Nitric Oxide-Mediated Inhibition of the Ability of Rickettsia prowazekii To Infect Mouse Fibroblasts and Mouse Macrophagelike Cells

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The role of the nitric oxide synthase (NOS) pathway in inhibiting the ability of Rickettsia prowazekii to initially infect (invade) mouse cytokine-treated, fibroblastic L929 cells and macrophagelike RAW264.7 cells and the ability of nitric oxide (NO) to damage isolated rickettsiae were investigated. Substantial amounts of nitrite (a degradation product of NO) were produced and the initial rickettsial infection was suppressed in cultures of L929 cells treated with crude lymphokine preparations (LK) or with gamma interferon (IFN-γ) plus tumor necrosis factor alpha (TNF-α) but not in L929 cell cultures treated with IFN-γ alone or TNF-α alone. The NOS inhibitors Nω-methyl-L-arginine and aminoguanidine both inhibited nitrite production and prevented the suppression of the initial rickettsial infection. Antibody-mediated neutralization of the IFN-γ in the LK also inhibited both nitrite production and suppression of the initial rickettsial infection. Cultures of RAW264.7 cells treated with IFN-γ plus lipopolysaccharide exhibited suppression of the initial rickettsial infection, and the suppression was relieved by aminoguanidine. Addition of oxymethemoglobin (a scavenger of extracellular NO) during the rickettsial infection alleviated the suppression of the initial rickettsial infection observed in appropriately treated L929 cells and RAW264.7 cells. In addition, the oxymethemoglobin restored the rickettsia-mediated, rapid killing of the treated RAW264.7 cells. Incubation of isolated rickettsiae with NO inhibited their ability to infect L929 and IFN-γ-treated RAW264.7 cells and to rapidly kill IFN-γ-treated RAW264.7 cells. In contrast, incubation of L929 cells with a solution that contained NO and/or degradation products of NO did not affect their ability to be infected by rickettsiae. The data are consistent with the hypothesis that NO released from appropriately stimulated potential host cells kills extracellular rickettsiae and thus prevents the rickettsiae from infecting the cells.

Rickettsia prowazekii, the etiologic agent of epidemic typhus, Brill-Zinsser disease, and flying-squirrel-associated typhus in humans, is an obligate intracytoplasmic bacterium. In vivo R. prowazekii grows within the endothelial cells that line the small blood vessels (39); this organism can also grow within macrophages (9).

The cytokines gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) alter the interactions between R. prowazekii and host cells such as fibroblasts, endothelial cells, and macrophages, and these cytokines may play roles not only in host defense but also in the pathogenesis of R. prowazekii infections (reviewed in references 26, 30, and 37). The effects of these cytokines on R. prowazekii infection in cultured cells include inhibition of rickettsial growth, killing of some of the rickettsiae, cytotoxic effects on the host cells, and (in some instances) inhibition of the initial infection of host cells by the rickettsiae (reviewed in reference 30).

Studies delineating the role of these cytokines in R. prowazekii infection in vivo are not available. However, studies of Rickettsia conorii infections in mice have demonstrated that both IFN-γ and TNF-α are important host defenses against infections with this Rickettsia species (7). How these cytokines protect the host is not completely understood; however, experimental evidence suggests the importance of nitric oxide synthase (NOS)-dependent alterations in rickettsia-host cell interactions (8, 31).

