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# The Role of Nitric Oxide in Experimental Murine Sepsis Due to Pyrogenic Exotoxin A-Producing *Streptococcus pyogenes*

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**Nitric oxide (NO) produced by inducible NO synthase (iNOS) mediates hypotension in endotoxemia. In this study, NO induction by a toxin-producing *Streptococcus pyogenes* isolate, H250, and by recombinant streptococcal pyrogenic exotoxin A (rSPEA) has been examined, both in vitro and in vivo. Streptococcal supernatants, but not rSPEA, induce production of nitrite by murine macrophages when both are coincubated with gamma interferon. Intraperitoneal injection of rSPEA did not cause significant production of NO. However, an elevated level of nitrate in serum was detected in a model of streptococcal fasciitis due to live H250. iNOS was localized to Kupffer cells, hepatocytes, and renal tubular cells by immunostaining. Administration of a NOS inhibitor, $N^G$-monomethyl-L-arginine (L-NMMA), reduced peak concentrations of nitrate in serum but did not affect survival. NO is induced by H250, both in vitro and in vivo, mainly via SPEA-independent mechanisms. In this model, iNOS is expressed predominantly in the liver. Furthermore, in this model L-NMMA is not protective.**

Nitric oxide (NO) produced by inducible NO synthase (iNOS) mediates hypotension in endotoxin-dependent animal models of infection with gram-negative organisms (32). NO is identical to endothelium-derived relaxing factor and can be induced by combinations of endotoxin and cytokines from rodent macrophages, endothelial cells, and, to a lesser extent, vascular smooth muscle cells (17). The role of NO in gram-positive infections has rarely been studied (1, 9, 23), and yet gram-positive infections constitute 40 to 60% of all cases of septic shock (7, 13). The recently recognized streptococcal toxic shock syndrome, which is caused by *Streptococcus pyogenes*, is characterized by profound hypotension and multiorgan failure (37). It may be, therefore, that NO plays a role in the pathogenesis of this syndrome.

Group A streptococci produce cell wall constituents, such as peptidoglycan and lipoteichoic acid, which are common to other gram-positive bacteria, in addition to M protein and a range of soluble enzymes and toxins, such as streptolysin O, streptococcal cysteine protease, and the pyrogenic exotoxins. Each of these components can induce cytokine production in vitro (2, 11, 12, 20, 34). Of particular interest are the superantigens, streptococcal pyrogenic exotoxin A (SPEA) and SPEC, mitogenic factor, and streptococcal superantigen. These exotoxins can initiate clonal T-cell proliferation and cytokine production in vitro, and such superantigens may be responsible for the shock and hypotension encountered clinically in streptococcal toxic shock syndrome (28, 35).

Recent epidemiological studies have suggested that $M1^+$ $SPEA^+$ strains of *S. pyogenes* are more likely to be associated with severe and fatal infections than other strains (29, 29a). In this study, we assessed the ability of $M1^+$ SPEA-producing *S. pyogenes* to induce NO in vitro, and then investigated the induction of NO in vivo, in a murine model of fasciitis and multiorgan failure due to the same organism.

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## MATERIALS AND METHODS

**Bacteria.** A scarlet fever M1T1 *S. pyogenes* isolate, H250, known to produce SPEA but not SPEC (genotyping by PCR performed by Streptococcal Reference Laboratory, Central Public Health Laboratory, Colindale, United Kingdom) was used in all experiments involving sepsis due to live streptococci. This isolate produces 2 to 3 μg of SPEA per ml in rich media, as measured by enzyme-linked immunosorbent assay (ELISA).

**Bacterial supernatants for tissue culture experiments.** For tissue culture, a fresh colony of H250 was inoculated into 20 ml of RPMI medium, supplemented with glutamine (Gibco-BRL, Paisley, United Kingdom) and 10% fetal calf serum (Labtech, Uckfield, United Kingdom). Bacteria were cultured at 37°C overnight to a density of $10^6$ CFU/ml, determined by plating serial dilutions of broth onto blood agar. Cultures were then sterilized with 0.45-μm-pore-size filters (Sartorius, Gottingen, Germany) and aliquoted prior to storage at −70°C until required for use. The same supernatant was used in all experiments. Sample aliquots were plated on blood agar and incubated at 37°C overnight to ensure sterility. The SPEA content of H250 supernatants was 600 ng/ml as measured by ELISA. Endotoxin contamination of a 1/10 dilution of bacterial supernatant was <5 pg/ml (sensitivity, 5 pg/ml), as measured by *Limulus* assay (Quadratech, Epsom, United Kingdom).

