Expression and Bactericidal Activity of Nitric Oxide Synthase in *Brucella suis*-Infected Murine Macrophages

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We examined the expression and activity of inducible nitric oxide synthase (iNOS) in both gamma interferon (IFN-γ)-treated and untreated murine macrophages infected with the gram-negative bacterium *Brucella suis*. The bacteria were opsonized with a mouse serum containing specific antibrucella antibodies (ops-*Brucella*) or with a control nonimmune serum (c-*Brucella*). The involvement of the produced NO in the killing of intracellular *B. suis* was evaluated. *B. suis* survived and replicated within J774A.1 cells. Opsonization with specific antibodies increased the number of phagocytized bacteria but lowered their intramacrophage development. IFN-γ enhanced the antibrucella activity of phagocytes, with this effect being greater in ops-*Brucella* infection. Expression of iNOS, interleukin-6, and tumor necrosis factor alpha (TNF-α) mRNAs was induced in both c-*Brucella*- and ops-*Brucella*-infected cells and was strongly potentiated by IFN-γ. In contrast to that of cytokine mRNAs, iNOS mRNA expression was independent of opsonization. Similar levels of iNOS mRNAs were expressed in IFN-γ-treated cells infected with c-*Brucella* or ops-*Brucella*; however, expression of iNOS protein and production of NO were detected only in IFN-γ-treated cells infected with ops-*Brucella*. These discrepancies between iNOS mRNA and protein levels were not due to differences in TNF-α production. The iNOS inhibitor 1-nitro-l-arginine methyl ester increased *B. suis* multiplication specifically in IFN-γ-treated cells infected with ops-*Brucella*, demonstrating a microbicidal effect of the NO produced. This observation was in agreement with in vitro experiments showing that *B. suis* was sensitive to NO killing. Together our data indicate that in *B. suis*-infected murine macrophages, the postranscriptional regulation of iNOS necessitates an additive signal triggered by macrophage Fcγ receptors. They also support the possibility that in mice, NO favors the elimination of *Brucella*, providing that IFN-γ and antibrucella antibodies are present, i.e., following expression of acquired immunity.

*Brucella* species are gram-negative, facultative, intracellular bacteria that can induce chronic infections in a wide range of mammalians, including humans and domestic ruminants. In humans, after they have invaded the reticuloendothelial system, the bacteria develop intracellularly within mononuclear phagocytes, and chronic infection generally results in fixation of infected macrophages in specific locations within the body (spleen, brain, heart, and bones). The disease is characterized by undulant fever, endocarditis, arthritis, and osteomyelitis (51). The pathophysiology of human infection differs in many respects from illness induced in domestic ruminants, where chronic infection results mainly in abortion in females and orchitis in males (15). In contrast to the case for the mammalians mentioned above, *Brucella* infection in mice is controlled and resembles septicemia (18), and mice are finally able to eliminate the bacteria a few weeks after infection or to maintain them at a very low level and prevent their further replication (34). These observations suggest specific interactions of *Brucella* organisms with the immune systems of the different hosts.

Host resistance to intracellular parasites is associated with the development of cell-mediated immunity and activation of macrophages to resist intracellular bacterial replication. Both phenomena are controlled by the production of cytokines, which occurs during infection. Among these cytokines, gamma interferon (IFN-γ) is a macrophage-activating factor which was shown to activate rodent macrophages to resist *Brucella* in vitro (24, 26) or in vivo (43, 53, 55). In addition, IFN-γ production was reported to be defective in *Brucella*-infected patients (38). IFN-γ primes murine macrophages to express inducible nitric oxide synthase (iNOS) (22), a cytosolic enzyme catalyzing the intracellular generation of short-lived nitric oxide radicals (NO) from the terminal guanido-nitrogen atom of L-arginine in response to an activation signal. NO was identified as the effector molecule in killing a range of intracellular pathogens (30, 33), including *Toxoplasma gondii* (1), *Leishmania* spp. (28, 29, 32), *Mycobacterium leprae* (2), *Mycobacterium tuberculosis* (12), *Legionella pneumophila* (44), and *Schistosoma mansoni* (23). The mechanism of this activity is still unknown, but one possibility is that during infection NO could combine with superoxide anion to generate the deleterious ONOO− anion (4, 57). Conversely, in humans, iNOS does not appear to be a component of the antimicrobial armature of mononuclear phagocytes (42), demonstrating a fundamental difference between human and mouse macrophages.

