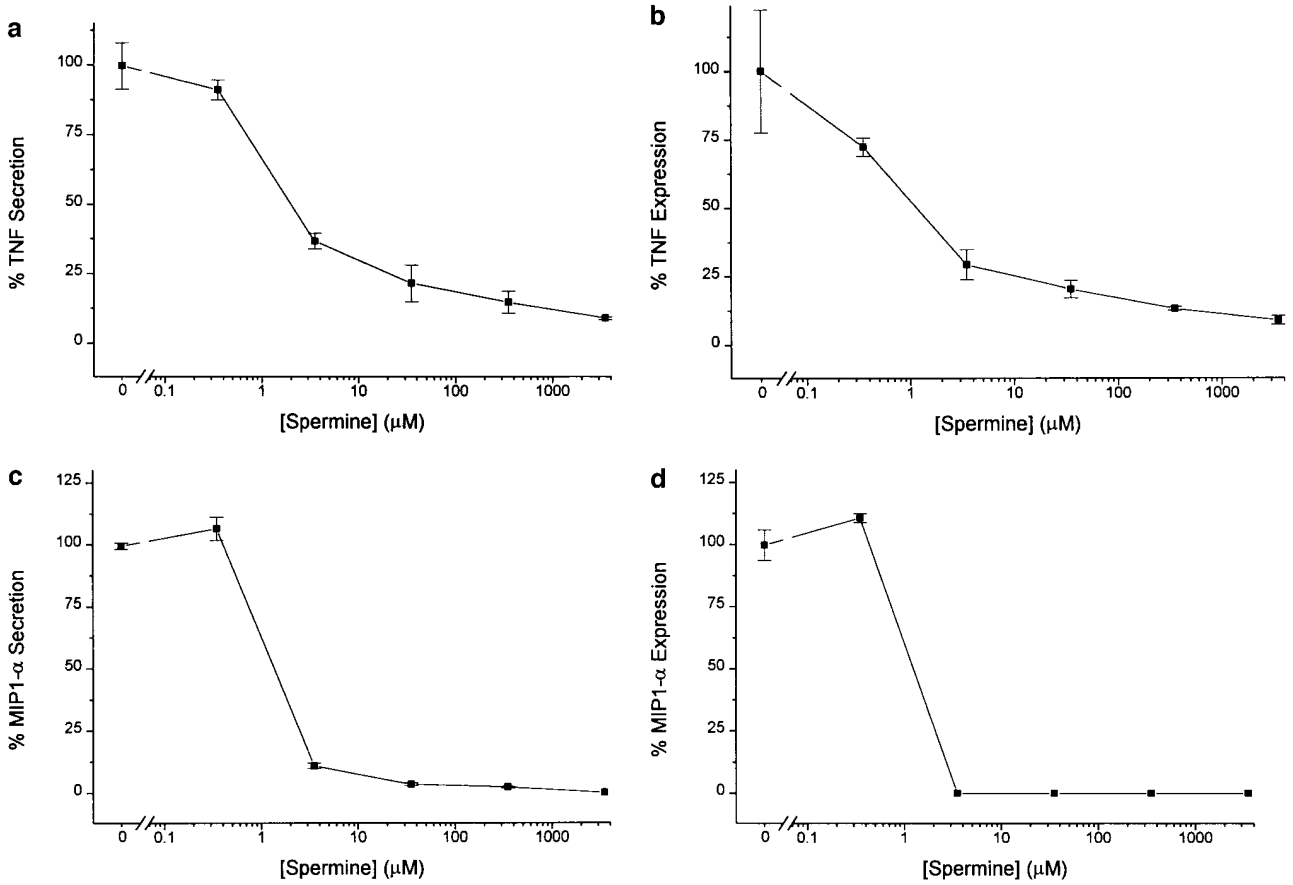

Figure 3. Spermine inhibition of TNF and MIP-1α protein in cell lysates and supernatants.Human PBMCs were exposed to spermine for 1 h, stimulated with LPS, and after 4 h of stimulation lysed as described in Materials and Methods. The levels of TNF and MIP-1α in the supernatants (*a* and *c*) and corresponding whole-cell lysates (*b* and *d*) are shown. Data are mean ± SEM.

