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Induction of macrophage parasiticidal activity by *Staphylococcus aureus* and exotoxins through the nitric oxide synthesis pathway

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SUMMARY

Murine peritoneal macrophages stimulated *in vitro* with killed Gram-positive bacteria *Staphylococcus aureus* or its membrane components in the presence of interferon-γ (IFN-γ) expressed high levels of nitric oxide (NO) synthase and produced large amounts of NO in a dose-dependent manner. This is not due to the contamination by Gram-negative endotoxin because the stimulatory activity was not affected by the addition of polymyxin B. The expression of the NO synthase and the synthesis of NO by macrophages stimulated with toxic shock syndrome toxin-1 (TSST), lipoteichoic acid (LTA) or killed whole *S. aureus* together with IFN-γ was inhibited by the glucocorticoid, dexamethasone or by the specific inhibitor of NO synthesis, L-*N*-iminoethyl-ornithine (L-NIO). The exotoxins together with IFN-γ also activated macrophages to kill the intracellular parasite *Leishmania major*. The leishmanicidal activity was completely inhibited by L-NIO.

INTRODUCTION

Nitric oxide (NO) is an important mediator of macrophage tumoricidal and antimicrobial activities.[1] In murine leishmaniasis, NO plays a crucial role in the killing of parasites *in vitro*[2] and *in vivo*.[3] Nitric oxide is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine by NO synthase of which there are two types. One is constitutive and is Ca^{2+}/calmodulin dependent. The other, inducible by immunological stimuli, is Ca^{2+} independent. The activity of these enzymes can be specifically inhibited by L-arginine analogues such as L-*N*-iminoethyl-ornithine (L-NIO) and the induction of the inducible enzyme can be abrogated by glucocorticoids such as dexamethasone (reviewed in ref. 4). Cytokines such as interferon-γ (IFN-γ)[5] and tumour necrosis factor-α (TNF-α)[6] act synergistically with Gram-negative bacterial endotoxin, lipopolysaccharide (LPS) in the induction of NO synthesis in murine macrophages.

In Gram-positive bacterial infections, various toxins including microbial superantigens (that cause activation of T lymphocytes), mimic the pathophysiological events observed in the bacterial infection.[7] Staphylococcal enterotoxins A and B (Ent. A and B), toxic shock syndrome toxin-1 (TSST), or the bacteria *Staphylococcus epidermidis* can induce haemodynamic alterations in animal models[8–10] and the release of interleukin-1 (IL-1), TNF-α and IL-6.[7,11–13] It was demonstrated recently that some of these exotoxins in combination with IFN-γ enhance the tumoricidal activity of macrophages.[13] We report here that TSST, lipoteichoic acid (LTA) and killed *S. aureus* in combination with IFN-γ induce murine macrophages to kill the protozoal parasite *Leishmania major* via the L-arginine-dependent pathway.

Abbreviations: Ent. B, enterotoxin B; IFN-γ, interferon-γ; L-NIO, L-*N*-iminoethyl-ornithine; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NO, nitric oxide; TNF-α, tumour necrosis factor-α; TSST, toxic shock syndrome toxin-1.

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MATERIALS AND METHODS

Materials

Murine recombinant IFN-γ and murine recombinant TNF-α were kindly provided by Dr G. Adolf (Ernst Boehringer-Institut fur Arzneimettel-Forshung, Vienna, Austria). LPS from *Escherichia coli* (026:B6) was obtained from Difco (West Molesey, U.K.). TSST, Ent. B, and LTA from *S. aureus* and polymyxin B were purchased from Sigma (Poole, U.K.). *Staphyloccus aureus* was grown to stationary phase (about 10^9 bacteria/ml) and killed with formalin. Appropriate dilutions were made on the day of the experiment. Dexamethasone was purchased from Merck Sharp & Dohme Ltd (Hertfordshire, U.K.), L-NIO was synthesized at The Wellcome Research Laboratory (Beckenham, U.K.). All tissue culture reagents were purchased from Gibco (Paisley, U.K.). All other reagents were purchased from Sigma.

Macrophages

Murine peritoneal macrophages were harvested from BALB/c mice (Harlan Olac Ltd, Bicester, U.K.) which had been injected i.p. 4 days previously with 2 ml of sterile thioglycollate solution [3% w/v in phosphate-buffered saline (PBS)]. The cells were maintained in culture medium RPMI (Gibco) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin.