Although a previous report indicated a minor role of NO in the intracellular killing of *Brucella abortus* by murine macrophages (25), we evaluated the expression and activity of iNOS in murine monocytic cells infected by *Brucella suis*. The involvement of NO radicals in antibrucella activity of infected macrophages was also determined. We report here that NO is one component of antibrucella activity but only in IFN-γ-treated murine macrophages infected with *Brucella* opsonized with antibrucella antibodies. Under natural conditions in in-
ected mice which generate IFN-γ and antibodies against the bacteria, Fcy receptor (FcγR)-mediated increases in NO therefore may affect the course of host protection and pathogenesis.

MATERIALS AND METHODS

Reagents. Actinomycin D and Nu-nitro-l-arginine methyl ester (l-NAME) were purchased from Sigma Chimie (Saint-Quentin Fallavier, France), and 3-morpholinosydnonimine hydrochloride (sin-1) was from Molecular Probes (Eugene, Ore.). Murine recombinant INF-γ (rIFN-γ) prepared in baculovirus was obtained from Invitrogen (San Diego, Calif.). Murine recombinant tumor necrosis factor alpha (rTNF-α) (reference no. 87/650) and murine anti-rTNF-α were from the National Institute for Biological Standards and Controls (Potters Bar, United Kingdom), and the metalloprotease inhibitor BB-1101 was a kind gift of A. J. H. Gearing (British Biotech, London, United Kingdom).

Bacterial strains and media. In our experiments, Escherichia coli K-12 JM109 (American Type Culture Collection, Rockville, Md.) and B. suis 503 (a human isolate obtained from M. Ramuz, Nimes, France) were used. Bacteria were grown at 37°C with vigorous shaking to stationary phase in tryptic soy broth (Gibco BRL Life Technologies, Cergy, France). The anti-Brucella serum was obtained from BALB/c mice immunized by four successive intraperitoneal injections of gentamicin-killed B. suis. Immunization was achieved by an intramuscular injection of 100 μl of heat-inactivated serum antibodies to the bacteria on 3 consecutive days. The bacteria or bacteria plus antibody were readded to the gentamicin-supplemented medium during the culture.

Preliminary experiments which evaluated ratios of bacteria to macrophages of from 10:1 to 3:1 showed that a ratio of 50:1 resulted in an optimal number of multiplication between 7 and 48 h after infection. The number of viable bacteria was observed, i.e., between 7 and 48 h after infection. The number of intracellular bacteria was measured at different times postinfection, and regression analyses were performed to calculate the rates of Brucella growth, which were then compared for different infections by using Student’s t test.

Quantitation of TNF-α and NO2- and l-citrulline measurements. The biological activity of TNF-α released in cell supernatants was evaluated by a cytotoxic assay performed with the TNF-α-sensitive murine fibroblast cell line L929. Levels of TNF-α were quantified by comparison with an rTNF-α standard from the National Institute for Biological Standards and Controls, as previously described (8-10). To assess the amount of NO produced, culture cell-lysates and supernatants were assayed for accumulation of the stable end product of NO, NO2-, which was measured by the Griess reaction (13). In some experiments, the l-citrulline concentration was also determined by the colorimetric reaction of carbodimido groups with diacetyl monoxime in acid solution, as previously described (13).