**Cytokines, antibodies, and reagents.** Gamma interferon (IFN-γ) used in tissue culture experiments was from Genzyme (Cambridge, Mass.). Lipopolysaccharides (LPS) of *Escherichia coli* O111 and polymyxin B were from Sigma (Poole, United Kingdom). Recombinant SPEA (rSPEA) was expressed and rabbit polyclonal anti-SPEA antibody was raised as previously described (27). The biological activity of rSPEA mirrored that of native SPEA purified from *S. pyogenes* (Toxin Technology, Sarasota, Fla.) and other bacterial superantigens in human lymphocyte mitogenesis and lymphokine assays. Endotoxin levels of 50 to 100 pg/μg of SPEA were measured by *Limulus* assay. Rabbit polyclonal antibody to rSPEA reacted with native SPEA in Western blot analysis, yielding a single specific band. Anti-SPEA immune rabbit serum neutralized the activity of 600 ng of rSPEA per ml in a human lymphocyte mitogenesis assay at a dilution of $1/10^5$.

**Cells and culture conditions.** Peritoneal macrophages were obtained from CD1 mice by thioglycolate injection, purified by adherence, and resuspended in Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, glutamine (Gibco-BRL), and 10% fetal calf serum (Labtech) (18). Cells were seeded at $10^5$/well in 96-well plates and allowed to settle for 6 h prior to stimulation with either 1/10 volume of bacterial supernatant or 10 to 10,000 ng of rSPEA per ml (with or without IFN-γ [100 U/ml]) overnight. Control stimulants included medium only, IFN-γ only, and 2 μg of LPS per ml plus IFN-γ. Polyclonal rabbit anti-SPEA serum was added to the stimulation mixture at a concentration of $1/10^3$, with preimmune serum from the same rabbit used as a control. Cells from single mice were used in individual experiments, and all

experiments with primary cells were conducted in the presence of polymyxin B at a concentration of 10 μg/ml.

RAW 264 murine macrophage and A7r5 rat aortic smooth muscle cells, obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, United Kingdom), were grown to confluence and then seeded at a density of $5 \times 10^5$ cells/well in 24-well plates. Eight hours after plating, adherent cells were stimulated by bacterial supernatant or rSPEA (with or without IFN-γ [100 U/ml]). Primary rat neonatal cardiomyocytes were seeded at a density of $10^5$ cells/well and cultured for 3 days prior to use, at which time cells were adherent and spontaneous beating had commenced, by using minor modifications of a previously published protocol (21). Cardiomyocytes were stimulated in the same way as cell lines. Cell-free supernatants from all stimulated cells were harvested and tested immediately for nitrite.

**Measurement of nitrite in culture supernatants.** Supernatants from tissue culture were mixed in equal volumes with Griess reagent, as previously described, and the optical density at 580 nm ($OD_{580}$) was measured following color change; standard concentrations of nitrite were used for comparison (10).

**Animal model of infection and experimental design. (i) Bacteria for the animal model.** A fresh colony of H250 was inoculated into Todd-Hewitt broth (Oxoid, Basingstoke, United Kingdom) and incubated for 8 h at 37°C. Bacteria were then harvested by centrifugation and resuspended in pyrogen-free saline, and bacterial density was determined by measuring $OD_{325}$. The suspension was then appropriately diluted to $2 \times 10^{10}$ CFU/ml with pyrogen-free saline, with standard growth curves used to relate measured $OD_{325}$ to bacterial concentration.

**(ii) Animal model.** Male 6- to 8-week-old CD1 mice (Charles River Laboratory, Margate, United Kingdom) weighing 22 to 25 g were used in all experiments.