Parasites

The maintenance, cultivation and isolation of the promastigote stage of parasite *L. major* (LV39) have been described in detail elsewhere.[14]

Macrophage activation

Peritoneal macrophages were cultured for 2 hr at 37° in an atmosphere of 5% CO_2 in air in 12-well Costar plates at 2×10^6 cells/2 ml culture medium. Non-adherent cells were removed and the adherent cells washed three times with pre-warmed medium. TSST, LTA, Ent. B, *S. aureus* were added at various concentrations to the cultures alone or in combination with recombinant murine (rm) IFN-γ (10 U/ml). LPS (10 ng/ml) was added in some cultures as positive controls. In some experiments, dexamethasone (20 μM) or L-NIO (200 μM) were added in the cultures 2 hr before the macrophage activation. For experiments testing the possible LPS contamination, IFN-γ and the various bacterial materials were incubated with polymyxin B (10 μg/ml) for 30 min at room temperature before adding to the cultures containing macrophages. Culture supernatants were collected 18 hr after activation for assay of NO_2^-. At the end of the incubation period, the cells were also tested for their expression of NO synthase as follows. They were washed with saline and 250 μl of 0·1 M HEPES buffer (pH 7·4 with 1 mM DTT and 1 mM EDTA) was added to each well. The cells were harvested with a disposable rubber policeman and sonicated. The samples were then centrifuged at 100,000 *g* for 10 min at 4° and the supernatants were assayed for NO synthase activity using the oxyhaemoglobin assay as described by Feelisch and Noack.[15] The results were expressed as pmol of NO generated/mg protein/min.

Measurement of NO_2^-

NO_2^- in the culture supernatant was determined by chemiluminescence as described previously.[16] This measurement reflects the level of NO produced by the cells.[17]

Leishmanicidal assay

This method based on the ability of surviving parasites to incorporate [^{3}H]thymidine, which is comparable to the microscopic examination of intact intracellular parasites, has been described in detail elsewhere.[18] In some experiments L-NIO (200 μM) was added to cultures 2 hr before the stimuli.

Statistical analysis

Statistical significance ($P < 0.05$) was analysed by Student's *t*-test. Results are expressed as means ± 1 SEM, $n = 3$–6.

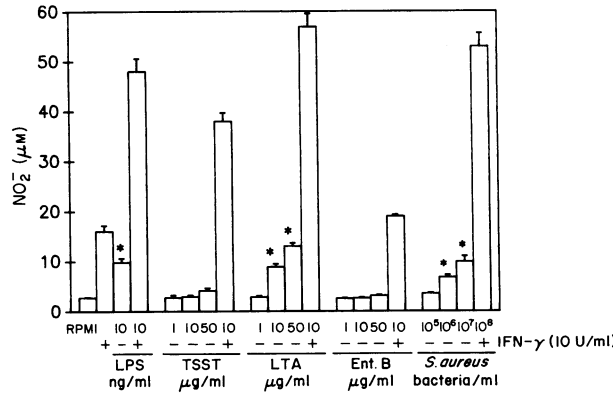

Figure 1. Levels of NO_2^- produced by peritoneal macrophages after culturing for 18 hr with different concentrations of TSST, LTA, Ent. B or killed *S. aureus* alone or in combination with IFN-γ (10 U/ml). LPS (10 ng/ml) was included as a positive control. Vertical bars = 1 SEM, $n = 3$. *$P < 0.05$ compared to controls (RPMI alone). Results are representative of three experiments.

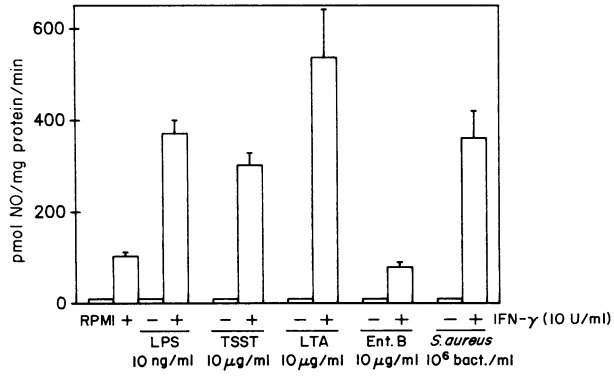

Figure 2. Levels of nitric oxide synthase in peritoneal macrophages after culturing for 18 hr with different concentrations of TSST, LTA, Ent. B or killed *S. aureus* alone or in combination with IFN-γ (10 U/ml). LPS (10 ng/ml) was included as a positive control. Vertical bars = 1 SEM, $n = 3$. Results are representative of three experiments.