Analysis of mRNA expression by reverse transcription-PCR (RT-PCR). Total RNA from either infected macrophage-like cells or control cells (2.5 × 105 cells per sample) was extracted with Trizol (Gibco BRL Life Technologies) as described by the manufacturer. The RT reaction was performed at 42°C for 90 min with 20 μg of total RNA, using murine Moloney leukemia virus reverse transcriptase (Gibco BRL Life Technologies and oligo(dT) (12-18 oligo(dT)-Gibco BRL Life Technologies) in the presence of 1 μg of [α-32P]dCTP (ICN, Orsay, France), for quantification of synthesized cDNA (7). One nanogram of each cDNA was amplified with 2.5 U of Gold Star polymerase (Eurogentec, Seraing, Belgium) and 1 μM specific primers. For TNF-α mRNA, the 5′ primer was 5′-TCT CAT CAG TTC TAC TGT GC-3′, the 3′ primer was 5′-GGG AGT AGA CAA GTA ACA AGC-3′, the amplon length was 212 bp; for iNOS mRNA, the 5′ primer was 5′-CTC TTC CGA AAG ACC ACA TGC-3′, the 3′ primer was 5′-GGG TTG GGT TGG TGA TGT AGC-3′, and the amplon length was 156 bp. The CDNs were amplified by repeated cycles of 90°C for 20 s, 60°C for 45 s, and 75°C for 45 s. A nonsaturating number of cycles (15 to 35) was adjusted for each cDNA. Amplification of β2-microglobulin (17 cycles) was used as a control. PCR products were run on 1.2% agarose gels supplemented with ethidium bromide, and their sizes were evaluated with molecular size standards (123-bp ladder; Gibco BRL Life Technologies).

Nitric oxide-mediated killing of B. suis. Direct killing of B. suis by NO radicals was examined as previously described for L. pneumophila (44). A total of 106 viable Brucella cells were suspended in 1 ml of RPMI containing 0.1, 0.5, or 2.5 mM sin-1 as a source of NO radicals (31). The bacterial suspension was incubated at 37°C for 24 h. After 4, 10, and 24 h of incubation, the number of viable bacteria in the medium supplemented with sin-1 and in controls were evaluated by CFU determination. NO2- concentrations in bacterial supernatants were quantified with Griess reagent as described above.

Analysis of iNOS protein. At 24 h after being infected, 1.5 × 106 J774A.1 cells were washed with PBS and lysed with radioimmunoprecipitation assay buffer (39). Nuclei were removed by centrifugation, and the cytosolic fraction was denatured by the addition of Laemmli buffer. Cell lysates were then subjected to electrophoresis on sodium dodecyl sulfate-7.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Poly-screen; Dupont NEN) with a semidry Millipore system. The membrane was blocked with 3% bovine serum albumin in PBS, incubated for 1 h with a rabbit anti-human iNOS serum (dilution, 1:2,000) (Alexis Corporation, San Diego, Calif.), washed with PBS-0.05% Tween, and revealed with a donkey anti-rabbit Ig horseradish peroxidase-conjugated antibody (Amersham France, Les Ulis, France) by using enhanced chemiluminescence reagents (Dupont NEN).

RESULTS

J774A.1 cell infection by B. suis. J774A.1 cells are currently used as a murine macrophage model. Measurements of bacterial CFU over a 72-h period established that phagocytized B. suis (c-Brucella) survived and, after a short period of time, replicated in these cells (Fig. 1A). The number of live bacteria within infected cells increased 200-fold in 48 h. Thereafter, due to the death of some infected macrophages and exposure of brucellae to antibiotic killing (24), the development of the bacteria appeared to be reduced. As previously shown for B. abortus (24, 25) and other Brucella species (8-10), opsonization of B. suis with an immune serum directed against the bacteria prior to infection (ops-Brucella) increased the number of phagocytized bacteria 20- to 30-fold (Fig. 1B). The number of intracellular bacteria then decreased significantly during the first hours and increased thereafter. Nevertheless, the bacterial growth was slightly slower than in the case of c-Brucella-infected cells, a result shown by comparison of the rates of bacterial multiplication between 7 and 48 h in both types of infection (P = 0.003).

