INFECTION AND IMMUNITY, Mar. 2000, p. 1740–1745 Vol. 68, No. 3
0019-9567/00/$04.00+0
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Lipopolysaccharides of *Brucella abortus* and *Brucella melitensis* Induce Nitric Oxide Synthesis in Rat Peritoneal Macrophages

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Received 28 October 1999/Returned for modification 16 November 1999/Accepted 6 December 1999

Smooth lipopolysaccharide (S-LPS) and lipid A of *Brucella abortus* and *Brucella melitensis* induced the production of nitric oxide (NO) by rat adherent peritoneal cells, but they induced lower levels of production of NO than *Escherichia coli* LPS. The participation of the inducible isoform of NO synthase (iNOS) was confirmed by the finding of an increased expression of both iNOS mRNA and iNOS protein. These observations might help to explain (i) the acute outcome of *Brucella* infection in rodents, (ii) the low frequency of septic shock in human brucellosis, and (iii) the prolonged intracellular survival of *Brucella* in humans.

Members of the genus *Brucella* are gram-negative bacteria that produce chronic infections in a large number of mammals, including humans (10). *Brucella* species are facultative intracellular pathogens which survive within a variety of cells, including macrophages, and the virulence of these species and the establishment of chronic infections by them are thought to be essentially due to their ability to avoid the killing mechanisms within macrophages (3, 36). The molecular mechanisms accounting for these properties are incompletely understood, and only some aspects of the processes involved have been identified as yet. *Brucella* does not evade phagocytosis by macrophages or neutrophils (6) but inhibits the degranulation of both primary and secondary neutrophil granules (30, 33, 34) and the myeloperoxidase-hydrogen peroxide-halide system (5, 7). The virulence of *Brucella abortus*, *Brucella melitensis*, and *Brucella suis* is associated with the smooth colony morphotype which contains the full lipopolysaccharide (LPS) (36, 38). The low biological activity induced by *Brucella* smooth LPS (S-LPS) compared with that produced by enterobacterial endotoxin might be one of the factors contributing to the survival of these pathogens in phagocytic cells (32). Further, a major proportion of the protective antibody response is directed against the O-chain component of the S-LPS (11) and *Brucella* LPS itself is a virulent factor because it is the main cause of the resistance of *Brucella* to lysosomal cationic proteins (15).

Nitric oxide (NO) has been shown to play an important role in diverse functions, including vasoregulation, neurotransmission, immune response regulation (23, 24, 37), and macrophage-mediated cytotoxic activity against tumor cells and a variety of pathogens, including bacteria, fungi, viruses, helminths, and protozoa (22). NO has been implicated in host defenses against intracellular pathogens and might play a role in persistent or latent infections (14). NO is derived from L-arginine in a reaction catalyzed by the enzyme NO synthase (NOS), of which three different isoforms have been identified (29). The inducible isoform of NOS (iNOS) is responsible for the high-output path of NO production involved in antimicrobial activity (28). iNOS expression is induced by proinflammatory cytokines such as gamma interferon (IFN-γ), tumor necrosis factor alpha, and interleukin 1 (IL-1), as well as by microbial products such as LPS and lipoteichoic acid (14). The purpose of this study was to determine whether *Brucella* S-LPS induces NO production in rat peritoneal macrophages and compare the effects of S-LPS from various *Brucella* species with that of *Escherichia coli* LPS. Since most biological effects of LPS have been associated with the lipid A moiety (25), we also investigated NO induction by *Brucella* lipid A. We report that *B. abortus* and *B. melitensis* S-LPS and lipid A induce NO production in rat peritoneal macrophages by a mechanism involving transcriptional up-regulation of the iNOS gene.