A modification of the Selbie thigh lesion test was used to initiate infection in 20 mice (22). Bacteria were inoculated into the right thigh by intramuscular (i.m.) injection of 0.1 ml of H250 bacterial suspension (90% lethal dose = $2 \times 10^9$ CFU/mouse). Mice developed thigh lesions due to fasciitis and myositis and a systemic illness within 24 h that was associated with multiorgan failure. Control animals received saline only.

To assess the effects of SPEA alone, 200 μg of rSPEA in 0.4 ml of saline was administered to nine mice by intraperitoneal (i.p.) injection, with control animals receiving sterile saline alone. Injection of 200 μg of rSPEA i.p. resulted in serum rSPEA levels of 5 to 10 μg/ml 5 h after injection, as measured by ELISA, though levels were undetectable at 10 h.

**Detection of nitrite and demonstration of iNOS.** Blood was obtained from animals by cardiac puncture at 0, 5, 15, and 25 h following i.m. infection or i.p. rSPEA injection and was centrifuged at 10,000 × *g*, and serum was separated for the nitrate-plus-nitrite assay. At 25 h, the remaining animals were sacrificed and tissues (thigh muscle, spleen, liver, kidney, small bowel, heart, and lung) were fixed in 1% paraformaldehyde for immunohistochemistry studies.

**Inhibition of NOS activity in vivo.** $N^G$-Monomethyl-L-arginine (L-NMMA) (Calbiochem, La Jolla, Calif.) dissolved in pyrogen-free saline was administered intravenously (i.v.) at 0 or 8 h following i.m. injection of H250 to groups of two to four animals to identify the timing and dose required to abrogate NO production. Doses of L-NMMA used were 0 to 600 mg/kg of body weight. Blood was obtained at 15 h by cardiac puncture, and serum was reserved for nitrate-plus-nitrite assay, as described above. In a separate experiment, 400 mg of L-NMMA per kg was administered i.v. to 20 mice 8 h after i.m. H250 inoculation, with control animals receiving saline only. Five mice from each group were sacrificed at 15 h for cardiac puncture and assay of serum nitrate-plus-nitrite levels.

**Serum nitrate-plus-nitrite assay.** Levels of nitrate plus nitrite in serum were measured as described by Evans et al. (4). Any grossly hemolyzed samples were not analyzed. Serum was diluted fourfold and kept on ice. Nitrate was reduced to nitrite by addition of 0.25 U of nitrate reductase (*Aspergillus* spp.) per ml incubated at 37°C for 30 min in the presence of 5 mM NADPH. Excess NADPH was removed by incubation with 33 mM sodium pyruvate and 16 U of lactate dehydrogenase per ml for a further 15 min at 37°C. Nitrite concentrations were then determined by mixing treated serum with Griess reagent in equal volumes, as described above. Values were corrected for the efficiency of conversion of nitrate to nitrite by measuring the conversion of standard concentrations of nitrate to nitrite.

**Immunohistochemical studies.** Polyclonal antibody to a synthetic peptide, corresponding to amino acid residues 47 to 71 of murine macrophage iNOS, was raised in rabbits. This antibody is known to be specific for mouse and rat macrophage and hepatic iNOS (3). Paraformaldehyde-fixed tissues were stained by the avidin-biotin-peroxidase complex method. Tissues were incubated successively with rabbit anti-iNOS, biotinylated goat anti-rabbit immunoglobulin G, and avidin-biotin-peroxidase (Vector Laboratories, Burlingame, Calif.) prior to development by the glucose oxidase–diaminobenzidine-nickel enhancement method (24).

**Statistical analysis.** Nitrate-plus-nitrite levels between different groups were compared by Kruskal-Wallis and Mann-Whitney U tests. *P* values of <0.05 were considered statistically significant.