Pretreatment of J774A.1 cells with 10 U of IFN-γ per ml did not significantly change the number of phagocytized c-Brucella or ops-Brucella (Fig. 1) but increased the bactericidal activity of the macrophages. This effect was relatively weak in c-Brucella infected cells and was greater in ops-Brucella infection. At 48 h following infection with ops-Brucella, the number of bacteria was 20-fold lower in IFN-γ-treated cells than in untreated cells. Compared to untreated cells, IFN-γ-treated cells showed an increase in the initial killing of the brucelle and then a...
increase in their further proliferation; the difference in rates of bacterial growth between 7 and 48 h were significant (P = 0.0037) (Fig. 1B).

As a control, we observed that nonpathogenic E. coli K-12 organisms were rapidly killed after phagocytosis whether J774A.1 cells were treated with IFN-γ (Fig. 1) or not (data not shown).

**iNOS, TNF-α, and IL-6 mRNA expression in infected J774A.1 cells.** To investigate a putative role of iNOS in Brucella infection, we first measured iNOS mRNA expression in infected mononcytic phagocytes by RT-PCR analysis. In parallel, we evaluated J774A.1 activation by measuring the expression of TNF-α and IL-6 mRNAs, two inflammatory cytokines induced in stimulated macrophages. To obtain comparable data on steady-state levels of mRNAs in the different samples, care was taken to optimize the amount of cDNA used in the PCR in order to highlight differences in mRNA levels. PCR analyses were performed with equal amounts of cDNA (1 ng, after verification that identical levels of β2-microglobulin mRNA were present in each sample) with a nonsaturating number of amplification cycles.

No expression of mRNAs encoding iNOS, TNF-α, or IL-6 was observed in resting J774A.1 cells despite a high number of amplification cycles during PCR experiments (e.g., 35 for iNOS

(not shown) (Fig. 2). IFN-γ pretreatment of the resting cells did not significantly change (or only slightly increased) steady-state levels of these mRNAs. J774A.1 cell infection with c-Brucella, ops-Brucella, or E. coli promoted efficient induction of iNOS, TNF-α, and IL-6 mRNAs, which were markedly increased in IFN-γ-pretreated cells. In J774A.1 cells incubated with the different infectious agents, amplicons corresponding to iNOS transcripts were barely detectable after 30 PCR amplification cycles, but they were easily observed in IFN-γ-pretreated cells (Fig. 2, compare lane 3 with lane 4 and compare lane 5 with lane 6). iNOS mRNA was induced at very similar levels in cells infected with c-Brucella or ops-Brucella, whether the cells were treated with IFN-γ or not (Fig. 2, compare lane 3 with lane 5 and compare lane 4 with lane 6). IFN-γ-pretreated cells infected with E. coli expressed a steady-state level of iNOS mRNA, slightly higher than that of cells infected with c-Brucella or ops-Brucella (Fig. 2, compare lane 7 with lanes 4 and 6).

**NO and citrulline production in infected J774A.1 cells.** To determine whether iNOS mRNA induction was correlated with NO production, NO2− accumulation in infected-cell supernatants was measured. Like uninfected control cells, J774A.1 cells not pretreated with IFN-γ showed negligible NO2− production 24 or 48 h after infection with c-Brucella or ops-Brucella (Fig. 3A) or even E. coli (not shown). In marked contrast, significant accumulation of NO2− was observed in supernatants of IFN-γ-treated J774A.1 cells infected with ops-Brucella or E. coli (Fig. 3A). This accumulation was optimal 48 h after infection: at 24 and 48 h, NO2− concentrations were, respectively, 15 and 25 μM in the case of ops-Brucella and 20 and 38 μM in the case of E. coli. We confirmed that the NO2− accumulation resulted from NO production by iNOS: (i) it was inhibited by L-NAME (48), and (ii) measurement of citrulline concentrations in 48-h cell supernatants demonstrated production of this metabolite which paralleled NO2− accumulation (Fig. 3B). Surprisingly, although similar levels of iNOS mRNA were induced in J774A.1 cells after infection with c-Brucella or ops-Brucella, no NO2− accumulation was measured in c-Brucella-infected-cell supernatants.