Pathogen-free Wistar rats (200 to 300 g) were used for all studies. *B. melitensis* 16M (biotype 1) and *B. abortus* 544 (biotype 1) smooth virulent strains were grown on *Brucella* broth (Difco Laboratories, Detroit, Mich.). Phenol-inactivated bacteria were harvested by centrifugation and washed twice with saline. S-LPS was extracted by the phenol-water method modified for *Brucella* organisms as described by Leong et al. (20), which allows the separation of *Brucella* S-LPS in the phenolic phase. Purification of crude S-LPS isolated in the phenolic phase was performed according to the procedure of Moreno et al. (26). The thiobarbituric method was used to measure 2-keto-3-deoxyoctonate (KDO) (2). Under these experimental conditions *B. melitensis* 16M purified S-LPS contained 0.79% KDO, *B. abortus* 544 purified S-LPS contained 0.70% KDO, and *E. coli* O:26,B:6 LPS (Sigma Chemical, St. Louis, Mo.) contained 1.6% KDO. Lipid A from *E. coli*, *B. abortus*, and *B. melitensis* was obtained by hydrolysis of purified S-LPS with 2% acetic acid at 100°C for 1 h (*E. coli*) or 5 h (*Brucella*) (25). Resident peritoneal cells were collected from the peritoneal cavity with cold phosphate-buffered saline as reported previously (4). Adherent macrophage monolayers were obtained by plating the cells in 24-well tissue culture plates at 1.5×10^6 cells/well for 2 h at 37°C. The production of NO was determined by the accumulation of nitrite measured by the Griess reaction (16). Statistical analysis was carried out using the unpaired Student t test for comparison of two means and one-way analysis of variance with the Bonferroni test for multiple comparisons of means. Statistical significance was set at a P value of <0.05.

Production of NO by rat peritoneal cells stimulated *Brucella* S-LPS and lipid A. Peritoneal cells stimulated with *E. coli*, *B. abortus*, and *B. melitensis* LPS generated nitrite in a dose-dependent manner (Fig. 1A). Since *E. coli* LPS is a microbial

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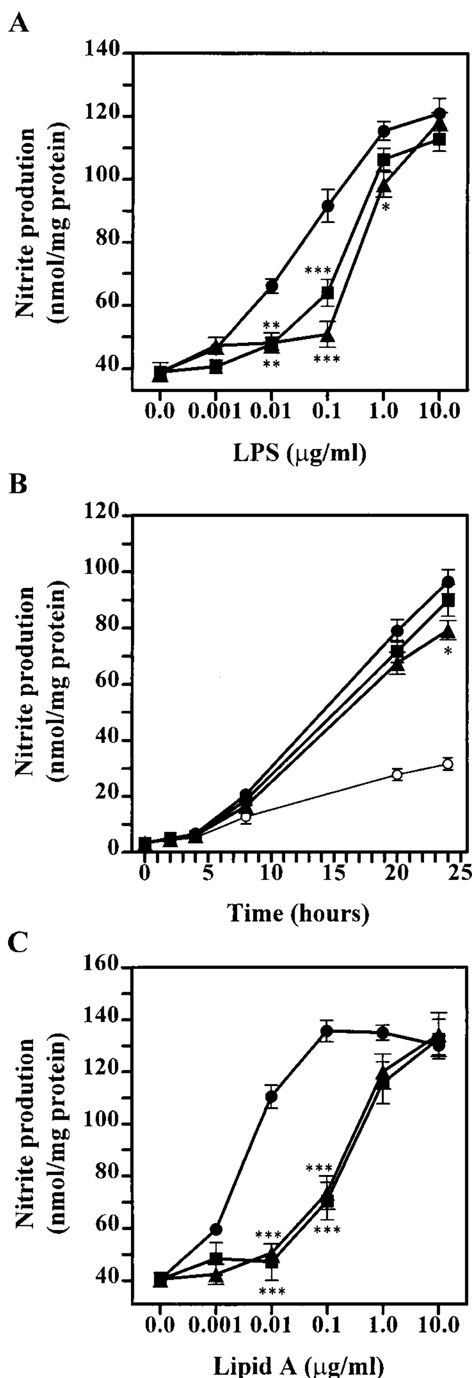