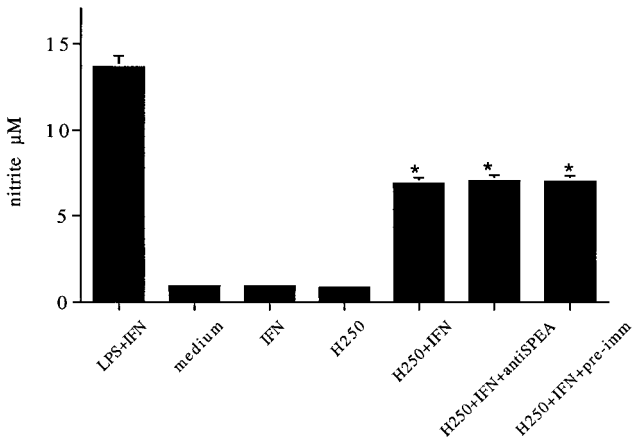


FIG. 1. Nitrite induction from CD1 macrophages stimulated with various treatments (shown on the *x* axis). Results show the means of data from four experiments; error bars show standard errors. Concentrations: LPS, 2 μg/ml; H250, 10% bacterial supernatant from *S. pyogenes* H250; IFN, 100 U/ml; anti-SPEA, $1/10^3$-diluted neutralizing rabbit anti-SPEA serum; pre-imm, $1/10^3$-diluted preimmune serum control. $P = 0.001$, Kruskal-Wallis test; levels marked with an asterisk are significantly elevated compared with the medium control. $P = 0.02$, Mann-Whitney U test.

## RESULTS

**In vitro induction of NO by H250 and rSPEA.** Supernatants of H250 alone did not induce production of nitrite from cells. However, induction of nitrite production was seen from peritoneal macrophages when cells were coincubated with IFN-γ (Fig. 1). This finding was also seen in stimulated RAW 264 cells (data not shown). Anti-SPEA-neutralizing serum failed to abrogate the induction of NO by H250 and IFN-γ in peritoneal macrophages. A7r5 cells and cardiac myocytes did not produce nitrite when stimulated with supernatants plus IFN-γ.

rSPEA did not induce NO production by CD1 macrophages at the concentrations used, even in the presence of IFN-γ (Fig. 2). rSPEA did not induce significant NO production from RAW 264 cells, A7r5 cells, or cardiac myocytes.

**In vivo nitrite production.** Significant elevation of levels of nitrate plus nitrite in serum from the baseline was detected at 15 h after infection with H250. Levels declined at 25 h, at which time deaths were beginning to occur, although the levels re-

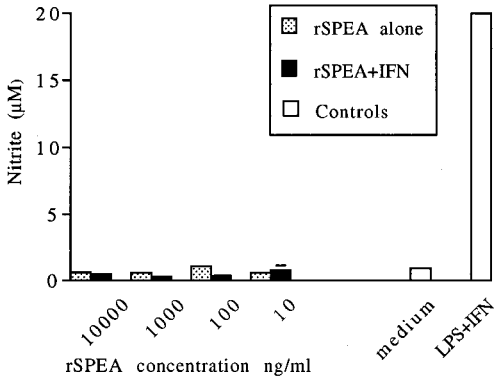


FIG. 2. Nitrite induction from CD1 macrophages stimulated with 10 to 10,000 ng of rSPEA per ml in the presence and absence of IFN-γ. LPS concentration, 2 μg/ml. Data show the mean level from three experiments at each rSPEA concentration, and error bars show standard errors, except for LPS plus IFN, where the mean of only two experiments is given.

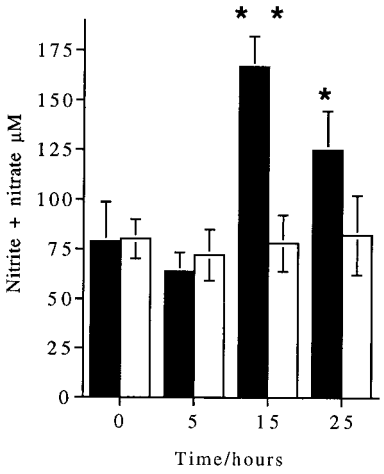


FIG. 3. Levels of nitrate-plus-nitrite production in serum during H250 sepsis (filled bars). Open bars show saline control data. Means and standard errors are shown at each time point ($n = 5$, except at 0 h, where $n = 3$). Significant differences from values at 5 h are marked: *, $P = 0.02$; **, $P = 0.008$ (Mann-Whitney U test).

mained higher than the baseline or 5-h values (Fig. 3). There was no rise in levels of nitrate plus nitrite in serum in animals receiving i.m. saline only. Injection of rSPEA i.p. did not significantly affect nitrate-plus-nitrite levels in serum, although there was a trend for them to rise from mean baseline levels of 78.7 μM (standard deviation, 34.9) to 138.1 μM (standard deviation, 57.4).