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**FIG. 1.** Intracellular behavior of B. suis and E. coli within J774A.1 cells. (A) and (C) and IFN-γ-treated J774A.1 cells (■ and ○). J774A.1 cells were infected with c-Brucella (■) and E. coli (○). The intracellular growth of bacteria was measured and expressed as CFU/well, as described in Materials and Methods. The results are from one representative experiment of four similar ones and are means ± standard errors of means for replicate infections performed concomitantly in the infection. **FIG. 2.** RT-PCR detection of IL-6, TNF-α, iNOS, and β2-microglobulin mRNAs in J774A.1 cells (lanes 1, 3, and 5) and IFN-γ-treated J774A.1 cells (lanes 2, 4, 6, and 7) infected with c-Brucella, ops-Brucella, or E. coli. J774A.1 cells or IFN-γ-treated J774A.1 cells were not infected (lanes 1 and 2) or were infected with c-Brucella (lanes 3 and 4), ops-Brucella (lanes 5 and 6), or E. coli (lane 7). At 6 h after the onset of infection, total RNAs from uninfected or infected cells were isolated. After an RT step, PCR were performed with 1 ng of cDNA. The PCR products, obtained as described in Materials and Methods after 17 cycles (β2-microglobulin), 30 cycles (iNOS), 25 cycles (TNF-α), and 22 cycles (IL-6), were analyzed on 1.2% agarose gels supplemented with ethidium bromide. The results are representative of three different experiments. NT, not treated.
iNOS expression in infected J774A.1 cells. To explain the differences between iNOS activation in IFN-γ-treated cells infected with c-Brucella, ops-Brucella, or E. coli, we measured iNOS expression by Western blot analysis in cell homogenates. Immunoblots demonstrated the appearance of a band at approximately 130 kDa in cells infected for 48 h with ops-Brucella or E. coli but not in cells infected with c-Brucella or in controls (Fig. 4). The intensity of the band was higher in E. coli-infected cells than in ops-Brucella-infected cells, which explained the differences in NO_2^- accumulation measured in cell supernatants.

TNF-α production in infected J774A.1 cells. B. suis phagocytosis triggered activation of genes of different cytokines (Fig. 2). Among these cytokines, TNF-α was reported to be an autologous activator of iNOS induction (28). We thus tried to determine whether differences in TNF-α production could result in differences in NO production. Table 1 shows that IFN-γ-treated J774A.1 cells infected with c-Brucella produced fourfold less TNF-α than the same cells infected with ops-Brucella. Nevertheless, when ops-Brucella infection occurred in the presence of a neutralizing anti-TNF-α antibody or 2.5 μM BB-1101, a metalloprotease inhibitor which prevented 80% of TNFα secretion (20), there was no significant inhibition of NO_2^- accumulation in the cell supernatants.

Direct killing of Brucella by NO. The ability of NO-generating agents to kill viable B. suis was examined. The bacteria were cultured in RPMI for 24 h in the presence of different concentrations of sin-1, a generator of NO radicals (31). B. suis survived and proliferated slightly (400% in 24 h) in RPMI. As shown in Fig. 5, sin-1 exerted a dose-dependent bactericidal effect on the bacteria. The lethal effect of sin-1 was correlated with a dose-dependent increase in the nitrite concentration in the bacterial supernatant. B. suis opsonisation did not change the ability of sin-1 to kill the bacteria (not shown).