FIG. 1. (A) Production of nitrite by rat peritoneal adherent cells stimulated with different concentrations of *E. coli* LPS (●), *B. abortus* S-LPS (▲), and *B. melitensis* S-LPS (■). Adherent rat peritoneal cells were stimulated with LPS at the concentrations indicated. NO_2^- production was assayed 24 h after stimulation. Data are the means ± the standard errors of the means of results of four experiments with duplicate samples. (B) Kinetics of nitrite synthesis by adherent peritoneal cells stimulated with 1 μg of *E. coli* LPS (●), *B. abortus* S-LPS (▲), or *B. melitensis* S-LPS (■) per ml. Cells were allowed to adhere to plastic dishes and then were stimulated as indicated. Open circles indicate cells without any stimulus. Data are the means ± the standard errors of the means of results of three experiments performed in duplicate. (C) Production of nitrite by rat peritoneal adherent cells stimulated with different concentrations of *E. coli* (●), *B. abortus* (▲), or *B. melitensis* (■) lipid A. Nitrite production was assayed after 24 h of incubation. Data are the means ± the standard errors of the means of results of four experiments with duplicate samples. The nitrite levels induced by *Brucella* S-LPS and lipid A were significantly different from those of *E. coli*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

product inducing NO production by murine macrophages (39), it was included as a control of our cellular system and for comparison with *Brucella* S-LPS induction. *E. coli* LPS induced a significant level of production of NO from a dose of 0.01 μg/ml, plateau values being reached for LPS doses of ≥1 μg/ml. *B. abortus* S-LPS showed significant levels of NO production at doses of >0.1 μg/ml and produced maximal levels at a dose of 10 μg/ml. *B. melitensis* S-LPS induced NO production at concentrations higher than 0.01 μg/ml, with maximal induction at doses of ≥1 μg/ml. These data indicate that *E. coli* LPS is the most potent inductor of NO, whereas there was no significant difference in the NO production levels induced by *B. abortus* and *B. melitensis* S-LPS. The generation of nitrite by *E. coli*, *B. abortus*, and *B. melitensis* LPS was time dependent (Fig. 1B), first showing a lag of ≈8 h before significant production could be assayed and then increasing up to 24 h and showing plateau levels at 48 and 72 h (data not shown). Because most of the biological effects of the LPS have been associated with the lipid A moiety, we studied the ability of these lipids to induce NO production. Lipid A from both *Brucella* stimulated the production of NO in a dose-dependent manner (Fig. 1C). *E. coli* lipid A was again a potent inductor, significant production of NO being detected with concentrations of lipid A as low as 0.001 μg/ml, whereas maximal production was observed at a concentration of ≈0.1 μg/ml. *B. abortus* and *B. melitensis* lipid A showed similar potencies to elicit NO induction, i.e., significant amounts of nitrite were obtained in the presence of a concentration of 0.1 μg/ml and maximal production was obtained with concentrations of ≥1 μg/ml. Evidence of the involvement of the L-arginine pathway was obtained with the NOS inhibitor N^G-methyl-L-arginine (L-NMA), which suppressed nitrite production induced by each LPS and lipid A (Fig. 2).

Induction of iNOS mRNA expression in adherent peritoneal cells stimulated with LPS and lipid A. To address the involvement of iNOS in response to *Brucella* S-LPS the expression of iNOS mRNA was studied by Northern blot analysis. For this purpose, total cellular RNA was prepared from cell cultures according to the guanidium isothiocyanate method (8). Aliquots of total RNA (10 μg) were denatured and then separated by electrophoresis (35). After capillary transference of the RNA to nylon membranes, the membranes were hybridized overnight (9) with a radiolabeled DNA probe specific for mouse macrophage iNOS. The membranes were rehybridized, with ^{32}P-labeled β-actin probe used as an internal control to demonstrate the integrity of the RNA, the equivalence of loading, and the specificity of mRNA induction. As shown in Fig. 3, cells incubated with *E. coli*, *B. abortus*, and *B. melitensis* LPS showed enhanced expression of iNOS mRNA compared to cells incubated with vehicle. *E. coli* LPS induced an increased expression of iNOS mRNA that was already detected at 2 h and that showed a peak at 8 h and an elevation above resting levels at 24 h (Fig. 4A and B). This time course of iNOS mRNA induction closely parallels nitrite production. *B. abortus* S-LPS also showed a time-dependent induction of iNOS mRNA (Fig. 4C and D), with increased levels after 2 h of stimulation and a maximal expression at 4 h. Incubation with *B. melitensis* S-LPS also increased iNOS mRNA in a time-dependent manner (Fig. 4E and F). Nitrite production induced by lipid A also correlated with iNOS mRNA expression. As shown in Fig. 3, the addition of *E. coli*, *B. abortus*, and *B. melitensis* lipid A enhanced iNOS mRNA expression, thus confirming the involvement of the lipid A moiety in this biological effect. Induction of iNOS mRNA was accompanied by the expression of iNOS protein, as judged from Western blot analysis. For this purpose, cell lysates were subjected to electrophoresis on a