serum-free conditions was similar to that measured in serum-containing media (serum-free $IC_{50} = 8 \pm 4$ μM) versus serum-containing media (10% FBS $IC_{50} = 20 \pm 15$ μM; $P > 0.05$). Although these experiments suggested that oxidation of spermine by polyamine oxidase was not required for TNF suppression in human PBMCs, we wished to confirm this interpretation by adding a pharmacological inhibitor of polyamine oxidase to serum-containing media and then measuring the effect of spermine on TNF synthesis. In these experiments, we measured spermine oxidase activity in bovine serum–containing media using HPLC detection of spermine, and in agreement with previous results (33), addition of aminoguanidine (2 mM) suppressed enzyme activity to <1% of controls. In these conditions of complete enzyme inhibition we also observed a spermine dose-dependent suppression of TNF synthesis (Fig. 5 *b*). The IC_{50} for spermine inhibition of TNF in the presence of complete polyamine oxidase inhibition with aminoguanidine ($IC_{50} = 8 \pm 6$ μM) was similar to that observed in controls, as measured in serum-containing media without addition of aminoguanidine ($IC_{50} = 20 \pm 15$; $P > 0.05$). Although these results suggested that spermine oxidation is not required for the cytokine suppressing activity of spermine, we wished to control for the possibility that an alternative oxidative mechanism might have resulted in the formation of spermidine in these cultures. Accordingly, we assessed the effects of spermidine on LPS-stimulated TNF synthesis. Although we did observe spermidine to inhibit TNF synthesis in human PBMCs, the measured IC_{50} (638 ± 143 μM) was significantly higher than for spermine itself ($P > 0.05$), suggesting that metabolism of spermine to spermidine is not the molecular basis for the observed cytokine inhibition by spermine. When considered together, these results indicate that the inhibition of proinflammatory cytokine synthesis in human PBMCs is directly attributable to spermine, and is not dependent upon the activity of polyamine oxidase.

Administration of Spermine In Vivo Suppresses Carrageenan-mediated Inflammation. The observations that spermine decreases proinflammatory cytokine production by LPS-stimulated human monocytes suggested that local accumulation of spermine at inflammatory sites could suppress the development of cytokine-mediated tissue responses. We investigated this mechanism in vivo by measuring the development of edema induced by injection of carrageenan into the footpad of mice, a widely used model of inflammation in which cytokine antagonists and other anti-inflammatory agents can effectively suppress the inflammatory response