Effect of an iNOS inhibitor on the intramacrophage development of Brucella. We assessed the putative role of NO production during B. suis infection by infecting IFN-γ-primed J774A.1 cells with ops-Brucella in the presence or absence of 3 mM L-NAME. Figure 6 shows that the intracellular multiplication of phagocytized bacteria was increased in IFN-γ-primed cells treated with L-NAME as compared to untreated IFN-γ-primed cells. L-NAME did not significantly affect phagocytosis of the bacteria (Table 2), but 48 h after infection, there were 20- to 30-fold more viable bacteria in L-NAME-treated cells than in untreated cells (Fig. 6). L-NAME inhibited the initial killing of the bacteria and accelerated its further development. The concomitant measurement of NO_2^- accumulation confirmed that in these experiments, L-NAME inhibited NO_2^- accumulation in supernatants of infected cells. In the various experiments, the NO_2^- concentration measured 24 h after the bacterium-cell contact decreased from 25 to 18 μM to 4.2 to 2.5 μM.

The effect of L-NAME on c-Brucella infection of IFN-γ-primed cells was also assessed. L-NAME affected neither the phagocytosis nor the further proliferation of the bacteria (Table 2; Fig. 6).

DISCUSSION

c-Brucella or ops-Brucella was phagocytized and proliferated in murine J774A.1 cells. Like in human macrophages (8, 10), bacterial opsonization substantially enhanced the phagocytosis process and promoted significant killing of the ingested bacteria and a slight diminution of their proliferation. Thus, 48 h after infection, the multiplication of the live bacteria was much lower in ops-Brucella-infected cells than in c-Brucella-infected...
TABLE 1. Effect of endogenous TNF-α production on nitrate accumulation in supernatants of B. suis-infected cells

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>TNF-α (pg/ml)</th>
<th>NO$_2^-$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>20 ± 10</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>IFN-γ + c-Brucella</td>
<td>1,200 ± 180</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>IFN-γ + ops-Brucella</td>
<td>5,200 ± 20</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>IFN-γ + ops-Brucella + anti-TNF-α</td>
<td>720 ± 40</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>IFN-γ + ops-Brucella + BB-1101</td>
<td>420 ± 50</td>
<td>20 ± 6</td>
</tr>
</tbody>
</table>

* IFN-γ-treated J774A.1 cells ($5 \times 10^5$) were infected with c-Brucella or ops-Brucella as described in Materials and Methods. When indicated, the culture medium was supplemented with 10 μg/mL neutralizing anti-TNF-α antibody per ml or 2.5 μM BB-1101 at onset of infection. At 24 h (TNF-α) or 48 h (NO$_2^-$) later, TNF-α and NO$_2^-$ concentrations in cell supernatants were measured. Data are the means ± standard deviations from three different experiments.

in line with in vivo studies showing that IFN-γ enhances the elimination of the bacteria in B. abortus-infected mice (43, 52).

The iNOS, TNF-α, and IL-6 mRNA expression levels highlighted that J774A.1 cells infected with c-Brucella or ops-Brucella were activated. Although ops-Brucella-infected cells expressed higher levels of TNF-α and IL-6 mRNAs than c-Brucella-infected cells, steady-state levels of iNOS mRNAs were very similar in both types of infection. This observation suggests that during infection in the absence of IFN-γ, FcγR triggering enhances macrophage activation and bactericidal activity without any participation of iNOS.

The IFN-γ priming of J774A.1 cells enhanced the expression of cytokine and iNOS transcripts induced during c-Brucella or ops-Brucella phagocytosis, confirming that IFN-γ stimulated the transcription of genes induced during infection (49). As iNOS mRNA expression was markedly increased independently of Brucella opsonization, the iNOS pathway could be involved in the resistance of IFN-γ-treated J774A.1 cells to c-Brucella and ops-Brucella infection. However, NO$_2^-$ and citrulline levels in cell supernatants and the Western blot analysis of iNOS in cell homogenates indicated that this was not possible: only IFN-γ-treated cells infected with ops-Brucella expressed functional iNOS. Together, our data show that the activation signals linked to c-Brucella phagocytosis were unable to promote the posttranscriptional events necessary for full expression of iNOS. NO production thus appears to be regulated at different levels, depending on distinct stimuli.