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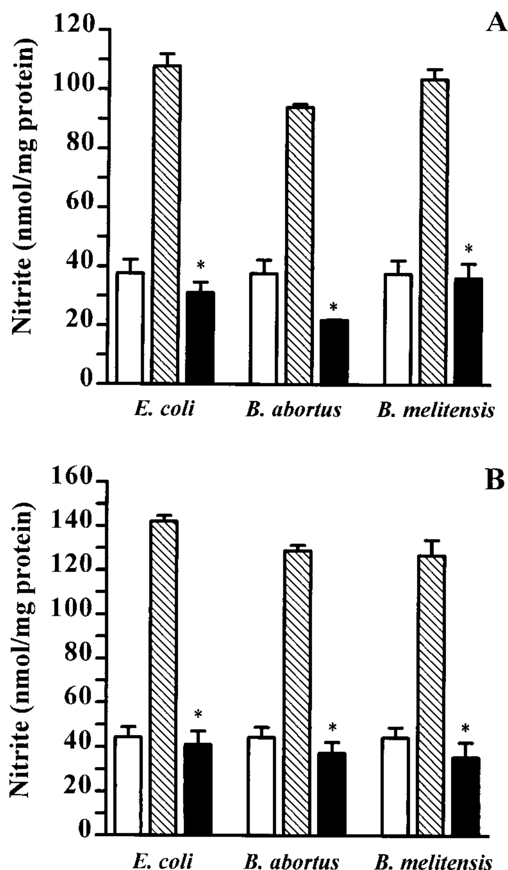

FIG. 2. Effect of L-NMA on nitrite production induced by *E. coli*, *B. abortus*, and *B. melitensis* LPS and lipid A. Adherent cells were incubated with 1 μg of the indicated LPS (A) or lipid A (B) per ml, in the presence (solid bars) or absence (hatched bars) of 0.5 mM L-NMA. Nitrite production was assayed after 24 h. Open bars show the production by control cells. Data are the means ± the standard errors of the means of results of three experiments performed in duplicate. The nitrite levels induced in the presence of L-NMA were significantly different from those induced with only the LPS or lipid A. *, $P < 0.001$.

sodium dodecyl sulfate-8% polyacrylamide gel, transferred to a nitrocellulose membrane, and then incubated with rabbit anti-mouse iNOS antibody (Calbiochem). The detection was performed using the enhanced chemiluminescence system (Amersham). Figure 5 shows the expression of iNOS protein in adherent peritoneal cells incubated with 10 μg of LPS from either class per ml.

Although the connection between NO and *Brucella* has been addressed by using whole bacteria and compared with the effect of *E. coli* (17), NO induction by *Brucella* S-LPS has not been reported. However, it has been observed that IL-12 depletion results in a reduced production of NO by spleen cells of mice infected with *Brucella*, thus indicating that LPS in intact *Brucella* stimulates macrophages to produce IL-12 and other costimulatory cytokines (44). It has been suggested that differences in the reactivities of LPS from *Brucella* and *E. coli* could explain the discrepancies in the capacities of these bacteria to induce NO release (17). Lipid A seems to be the active portion of *Brucella* S-LPS molecule associated with NO production, although it is ≈100-fold less potent than *E. coli* lipid A, a result in agreement with the relative potency reported by Rasool et al. (32), who compared the effect of *Brucella* and *Salmonella* LPS on nitroblue tetrazolium reduction and lysozyme release by neutrophils.