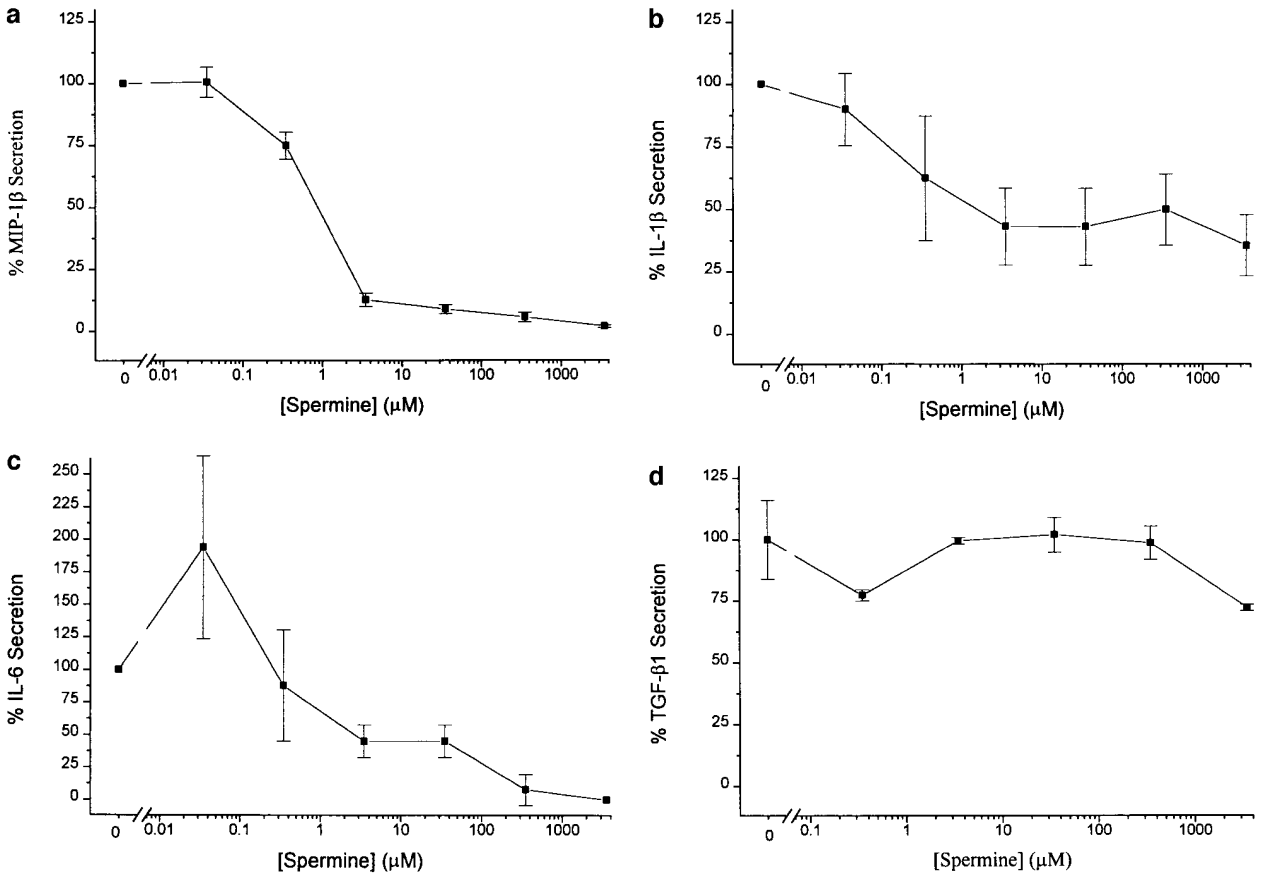

Figure 4. Spermine inhibition of MIP-1β, IL-1β, and IL-6, but not TGF-β from human PBMCs. Human PBMCs were pretreated with spermine for 60 min, then stimulated with LPS (100 ng/ml). 4 h after LPS treatment, supernatants were assayed by ELISA for: *a* MIP-1β; *b* IL-1β; *c* IL-6; and *d* TGF-β. Data are mean ± SEM of three experiments. Control cytokine levels: MIP-1β, 19 ng/ml; IL-1β, 32 pg/ml; IL-6, 6,000 pg/ml; and TGF-β, 680 ± 133 pg/ml.

(15, 34, 35). Administration of spermine directly into the carrageenan-injected paw significantly suppressed the development of swelling (Fig. 6). The anti-inflammatory effects were spermine dose-dependent, and maximally inhibited footpad swelling by 48% as compared with vehicle-treated controls ($P < 0.05$). Because the development of acute edema in this model is dependent upon the activity of cytokines (and independent of LPS), these results now suggest that the local accumulation of spermine in tissues can suppress the development of injurious inflammation in vivo.

Discussion

Some 44 years ago, a search for a natural product in animal tissues capable of suppressing the growth of tubercle bacilli ultimately led Hirsch and Dubos to discover that spermine was the active anti-mycobacterial principle (36). This seminal work revealed a potential mechanism through which the cytotoxic activity of a ubiquitous polyamine could protect the host during invasive infection. Because it was already known that spermine concentrations were significantly elevated in tissues during infectious, neoplastic, and inflammatory diseases (e.g., tuberculosis, pneumonia, cancer), they proposed a direct role for spermine in limiting the growth or spread of an infectious agent or tumor (36). Our present results now support an additional mechanism by which spermine can participate in the host response to infection or invasion, by counterregulating proinflammatory cytokine synthesis.

The proinflammatory cytokines TNF, IL-1, IL-6, MIP-1α, and MIP-1β occupy a pivotal role in stimulating the early stages of acute inflammation, including recruitment and activation of inflammatory cells, stimulation of endothelial cell activation, and direct cytotoxicity (5, 37–39). Whereas these inflammatory events can be critical to the ultimate recovery from infection or injury, normal counterregulatory mechanisms are also critical to the success of the immune response, because the inappropriate or excessive production of proinflammatory cytokines ultimately can lead to the development of shock and tissue injury (3, 4, 40). Previously, extensive investigations of counterregulatory immune mechanisms have focused on the cytokine inhibitory roles of the glucocorticoid hormones, the anti-inflammatory cytokines TGF-β and IL-10, and the prostaglandin PGE2 (8–12, 14, 41–43). Together, these studies demonstrate the critical central role occupied by counterregulating signals that serve to dampen the immune response. The present results now indicate that spermine counterregula-