**Distribution of iNOS in H250 sepsis by immunochemistry.** iNOS was distributed predominantly in Kupffer cells and hepatocytes of infected animals, though not in controls (Fig. 4). Renal tubular staining was also evident (Fig. 5). Preimmune serum did not stain infected tissues, and the staining could be inhibited by the presence of excess peptide. iNOS was not demonstrated in vascular smooth muscle or macrophages, nor was it found in saline-treated control animals.

**Protection studies using L-NMMA.** Administration of L-NMMA i.v. at the time of infection (0 h) did not reduce peak (15-h) nitrate-plus-nitrite levels significantly, even when doses of 600 mg/kg were used (data not shown). However, by delaying L-NMMA treatment until 8 h, peak (15-h) nitrate-plus-nitrite levels could be reduced to near background without apparent toxicity by using a dose of 400 mg/kg (Fig. 6). In an experiment to assess whether a single dose of 400 mg of L-NMMA per kg at 8 h had any protective effect in this model, no overall benefit in survival was observed compared with the survival rate for saline-treated similarly infected controls (data not shown). This occurred despite the fact that 15-h serum nitrate-plus-nitrite levels were significantly reduced from a mean value of 167 μM to a mean value of 105.2 μM ($P = 0.05$).

## DISCUSSION

The results of this study show that *S. pyogenes* H250, an $M1^+$ $SPEA^+$ group A streptococcus, induces iNOS and causes production of NO both in vitro and in vivo. SPEA is, however, unlikely to be the component responsible for this process. In this model, L-NMMA was unable to protect animals from death.

The mechanism by which H250 supernatants induced NO production from cultured macrophages cells is unclear. NO induction was not due to LPS contamination. Firstly, the

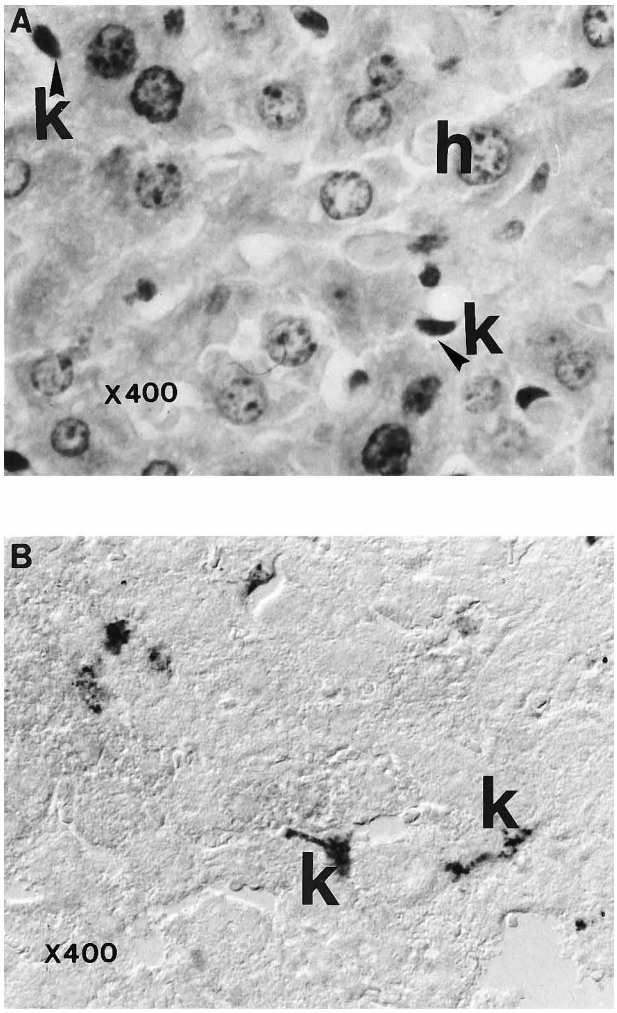


FIG. 4. (A) Hematoxylin- and eosin-stained section of liver from an H250-infected mouse, showing densely staining apoptotic hepatocytes. Normal hepatocyte (h) and Kupffer cells (k) are shown. (B) Immunochemical staining of Kupffer cells (k) for iNOS performed on an adjacent tissue section.