Since NO is a potent vasodilator, the overproduction of which has been shown to occur during the host inflammatory response associated with septic shock (13, 31), the reduced ability of *Brucella* S-LPS to induce NO production could explain the low frequency of septic shock and multiorgan failure observed in brucellosis (19) and the occurrence of clinical episodes similar to those observed upon infusion of small amounts of endotoxin (40). However, before extending these results to pathophysiological conditions, it should be taken into account that peritoneal macrophages are not the only contributors to host response during endotoxin-induced injury in vivo and the in vitro approach may overlook the contribution of other major players acting in vivo (1).

Expression of iNOS mRNA in J774A.1 cells infected with *B. suis* has been reported; however, both iNOS protein and NO production were detected only upon treatment with IFN-γ and antibrucella-specific antibodies (17). Since the stimulation of receptors for the Fc portion of immunoglobulin G (FcγR) can induce iNOS expression in rat macrophages (4), and immune complexes of different isotypes increase nitrite levels in murine macrophage-like J774.16 cells treated with IFN-γ (27), it

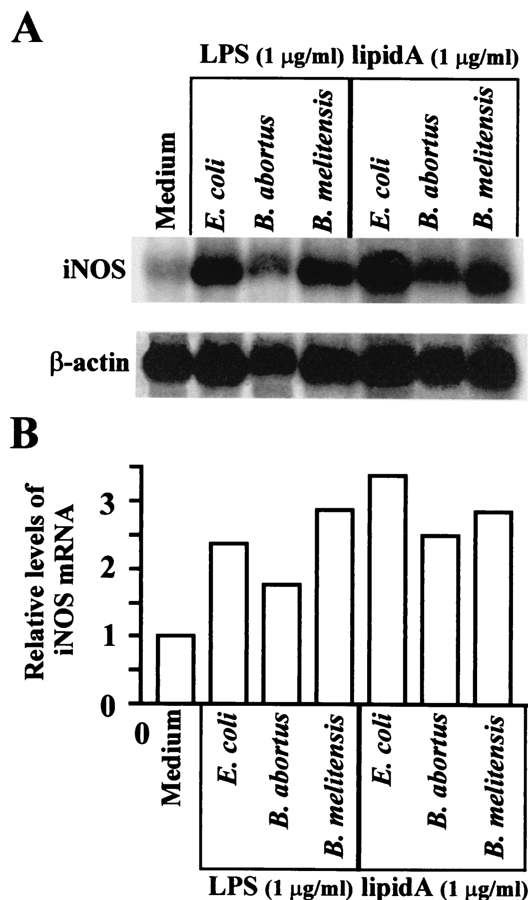

FIG. 3. Effect of *E. coli*, *B. abortus*, and *B. melitensis* LPS and lipid A on iNOS mRNA. Adherent cells were treated with 1 μg of LPS or lipid A per ml. (A) Total RNA was isolated after 24 h of stimulation and evaluated by Northern blot analysis with ^{32}P-labeled iNOS and β-actin DNA probes. The relative level of NOS mRNA expression was determined for each lane after normalization to the respective β-actin signal. The iNOS signal obtained in the absence of LPS was assigned a value of 1 to allow calculation of a relative level of mRNA expression for all other treatments. (B) The histogram shows the densitometric analysis of the autoradiograph shown in panel A. This is a representative experiment of four similar ones.