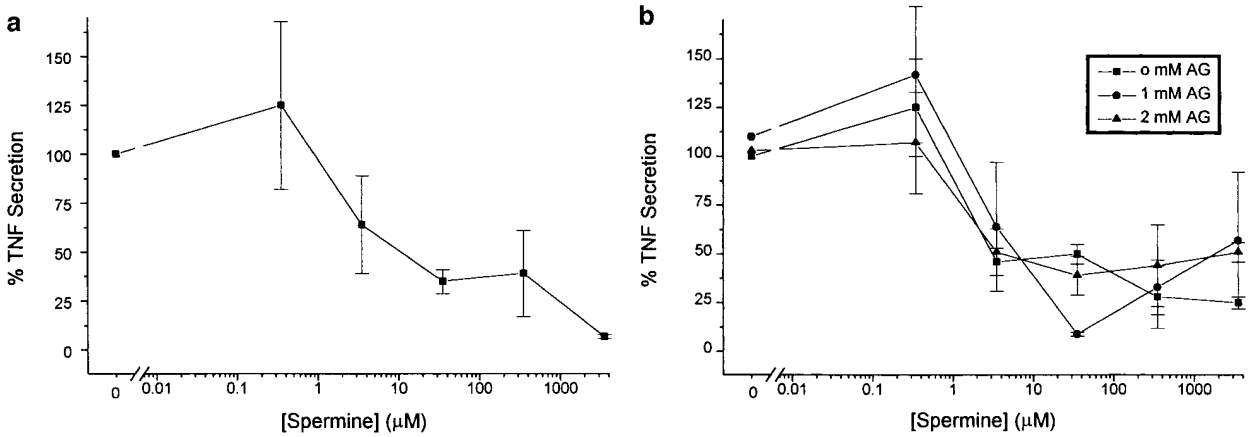

Figure 5. Spermine suppression of TNF synthesis in serum-free media and in the presence of polyamine oxidase inhibition. (*a*) PBMCs were cultured in serum-free media (OPTI-MEMI), stimulated with LPS for 4 h, then TNF levels in the media measured by ELISA as described. (*b*) Human PBMCs were cultured in RPMI-1640 containing FBS (10%) with the addition of aminoguanidine (0, 1, or 2 mM) as shown. 4 h after LPS treatment, conditioned supernatants were assayed for TNF by ELISA as described above.The data shown are from a representative experiment (repeated twice with similar results). Each point represents mean ± SEM from six replicates.

tion of proinflammatory cytokine production offers another level of molecular regulation capable of reducing the injurious activity of a local immune response.

In agreement with its proposed cytokine counterregulatory role, spermine-mediated cytokine inhibition was specific, reversible, and time dependent, therefore enabling an innate mechanism in which the affected monocytes can recover their cytokine producing function to participate in subsequent immune responses. Because spermine effectively inhibited cytokine synthesis in serum-free conditions, and in the presence of the polyamine oxidase inhibitor aminoguanidine, oxidative metabolism of spermine is not required for the molecular mechanism of cytokine counterregulation. Spermine levels required to suppress cytokine synthesis are readily achieved in vivo, because high tissue concentrations (500 μM to 2 mM) have been reported in tumors, and in patients infected with bacteria, mycobacteria, and viruses (36, 44–47). Because elevated polyamine levels have also been implicated in the immunosuppressive state associated with pregnancy and fetal development (24, 48, 49), it is now interesting to consider whether the mechanism of immunosuppression in these earlier observations is attributable at least in part to spermine-mediated counterregulation of proinflammatory cytokines. For instance, elevated spermine levels found in amniotic fluid might dampen the production of TNF and IL-1β, and thereby prevent the onset of abortion mediated by cytokine release in the amnion (50). The present study did not address the role of spermine on T cell cytokine synthesis, but earlier work by Flescher and colleagues (51) suggested that spermine does not inhibit IL-2 production in PHA-stimulated monocytes. Rather, inhibition of IL-2 synthesis was observed by a combination of spermidine, polyamine oxidase, and hydrogen peroxide, leading them to conclude that oxidative metabolism of spermidine, but not spermine, can inhibit T cell IL-2 production (51). In contrast, we found that spermidine was a poor inhibitor of monocyte/macrophage proinflammatory cytokine synthesis, and that spermine was an effective inhibitor even in the absence of polyamine oxidase activity. When considered together, these findings now suggest that spermine suppression of monocyte proinflammatory cytokine synthesis is specific and direct.