amount of LPS due to bacterial supernatants present in tissue culture (<5 pg/ml) would be insufficient to induce NO production by murine macrophages, even in the presence of IFN-γ. At least 500 ng of LPS per ml is required to induce NO from RAW 264 cells, and this figure is reduced to only 500 pg/ml when IFN-γ is present (our unpublished observations). Moreover, the concentration of polymyxin B used in the experiments was sufficient to neutralize 500 ng of LPS per ml when added to RAW 264 cells in the presence of IFN-γ (data not shown). Pyrogenic exotoxins can, however, enhance susceptibility to endotoxin, although the mechanisms leading to this are unclear (2). Cell wall components present in supernatants of gram-positive bacteria, such as lipoteichoic acid, can lead to NO production in tissue culture (16). It is likely that such components induce NO through their ability to induce production of proinflammatory cytokine, in particular the production of tumor necrosis factor alpha (TNF-α) (12, 33, 36). This may, in part, explain why bacterial supernatants added directly to A7r5 smooth muscle cells and purified myocardial cells did not induce NO production. These cells produce iNOS and nitrite when stimulated directly with cytokines such as TNF-α and

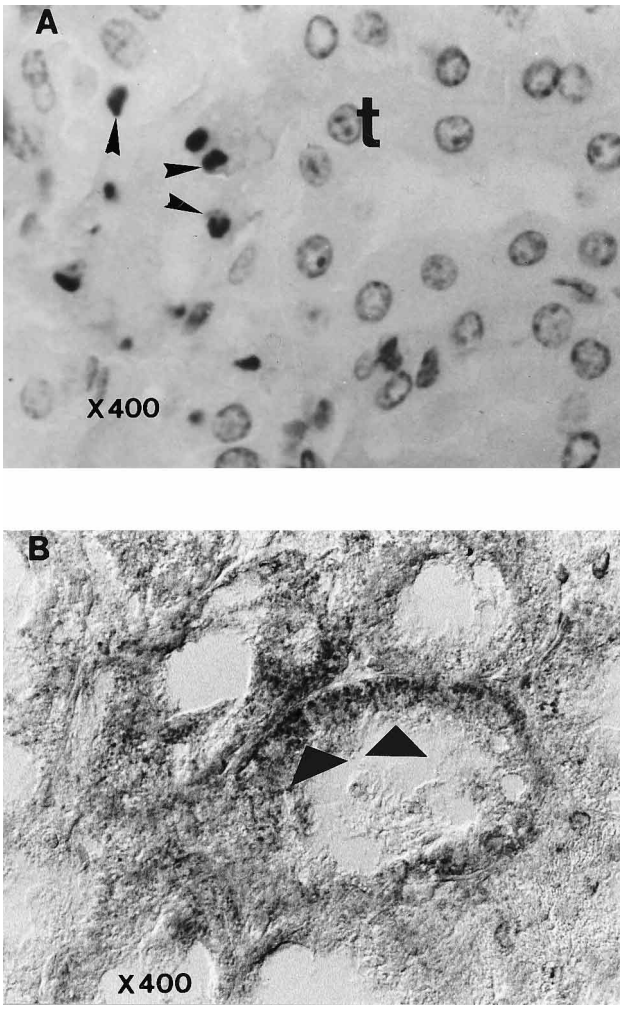


FIG. 5. (A) Hematoxylin- and eosin-stained renal tissue section from an H250-infected mouse. A normal tubular cell (t) and dead tubular cells (arrows) are shown. (B) Immunochemical staining of adjacent renal tissue. Tubular cells stain for iNOS (arrows).

interleukin-1 (IL-1) (3, 25). Unlike macrophages, they are not thought to produce proinflammatory cytokines themselves.