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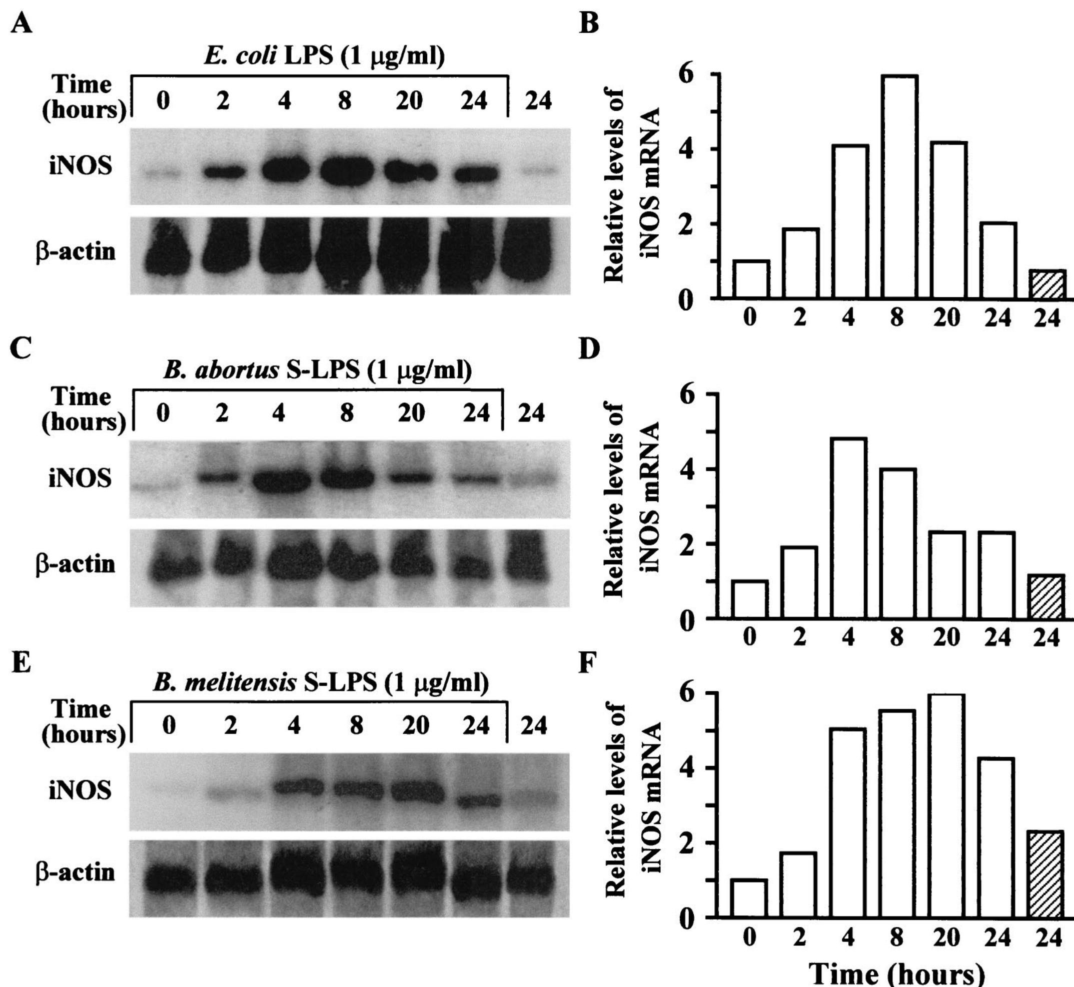

FIG. 4. Time course of iNOS mRNA induction by *E. coli* LPS (A and B), *B. abortus* S-LPS (C and D), and *B. melitensis* S-LPS (E and F). Total RNA was isolated at the indicated times and used for Northern blot analysis with ^{32}P-labeled iNOS and β-actin DNA probes. The relative level of iNOS mRNA expression was determined for each lane after normalization to the respective β-actin signal. The iNOS signal at 0 h was assigned a value of 1 to allow calculation of a relative level of mRNA expression for all other treatments. Results of scanning densitometric analysis of the autoradiographs in panels A, C, and E are presented in the histograms of panels B, D, and E (hatched bars indicate cells incubated for 24 h in the absence of LPS). These results are representative of those from three similar experiments.

seems likely that stimulation of FcγR could be the mechanism accounting for the induction of iNOS under these conditions. The synergistic inductive contribution of IFN-γ (17) agrees with the current paradigm of transcriptional regulation of iNOS. In fact, two regions have been identified in the promoter region of the mouse gene for macrophage iNOS (21): region I contains LPS-related responsive elements, including a binding site for nuclear factor–IL-6 and the κB binding site for activation of NF-κB, and region II contains motifs for binding IFN-related transcription factors such as interferon regulatory factor-1. Since NF-κB is involved in the induction of iNOS by LPS (43) and FcγR (4), these findings show the high degree of functional cooperation between microbial components and host immune response in the induction of the transcriptional activation implicated in antimicrobial actions.