Many informative studies of how IFN-γ and/or TNF-α do (and do not) alter R. prowazekii-host cell interactions have employed fibroblasts and macrophagelike cell lines (reviewed in references 26, 30, and 37). Such studies allow for evaluation of the effects of cytokines on rickettsial infections and the mechanisms of these effects in experimental systems that are easily manipulated and not affected by many of the variables inherent in other, more complex systems. Moreover, some questions about the effects of cytokines on rickettsia-host cell interactions that can be answered through studies with cultured cells would be very difficult (or even impossible) to answer through studies with intact animals. Studies with cultured cells demonstrated that multiplication of R. prowazekii is inhibited in mouse L929 cells treated with IFN-γ and/or TNF-α and that the combination of IFN-γ and/or TNF-α treatment and R. prowazekii infection has a cytotoxic effect on the host cells (20, 28). In addition, such investigations determined that a lack of the amino acid tryptophan does not explain the ability of IFN-γ to limit the growth of R. prowazekii in either mouse L929 cells (in which tryptophan is not depleted in response to IFN-γ) or human fibroblasts (in which reconstitution of the depleted tryptophan pool in IFN-γ-treated cells does not alleviate the inhibition of rickettsial growth) (25). Furthermore, such studies revealed (i) that both NOS-dependent and NOS-independent mechanisms are involved in suppressing the growth of R. prowazekii in mouse L929 cells treated with IFN-γ plus TNF-α (28), (ii) that the inhibition of growth of R. prowazekii observed in L929 cells treated with either IFN-γ alone or TNF-α alone is largely NOS independent (28), (iii) that the mechanism responsible for IFN-γ-induced, NOS-independent inhibition of growth of R. prowazekii in mouse L929 cells is likely to exist also in IFN-γ-treated human fibroblasts (30), and (iv) that the cytotoxic effects on host cells brought...
The effects of cytokine treatment on R. prowazekii-host cell interactions are observed in host cells infected with either the virulent Breinl strain or the avirulent Madrid E strain of R. prowazekii.

Under some conditions, the ability of R. prowazekii to initially infect (invade) host cells is suppressed after the latter are treated with cytokines (23, 24, 26). For example, if L929 cells are treated for 24 h with high concentrations of lymphokines (LK) (supernatant fluids collected from cultures of antigen- or mitogen-stimulated mouse spleen cells), the initial infection (invasion) of the host cells by R. prowazekii (Madrid E and Breinl strains) is reduced (24, 26). Curiously, IFN-γ alone does not cause suppression of the initial infection in L929 cells; however, neutralization of the IFN-γ in the LK abolishes the ability of the LK to cause suppression (24, 26). It has not been determined if TNF-α alone (or in combination with IFN-γ) causes suppression of the initial R. prowazekii infection in L929 cells.

In RAW264.7 cell cultures pretreated with a low concentration of IFN-γ plus lipopolysaccharide (LPS), decreased R. prowazekii (Madrid E)-mediated, rapid killing of the RAW264.7 cells is associated with increased production of nitric oxide (NO), and addition of NOS inhibitors restores the R. prowazekii-mediated killing of the RAW264.7 cells in such cultures to a level comparable to that observed in RAW264.7 cultures pretreated with a low concentration of IFN-γ alone (29). Because the initial rickettsial infections were not monitored in these experiments, it is not known if the decreased R. prowazekii-mediated, rapid killing of the RAW264.7 cells observed in cultures treated with IFN-γ plus LPS is associated with lower initial rickettsial infection. However, macrophage-like RAW264.7 cell cultures pretreated with high concentrations of LK or IFN-γ exhibit markedly lower initial R. prowazekii (Breinl) infections and significantly less rickettsia-mediated, rapid killing of the RAW264.7 cells than comparably infected RAW264.7 cell cultures that have been pretreated with low concentrations of LK or IFN-γ (23). The possible role of the NOS pathway in mediating these suppressive effects of high concentrations of cytokines has not been evaluated.

Infection (invasion) of a host cell by R. prowazekii requires metabolic activity on the part of both the host cell and the rickettsia (32, 33). The infection process involves both phagocytosis and a phospholipase A activity, which is presumed to allow the rickettsia to escape from the phagosome into the cytoplasm of the host cell (36). Since R. prowazekii is an obligate intracellular bacterium, preventing it from infecting a host cell prevents it from growing.

Because cytokine-induced inhibition of the initial infection of potential host cells by R. prowazekii may represent an important antirickettsial host defense, we sought to obtain information about the mechanism of this inhibition by evaluating the possible role of the NOS pathway. This study reports the NOS dependence of cytokine-induced suppression of the initial R. prowazekii infection in L929 cells and RAW264.7 cells and implicates extracellularly released NO and/or NO products in causing the suppression. Furthermore, experiments with isolated rickettsiae exposed to NO in the absence of host cells permitted evaluation of the effects of NO on the rickettsiae themselves. The results provide evidence that isolated rickettsiae exposed to NO are killed, as judged by their reduced ability to infect L929 cells and their decreased capacity for infecting and rapidly killing IFN-γ-treated RAW264.7 cells.