RESULTS

Activation of the NO pathway by exotoxins

Peritoneal macrophages incubated for 18 hr with TSST or Ent. B at concentrations up to 50 μg/ml expressed little or no NO synthase or NO (Figs 1 and 2). The supernatants of macrophages cultured with LTA (up to 50 μg/ml), or killed *S. aureus* (10^5–10^7 bacteria/ml) contained modest but detectable amounts of NO_2^-. However, in the presence of IFN-γ, TSST, LTA and *S. aureus* induced the macrophages to produce large amounts of NO_2^- (Fig. 1) and express high levels of NO synthase (Fig. 2). A similar synergistic effect with IFN-γ was observed with LPS which was approximately 1000 times more effective than the exotoxins on a w/w basis. The induction of NO synthase by the

Table 1. Effects of polymyxin B on the induction of NO synthesis

Treatment of macrophages	NO_2^- (μM)
Medium only	14±0·1
LPS+IFN-γ	125±3·0
LPS+IFN-γ+polymyxin B	**42±0·6**
LTA+IFN-γ	134±3·0
LTA+IFN-γ+polymyxin B	133±1·5
S. aureus+IFN-γ	75±1·2
S. aureus+IFN-γ+polymyxin B	82±1·8
TSST+IFN-γ	62±2·2
TSST+IFN-γ+polymyxin B	56±4·0

For details see Materials and Methods. LPS, 10 ng/ml; IFN-γ, 10 U/ml; *S. aureus*, 10^6 bacteria/ml; LTA, TSST and polymyxin, 10 μg/ml. Figure in bold type is significantly different from control ($P<0·01$).

Gram-positive bacterial materials in the presence of IFN-γ was not likely to be due to LPS contamination, since the addition of polymyxin B, which effectively inhibited the activity of LPS, had no effect on the induction of NO synthesis by IFN-γ plus LTA, TSST or *S. aureus* (Table 1). Ent. B was inactive in all the cultures.

Effect of dexamethasone and L-NIO on NO synthesis

The induction of NO synthase and NO_2^- production by macrophages stimulated with LPS, TSST, LTA or *S. aureus* in combination with IFN-γ were significantly inhibited by the addition of dexamethasone (20 μM) or L-NIO (200 μM) in the cell cultures 2 hr before the stimulation (Fig. 3).

Killing of intracellular *L. major* by macrophages activated by *S. aureus* and its toxins

LPS, LTA or *S. aureus* but not TSST alone induced small but significant leishmanicidal effects. This is directly correlated with the ability of these materials to induce the synthesis of NO (Fig. 1). The leishmanicidal activities were markedly enhanced by the presence of IFN-γ in a dose-dependent manner and were completely inhibited by the addition of 200 μM of L-NIO (Fig. 4).

DISCUSSION

Data reported here demonstrate that exotoxins TSST, LTA or killed *S. aureus*, in combination with IFN-γ are able to activate murine macrophages to express high levels of NO synthase and to kill the intracellular parasites *L. major*. The parasiticidal activity was mediated by NO synthesized by the activated macrophages, for there was a direct correlation between the leishmanicidal activity and NO synthesis, and both the functions were inhibited by the specific NO synthase inhibitor, L-NIO.

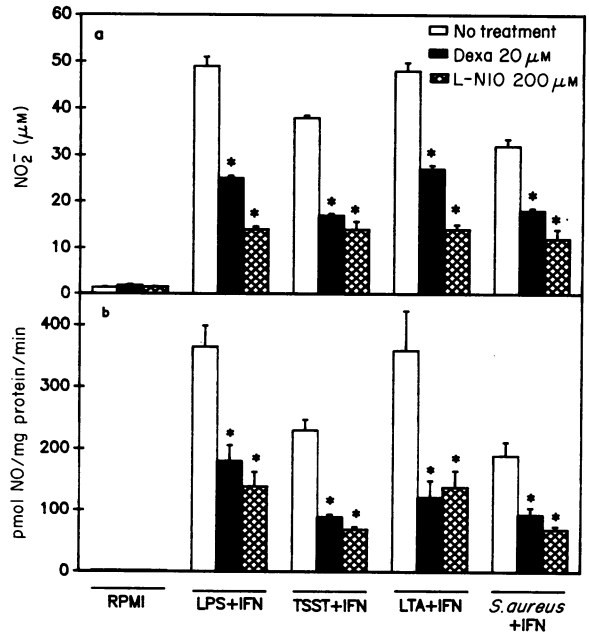

Figure 3. Effect of dexamethasone (Dexa) or L-NIO on (a) the synthesis of NO_2^-, and (b) induction of NO synthase in peritoneal macrophages by LPS (10 ng/ml), TSST (10 μg/ml), LTA (10 μg/ml) or *S. aureus* (10^6 organisms/ml). IFN-γ (10 U/ml) was present in all cultures. Dexamethasone (20 μM) or L-NIO (200 μM) were added to the cultures 2 hr before the stimuli. The levels of NO_2^- produced and the expression of NO synthase were determined at the end of an 18-hr culture period. Vertical bars = 1 SEM, $n=3$. $^*P<0·05$.