Concentrations of rSPEA which were representative of SPEA levels known to prevail in streptococcal broth and local

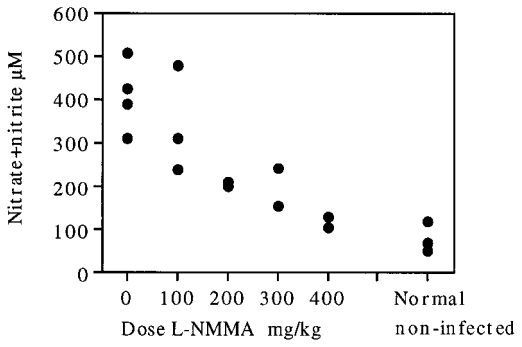


FIG. 6. Dose effect of i.v. L-NMMA given 8 h after H250 administration on serum nitrate-plus-nitrite levels measured 15 h after H250 administration. Results for individual mice are shown.

infection (14) did not induce NO production from macrophages, even in the presence of IFN. This was surprising because a related staphylococcal protein, toxic shock syndrome toxin 1, had previously been shown to induce NO production from a murine monocyte cell line (38). This contrast may reflect differences between cell types used or differences in the toxins themselves. It is noteworthy that the concentrations of toxic shock syndrome toxin 1 necessary for NO induction in that earlier study were around 2 μg/ml, and the possibility of endotoxin or other contamination was not discussed. The production of proinflammatory cytokines, such as TNF-α and IL-1, by purified monocytes stimulated with contaminant-free superantigen is known to require the presence of T lymphocytes (8, 15). While rSPEA cannot lead to NO production by purified monocytes, it may be able to induce NO from a mixed cell population and can induce NO production from adequately stimulated CD1 murine splenocytes (26).

Induction of iNOS and production of NO in an animal model of sepsis caused by gram-positive organisms has not, to our knowledge, been previously investigated. In H250-infected mice, nitrate-plus-nitrite levels at 15 h were significantly elevated compared with levels at 0 and 5 h. Indeed, in pilot experiments, we detected 15-h nitrate-plus-nitrite levels of 500 μM in H250-infected CD1 mice and up to 1,000 μM in H250-infected female BALB/c mice, demonstrating that the degree of NO production can vary substantially, depending on the precise experimental conditions and species used.

Compared with previous models of infection with gram-negative organisms and endotoxemia, in which peak NO production occurs at around 7 h, this model showed a delayed rise in NO due to H250 infection, perhaps reflecting its more prolonged time course of sepsis (5). Moreover, despite the apparent need for IFN during NO production by H250-stimulated monocytes in vitro, we were unable to detect serum IFN by ELISA during sepsis. This may also reflect the more gradual inflammatory response in this model compared with i.v. models of infection or may be the result of localized, compartmentalized cytokine production.

Despite its use at a dose sufficient to yield serum SPEA concentrations in the range attained during H250 sepsis (27), rSPEA failed to induce significant amounts of NO when administered by i.p. injection. This suggests that the production of NO in this model of streptococcal sepsis is unlikely to be due to SPEA. A modest rise in the level of nitrate plus nitrite in serum was, however, seen at 25 h. While rSPEA does not induce NO from purified monocytes, it might be expected to induce NO in vivo, due to the presence of a variety of cell types in the whole animal and the induction of a systemic inflammatory response. A related toxin, staphylococcal enterotoxin B, induces large amounts of NO when administered i.p. to staphylococcal enterotoxin B-responsive mice, and this appears to exert a protective counterregulatory effect on the subsequent production of TNF-α (6). CD1 mice are an outbred strain and would be expected to have a range of T-cell receptor variable β chains and H-2 molecules. While CD1 splenocytes proliferate and can produce TNF-α, IL-6, and nitrite in response to rSPEA in vitro (our unpublished data), it may be that CD1 lymphocytes respond differently to SPEA in vivo and less vigorously than cells from superantigen-responsive inbred strains. Levels of SPEA attained in vivo may be insufficient or too short lived for lymphocyte activation. It is also possible that rSPEA-induced in vivo responses are compartmentalized and not detectable systemically. Indeed, in separate experiments, intraperitoneal rSPEA, in contrast to sepsis with live H250 organisms, failed to induce systemic production of IL-6.