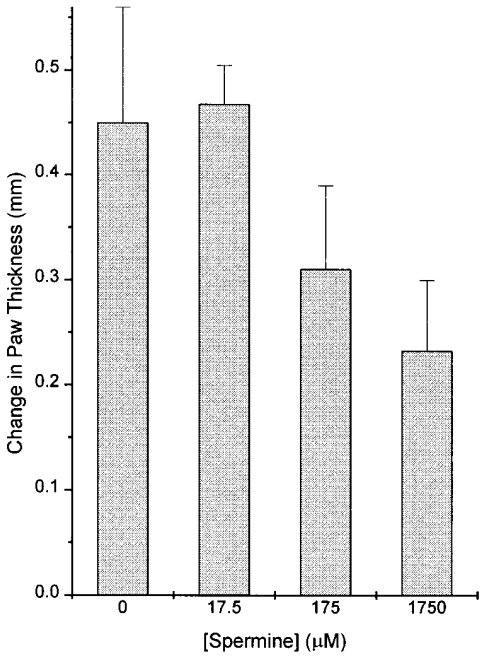

Figure 6. Spermine suppresses carrageenan-induced inflammation in mice. Carrageenan (0.2%) was injected into the left rear footpad of C3H/HeN mice with the concentration of spermine indicated in 50 μl; ; vehicle (1× PBS) was injected into the right hindpaw. 28 h later, the footpad thickness was measured with a caliper, and the difference between right and left hindpaw determined. Data are mean ± SEM (mm) from three experiments, with $n = 5$ animals in each experimental group.

It will be of interest to investigate whether spermine inhibition of cytokine translation is dependent upon cellular signaling pathways that are shared with other cytokine counterregulators, and whether they converge on some key regulatory step(s). We have considered the hypothesis that spermine might act via a molecular pathway shared with the glucocorticoids, but the following observations suggest that spermine-mediated cytokine counterregulation is independent of glucocorticoid signaling. (*a*) Spermine inhibition of TNF and MIP-1α occurs posttranscriptionally, whereas glucocorticoids inhibit both transcription and translation (7); (*b*) spermine retains its counterregulatory activity in the presence of IFN-γ, but IFN-γ overrides the suppressive activity of glucocorticoids (52); and (*c*) the recovery of monocyte TNF synthesis in spermine washout experiments is more rapid than that predicted for glucocorticoid-mediated inhibition (53). These observations suggest that spermine-mediated cytokine counterregulation occurs by a molecular pathway that is distinct from glucocorticoids. It will also be of interest to address whether the inhibitory effects of spermine in monocytes/macrophages are dependent upon an as yet unidentified monocyte/macrophage receptor or binding protein at the cell surface, and whether spermine transport into the monocyte/macrophage is required for interaction with an intracellular target.

Previous studies indicate that LPS stimulation of monocytes activates spermine uptake via a protein kinase C (PKC)–dependent mechanism (15, 54–57). In agreement with these results, we have observed enhanced spermine uptake after LPS stimulation in human PBMCs (data not shown). It is plausible that LPS-stimulated spermine uptake can participate in the observed suppression of proinflammatory cytokine synthesis. It should be noted that the action of spermine in suppressing cytokine synthesis is not simply attributable to neutralizing LPS via a charge effect, because LPS-stimulated signaling in the presence of spermine still produced significant increases in the levels of TNF and MIP-1α mRNA (Fig. 2). Moreover, spermine effectively suppressed the development of edema induced by carrageenan in vivo, an acute inflammatory response that is dependent upon cytokine production but independent of LPS (15, 34, 35).

Spermine counterregulation of proinflammatory cytokine synthesis was specific, because the suppression of MIP-1α, MIP-1β, and IL-6 approached 100%, but maximal suppression of TNF and IL-1β failed to exceed 75%. In further agreement with its proposed role as an anti-inflammatory mediator, spermine failed to suppress TGF-β, a potent anti-inflammatory cytokine (8). Consideration of these results leads to the appreciation that TNF and IL-1β each occupy pivotal beneficial roles in the immune response, so that the proposed mechanism of spermine in differentially counterregulating cytokine release also enables the continued production of beneficial, or perhaps necessary, low levels of these two mediators. Even in the presence of the very high spermine levels found in diseased tissues, low level production of TNF and IL-1 could persist to benefit the host by stimulating antimicrobial and antiviral immune responses, promoting tissue regeneration, and facilitating wound healing (5). Meanwhile, the uninhibited release of TGF-β can participate in further counterregulation and containment of the local immune response.

We conclude that spermine counterregulation of cytokine production is a mechanism for locally suppressing the acute, and potentially injurious tissue response to inflammation in vivo. Because spermine is released from dying and injured cells during infection, injury, ischemia, and inflammation, it is suitably positioned as a feedback signal to suppress further damage from cytokine excess. Tissue spermine levels predictably reflect the extent of surrounding tissue injury, thereby providing a sensitive counterregulatory signal to prevent excessive activation of macrophages. This molecular counterregulatory feedback loop would be anticipated to predominate locally at tissue sites of inflammation, and not systemically as is the case with the glucocorticoids and the anti-inflammatory cytokines.

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