A recent report[19] shows that TSST alone stimulated the release of a modest level of NO_2^- by a macrophage cell line, J774. In our study, the amounts of NO_2^- released (up to 4 nmol/10^6 cells; Fig. 1) by cells stimulated with TSST and Ent. B alone were comparable to that reported by this previous study[19] and were indistinguishable from those produced by unstimulated cells. In contrast with other reported data,[13] we were unable to find a synergistic effect of Ent. B with IFN-γ to induce NO_2^- production. Consistent with our finding is a recent report that the toxic effects of Ent. B *in vivo* are not mediated by macrophages.[10]

Most of the clinical, laboratory and pathological abnormalities observed during the course of Gram-positive shock are similar to those of endotoxin shock, and are probably due to a common effector pathway involving a range of inflammatory mediators.[20] It was recently demonstrated that NO plays an important role in the haemodynamic alterations observed in endotoxin shock.[21] The inhibitory effect of glucocorticoids on the induction of NO synthase by different cytokines in combination with LPS has been demonstrated previously.[4,22] The induction of NO synthase by Gram-positive bacteria and their toxins was similarly found here to be inhibited by dexamethasone. Our results therefore suggest that NO may have a similar function in Gram-positive shock and the beneficial effects of glucocorticoids in the treatment of shock may be partly due the inhibition of the induction of NO synthase.[23]

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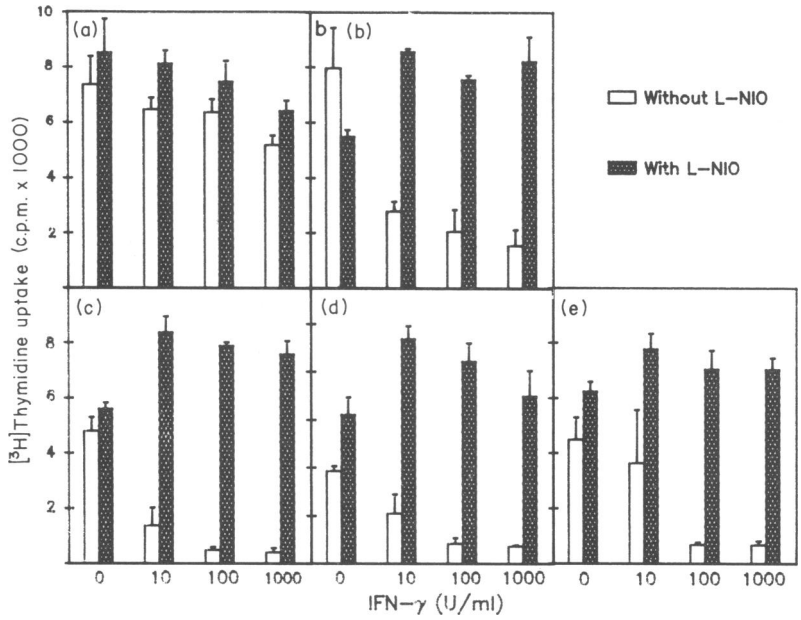

Figure 4. Leishmanicidal activity of macrophages by IFN-γ in medium alone (a), or plus TSST (10 μg/ml) (b), (10 μg/ml) (c), *S. aureus* (10^6 organisms/ml) (d) or LPS (10 ng/ml) (e), in the presence (■) or absence (□) of L-NIO (200 μM). Vertical bars = 1 SEM, $n = 3$.