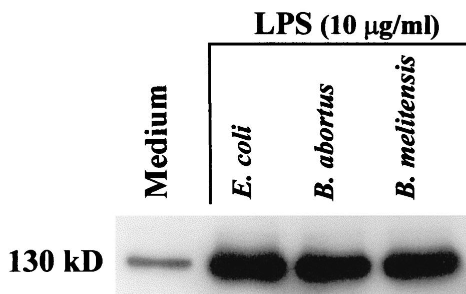

FIG. 5. Analysis of iNOS protein in peritoneal macrophages treated with *E. coli*, *B. abortus*, and *B. melitensis* LPS. Adherent cells were incubated with 10 μg of LPS per ml for 24 h. Cell lysates containing 40 μg of protein were analyzed by Western blotting with a rabbit anti-mouse iNOS antibody and peroxidase-conjugated anti-rabbit immunoglobulin G. These results are representative of three experiments.

Evidence of NO-dependent antimicrobial activity by human macrophages against parasites, fungi, bacteria, and viruses is now available (41), although significant differences between murine macrophages and human monocytes or macrophages have been stressed (12). Whereas rodent macrophages respond vigorously to a combination of IFN-γ and LPS, these stimuli induce the expression of iNOS in human cells but do not elicit any production of nitrogen derivatives, suggesting posttranslational regulation of NO synthesis in human cells (42). Addressing the effect of NO on *Brucella* survival seems of interest since *Brucella* stays alive in phagocytic cells; however,

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this is a controversial issue as yet. Zhan et al. (45) showed a vigorous production of NO by spleen cells of *B. abortus*-infected mice, compared to that of both spleen cells from noninfected mice and spleen cells from IL-12-depleted mice. When heat-killed brucellae were used as a stimulus for spleen cells from *B. abortus*-infected mice, the culture supernatants contained higher levels of nitrite than those for which an unrelated antigen was used (44). This could be explained by the activating effect of IFN-γ on NO production, but this could also be accounted for by the induction of NO by brucella LPS. The involvement of NO in the antibrucella activities of macrophages has also been suggested by pharmacological experiments with L-NMA (18), since this treatment increased significantly the number of brucellae recovered from macrophages infected with *B. abortus* isolated from the peritoneal cavity. However, when L-NMA was added to macrophages activated with IFN-γ prior to the infection with brucellae, a decrease of the number of CFU was observed, thus suggesting that NO might down-regulate the production of reactive oxygen intermediates. A corollary to these findings is that reactive oxygen intermediates might play a major role in antibrucella activities, whereas NO plays a minor one. In contrast, a direct killing of *B. suis* by NO and an increase of the intracellular development of *Brucella* by iNOS inhibitors in IFN-γ-treated J774A.1 cells infected with *B. suis* have been reported (17). In summary, *B. abortus* and *B. melitensis* S-LPS induce NOS in rat peritoneal macrophages. The ensuing NO production could explain why *Brucella* infection is controlled in mice, unlike human brucellosis, which tends to be chronic. The low *Brucella* S-LPS and lipid A NO production observed at low concentrations compared with LPS and lipid A *E. coli* induction could explain the low frequency of septic shock in human brucellosis and could contribute to explaining the long intracellular survival of *Brucella*.

We thank I. Moriyón, from the University of Navarra, for performing the KDO assays of LPS.

This work was supported by grants FIS 96/1017, SAF96-0144, and SAF98-0176.

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Editor: E. I. Tuomanen

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