Experiments involving inhibitors of TNF-α completely ruled out the possibility that differences in NO secretion between c-Brucella and ops-Brucella infection of IFN-γ-treated cells resulted from differences in TNF-α production. Lipopolysaccharide (LPS) is a potent inducer of NO in IFN-γ-primed
lated for each experiment. Results represent means of times as described in Materials and Methods, and the survival index was calculated for each experiment. Results represent means of ± standard errors from two different experiments.

murine macrophages (49). Nevertheless, differences in the reactivities of LPS from Brucella and E. coli explain the discrepancies in the capacities of the bacteria to induce NO release: LPS from Brucella is 100- to 1,000-fold less reactive than LPS from E. coli or Salmonella (8, 21). This suggests that during phagocytosis, Brucella LPS is not the main agent responsible for murine macrophage activation. FcγR triggering accounts for the differences observed in macrophage infection by c-Brucella or ops-Brucella. FcγRs are associated with mitogen-activated protein kinases which control the expression of several genes relevant to macrophage activation at multiple levels (27, 45). Thus, it is possible that in ops-Brucella infection, FcγR ligation affects iNOS expression at a posttranscriptional level, through activation of these signalling molecules.

The dose-dependent bactericidal effect of sin-1 demonstrated that, at physiological concentrations, NO has a critical role in the direct killing of Brucella. In ops-Brucella-infected, IFN-γ-treated J774A.1 cells, the competitive inhibitor of iNOS (48) l-NAME, which did not affect phagocytosis, increased the intracellular development of Brucella. Thus, it is likely that in the absence of l-NAME, the production of NO significantly reduces the intracellular number of bacteria able to replicate. Conversely, l-NAME did not affect the growth of bacteria in c-Brucella-infected cells which did not display a functional iNOS. Finally, based on our findings, previous observations for other intracellular parasites (30–33) can now be extended to Brucella: in murine macrophages, Brucella is susceptible to killing by NO production. Nevertheless, this phenomenon requires opsonization of the bacteria with antibrucella antibodies before infection.

In vitro conditions, the production of NO clearly did not have a total lethal effect on the infecting inoculum. B. suis could partially escape the NO effect, as the NO concentrations to which the bacteria were exposed in the early phase of infection were too low. Once in the phagosome, Brucella could develop adaptive physiological changes that decreased its susceptibility to NO, which explains that it starts to multiply even if NO is still produced. Previous experiments using scavengers of reactive oxygen intermediates indicated that superoxide (O2−) and hydrogen peroxide were partially involved in the antibrucella activity of IFN-γ-treated J774A.1 cells ingesting opsonized B. abortus (25). These data indirectly demonstrated O2− production. Since NO and O2− are simultaneously synthesized in IFN-γ-treated cells infected with ops-Brucella, these radicals could interact together and produce peroxynitrite (ONOO−) (37), as in phorbol myristate acetate-activated Kupffer cells (47), endothelial cells (41), human neutrophils (16), and IFN-γ-treated mouse macrophages activated with immune complexes (36). Thus, NO could participate in the antibrucella activity of IFN-γ-treated macrophages ingesting ops-Brucella through the formation of ONOO−, a highly microbicidal radical (4, 6, 11, 19, 57).

Our results agree with a recent report of Mozaffarian et al. (35) indicating that in IFN-γ-treated J774A.1 cells, ligation of FcγRI, FcγRII, or FcγRIII induced NO production as well as superoxide release (5). In contrast, the data seem to conflict with the report of Jiang et al. (25) that NO was not produced in IFN-γ-treated J774A.1 cells infected with opsonized B. abortus. In fact, the differences between the results of those authors and ours might be explained by the two models studied: (i) Jiang et al. infected cells with B. abortus S19, a vaccine strain, while our experiments were performed with the virulent strain B. suis 503, and (ii) the bacteria were opsonized with B. abortus S19 immune bovine serum, whereas we used a serum from B. suis-immunized mice. The isotypes and quantities of antibodies could therefore have been very different in the two sera. Differences in the intramacrophage development of ingested Brucella have previously been reported to depend on the opsonization serum (26, 50), with the virulence of the Brucella strains used to prepare antisera being important. Phagocytized B. abortus was more easily eliminated when it was opsonized with an antisera raised against virulent B. abortus 2038 than when it was opsonized with B. abortus S19 antiserum (26). These differences were explained by the concentrations of specific antibodies of the IgG2a and IgG2b isotypes in B. abortus 2038 antiserum (50).