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REFERENCES

1. Hibbs J.B., Jr, Taintor R.R., Varvin Z., Granger D.L., Drapier J.-C., Amber I.J. & Lancaster J.R., Jr. (1990) Synthesis of nitric oxide from a terminal guanidino atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. In: *Nitric Oxide from L-arginine: a Bioregulatory System* (eds S. Moncada and E. A. Higgs), p. 189. Excerta Medica, Amsterdam.
2. Green S.J., Meltzer M.S., Hibbs J.B., Jr & Nacy C.A. (1990) Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *J. Immunol.* **144,** 278.
3. Liew F.Y., Millott S., Parkinson C., Palmer M.J. & Moncada S. (1990) Macrophage killing of *Leishmania* parasites *in vivo* is mediated by nitric oxide from L-arginine. *J. Immunol.* **144,** 4794.
4. Moncada S., Palmer R.M.J. & Higgs E.A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109.
5. Stuehr D.J. & Marletta M.A. (1987) Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon-γ. *J. Immunol.* **139,** 518.
6. Ding A.H., Nathan C.F. & Stuehr D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J. Immunol.* **141,** 2407.
7. Fleming S.D., Iandalo J.J. & Chapes S.K. (1991) Murine macrophage activation by Staphylococcal exotoxins. *Infect. Immun.* **59,** 4049.
8. Bone R.C. (1991) The pathogenesis of sepsis. *Ann. Int. Med.* **115,** 457.
9. Wakabayashi G., Gelfand J.A., Jung W.K., Connolly R.J., Burke J.F. & Dinarello C.A. (1991) *Staphylococcus epidermidis* induces complement activation, tumor necrosis factor and interleukin-1, a shock-like state and tissue injury in rabbits without endotoxemia. *J. clin. Invest.* **87,** 1925.
10. Miethke T., Wahl C., Heeg K., Echtenacher B., Krammer P.H. & Wagner H. (1992) T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J. exp. Med.* **175,** 91.
11. Parsonnet J., Hichman R.K., Eardley D.D. & Pier G.B. (1985) Induction of Human Interleukin-1 by toxic shock-syndrome toxin-1. *J. infect. Dis.* **151,** 514.
12. Bhakdi S., Klonisch T., Nuber P. & Fischer W. (1991) Stimulation of monokine production by lipoteichoic acids. *Infect. Immun.* **59,** 4614.
13. Fast D.J., Shannon B.J., Herriott M.J., Kennedy M.J., Rummage J.A. & Leu R.W. (1991) Staphylococcal exotoxins stimulate nitric oxide-dependent murine macrophage tumoricidal activity. *Infect. Immun.* **59,** 2987.
14. Liew F.Y., Howard J.G. & Hale C. (1984) Prophylactic immunisation against experimental leishmaniasis. III. Protection against fatal *Leishmania tropica* infection induced by irradiated promastigotes involves Lyt-1^+2^- T-cells that do not mediate cutaneous DTH. *J. Immunol.* **132,** 456.
15. Feelisch M. & Noack E.A. (1987) Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.* **139,** 19.
16. Palmer R.M.J., Ferrige A.G. & Moncada S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327,** 524.
17. Marletta M.A., Yoon P.S., Iyengar R., Leaf C.D. & Wishnok J.S. (1988) Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27,** 8706.
18. Liew F.Y., Millott S., Li Y., Lelchuk R., Chan W.L. & Ziltener H. (1989) Macrophage activation by interferon-gamma from host protective T-cell is inhibited by interleukin (IL)-3 and IL-4 produced by disease-promoting T-cells in leishmaniasis. *Eur. J. Immunol.* **19,** 1227.

19. Zembowicz A. & Vane J.R. (1992) Induction of nitric oxide synthase activity by toxic shock syndrome toxin 1 in a macrophage–monocyte cell line. *Proc. natl. Acad. Sci. U.S.A.* **89,** 2051.
20. Natanson C., Danner R.L., Elin R.J., Hosseini J.M., Peart K.W., Banks S.M., MacVittie T.J., Walker R.I. & Parrillo J.E. (1989) Role of endotoxemia in cardiovascular dysfunction and mortality. *Escherichia coli* and *Staphylococcus aureus* challenges in a canine model of human shock. *J. clin. Invest.* **83,** 243.
21. Wright C.E., Rees D.D. & Moncada S. (1992) The protective and pathological role of nitric oxide in endotoxin shock. *Cardiovasc. Res.* **26,** 48.
22. Rosa M.D., Radomski M., Carnuccio R. & Moncada S. (1990) Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. *Biochem. biophys. Res. Commun.* **172,** 1246.
23. Galanos C. & Freudenberg M.A. (1990) Tumor necrosis factor mediates entoxin shock: the protective effects of antibodies and cortisone. In: *Tumor Necrosis Factor: Structure, Mechanism of Actions, Role in Disease and Therapy* (eds B. Bonavida and G. Granger), p. 187. Karger, Basel.