Increased production of NOS, the enzyme which mediates

the release of NO, is likely to be responsible for the observed rise in levels of nitrate plus nitrite in serum during H250 sepsis. The increase in nitrate-plus-nitrite levels in the animal model was inhibited by L-NMMA, an inhibitor of both iNOS and constitutive NOS (cNOS). Moreover, the time course of NO production, both in vitro and in vivo, suggests that this process is inducible. Immunochemical staining of tissues from infected animals demonstrated that iNOS was detectable in infected animals but not in controls. In a rat model of endotoxemia, it was shown that macrophages distributed throughout several organs were the predominant sites of iNOS expression (3). In contrast, Kupffer cells and hepatocytes were the predominant sites of iNOS expression during H250 sepsis.

Histological examination of liver tissue from infected animals showed widespread changes of hepatocyte death due to apoptosis, without inflammatory cell infiltrate, all of which is suggestive of toxic damage. iNOS was also detected in renal tubular cells which, on histological examination, demonstrated changes due to apoptosis, raising the possibility that iNOS expression may be associated with cell death. To our knowledge, this is the first time that iNOS has been demonstrated in a model of sepsis with live bacteria. Moreover, this study provides an opportunity to compare iNOS induction in models of infection with gram-positive and gram-negative organisms. Tissue culture studies suggest that endotoxin- and cytokine-stimulated macrophages and vascular smooth muscle cells are potent sources of iNOS. The absence of widespread macrophage iNOS staining in this model may explain why serum nitrate-plus-nitrite levels measured are lower than levels measured in models of endotoxemia. The antibody used in our immunohistochemical studies is known to detect iNOS in a variety of cell types (3, 25). In this model, at least, the predominant source of iNOS appeared to be hepatic.

Despite induction of iNOS and increased NO production during H250 sepsis, the NOS antagonist L-NMMA was unable to protect mice from death, although it did cause a significant reduction in peak levels of nitrate plus nitrite. These results suggest that excess production of NO is not a dominant cause of death in this model, although the use of a delayed bolus of L-NMMA cannot wholly exclude the importance of NO throughout the course of sepsis. Indeed, the short half-life of L-NMMA may mean that continuous infusions prove to be a more efficacious mode of administration. In some models, inhibitors of NOS can protect animals from loss of vascular responsiveness and death due to endotoxin and sepsis due to gram-negative organisms (31, 32).

L-NMMA inhibits both iNOS and cNOS isoforms, although it is the iNOS activity which is thought to mediate hypotension and death in septic animals. It may be that concomitant inhibition of cNOS and iNOS is harmful and that more-specific inhibitors of iNOS are required. NO is known to assist in the killing of phagocytosed microorganisms in rodents, and therefore inhibition of NOS could have harmful consequences during sepsis with live bacteria (19). It is noteworthy that NO exerts a counterregulatory effect on Th1-type cytokine production in mice; inhibition of NOS may therefore be harmful, particularly in models of sepsis characterized by Th1 responses, such as superantigen shock (6, 30). To address the possibility that L-NMMA might induce SPEA-triggered lethality during streptococcal sepsis by enhancing cytokine production in our model (6), we performed three different experiments. Firstly, we measured TNF-α, IFN-γ, and IL-6 levels in mice treated with L-NMMA during H250 sepsis but found no difference between treated mice and controls. We then administered L-NMMA to CD1 mice that had been treated with 200 μg of rSPEA i.p. but found no difference between test mice and saline-treated controls. Finally, L-NMMA was also unable to protect CD1 mice infected with a non-SPEA-, non-SPEC-producing *S. pyogenes* strain (data not shown).

This study adds to the evidence that the characteristics of NO production in sepsis with live bacteria may be quite different from the production of NO induced by toxin injection or tissue culture. The results also highlight the importance of considering sepsis caused by both gram-positive and gram-negative organisms when therapeutic agents for septic shock are being designed, as patterns of inflammatory response may be quite different. Agents designed to combat sepsis due to gram-negative organisms may not necessarily be appropriate for shock due to gram-positive organisms.

## ACKNOWLEDGMENTS

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