The ability of heat-inactivated B. abortus to induce IgG2a in immunized mice showed that the immunization process stimulates the cells to produce IFN-γ, this cytokine being necessary for IgG2a switching (17). Thus, in Brucella-immunized mice where IFN-γ and antibodies of the IgG2a isotype are already present (54) and can be rapidly elevated to a high level, phagocytosis of Brucella could lead to NO synthesis which accelerates

| Table 2. Comparison of Brucella phagocytosis in IFN-γ-treated J774A.1 cells infected in the presence or absence of l-NAME* |
|-----------------|-----------------|-----------------|-----------------|
| Expt | Without l-NAME | With l-NAME | |
| | ops-Brucella | c-Brucella | ops-Brucella | c-Brucella |
| 1 | 35,250 ± 4,630 | 1,250 ± 200 | 39,100 ± 5,300 | 1,320 ± 180 |
| 2 | 55,420 ± 5,330 | 2,800 ± 220 | 50,600 ± 3,480 | 3,100 ± 290 |
| 3 | 42,700 ± 3,750 | 3,050 ± 250 | 40,230 ± 4,300 | 2,870 ± 210 |
| 4 | 28,980 ± 3,280 | 1,240 ± 150 | 31,280 ± 2,970 | 1,500 ± 170 |

* J774A.1 cells (4 × 10⁴/well) were treated overnight with 10 U of IFN-γ per ml. They were then preincubated with 3 mM l-NAME or without l-NAME and infected with c-Brucella or ops-Brucella in the absence or presence of the iNOS inhibitor. The number of phagocytized Brucella cells accumulated in the cells at time zero of the infection was then evaluated, as described in Materials and Methods, and expressed as CFU/well. Values obtained in four different experiments (means ± standard deviations for two wells) are reported.
the elimination of the pathogen. The synergistic activation of phagocytes by IFN-γ and Brucella opsonized with antibodies observed in our in vitro system could therefore be involved in the resistance of mice to Brucella infection through NO production in immunized mice or in mice infected for several weeks with the live bacteria. Such an effect explains the facts that when passively administered, Brucella antibodies of the IgG2a subtype prevent the establishment of B. abortus infection in mice (50) and that protective immunity against Brucella is mediated by antibodies as well as by cell-mediated immune responses (3, 14). In contrast, the establishment of the bacterium within its host in primary infection could be facilitated by the absence of NO production.

NO production is not the sole protective function regulating the antibrucella activity of the phagocytes. TNF-α (10, 25, 56) and/or IFN-γ (24, 53) (Fig. 1) exert a protective effect, by mechanisms still unknown, in primary infection where NO is not produced. Other cytokines, like IL-1 (25, 52) and IL-12 (56), play an important role. These cytokines could also participate in the antibrucella activity by an NO-independent mechanism in ops-Brucella-infected macrophages. Moreover, the direct effect of O₂⁻ must be considered (5).

We did not obtain any evidence of NO production in B. suis-infected human mononuclear phagocytes, regardless of the conditions assessed. In parallel, we observed no effect of t-NNAME on Brucella development in infected human macrophages (not shown). In fact, despite some recent results (46), the role of iNOS in the antimicrobial armature of these cells is still controversial (42). Moreover, in humans, NK and T lymphocytes from Brucella-infected patients were reported to have defective IFN-γ production (38, 40). The differences in iNOS participation should be taken into account in understanding why Brucella infections can become chronic in humans and ruminants but not in mice.

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