Materials and Methods

Cell cultures. Mouse L929 cells (originally purchased from Flow Laboratories) were grown in Eagle minimal essential medium supplemented with 10% newborn bovine serum. Mouse macrophage-like RAW264.7 cells (Mediatech, Midlothian, Va.) were grown in Dulbecco modified Eagle medium (Mediatech, Washington, D.C.) supplemented with 10% defined, iron-supplemented bovine calf serum (Hyclone Laboratories, Logan, Utah). As determined by the manufacturers, the medium and serum used for growing the cells contained LPS levels of ≤0.025 and 0.0125 ng/ml, respectively. The L929 cells were grown in an atmosphere of 5% CO2 in air at 34°C; the RAW264.7 cells were grown in an atmosphere of 5% CO2 in air at 37°C. During and after infection with rickettsiae, all cells were incubated at 37°C.

Rickettsiae. Rickettsiae were purified from the yolk sacs of embryonated chicken eggs inoculated with R. prowazekii Madrid E (yolk sac passage 281) as previously described (22). Samples of rickettsial suspensions in sucrose-phosphate-glutamate-magnesium solution (SPG-Mg) (8.18 mM sucrose, 3.76 mM KH2PO4, 7.1 mM K2HPO4, 4.9 mM potassium glutamate, and 10 mM MgCl2) (3) were stored at −80°C for use in later experiments. The numbers of viable rickettsiae were estimated by their hemolytic activity (34).

Cytokines, monoclonal antibody against murine IFN-γ, hemoglobin, NO, and other reagents. Crude LK were prepared by culturing the spleen cells of Listeria monocytogenes-challenged C57BL/6J mice in the presence of concanavalin A for 24 h according to a method modified from that of Havell (11), as previously described (20). After filtration, samples of the culture supernatant fluids were stored at −80°C until use. Recombinant murine IFN-γ derived from Escherichia coli (19×103 or 4.7×104 U/mg) and recombinant murine TNF-α derived from E. coli (1.2×104 U/mg) were generously provided by Genentech, Inc., South San Francisco, Calif. The IFN-γ preparations had LPS levels of 25 and 365 ng/ml, respectively, as determined by the manufacturer. Monoclonal antibody (rat immunoglobulin G1) against murine IFN-γ (11,000 neutralizing units per ml) was purchased from LEE BioMolecular Research Laboratories, Inc. (San Diego, Calif.). The antiviral activities of the crude LK (1,680 U/ml) and the recombinant IFN-γ were determined by a cytopathic effect inhibition assay as previously described (21, 27). The activity of the TNF-α was verified by assaying its ability to kill actinomycin D-treated L929 cells as described by Havell (11). TNF activity was not detected in the LK. We determined by titration that 3 U of anti-mouse IFN-γ antibody completely neutralized 4 U of murine IFN-γ. LPS prepared from E. coli O111:B4 by the phenol extraction procedure, aminoguanidine (bicarbonate salt), Nω-monomethyl-L-arginine, and bovine hemoglobin (twice crystallized and consisting primarily of methemoglobin) were purchased from Sigma Chemical Co., St. Louis, Mo. Oxymethoglobin was prepared according to the method of Murphy and Noack (14). NO (99%) was purchased from Gulf States Airgas, Inc. (Theodore, Ala.).

Deoxygenated solutions and NO solutions. Distilled water and SPG-Mg solution were deoxygenated in glass vials by applying a vacuum (700 mm Hg) for 30 min; then they were bubbled with N2 gas for 30 min. A stock solution of approximately 0.5 mM NO was prepared fresh on the day of each experiment by addition of mL of NO (in a gas-tight syringe) to a sealed vial containing 2 mL of deoxygenated, distilled water. The NO solution was then diluted in deoxygenated SPG-Mg as required. The concentration of NO in the deoxygenated SPG-Mg was determined spectrophotometrically by the oxymethoglobin method (14).

Incubation of cells with cytokines, assay of NO for nitrite, infection of cells with rickettsiae, assessment of the initial rickettsial infection, and determination of host cell viability. L929 cells were exposed to 5,000 rad of X irradiation as previously described (22) to inhibit their multiplication. The cells were then adjusted to a concentration of 2×105 cells per ml in serum-supplemented medium (MS), planted in 24-well plates that contained coverslips (600 μm per well), and incubated at 34°C overnight. The medium was then removed, and each well was given 400 μl of MS alone or MS plus cytokines and/or other additions as required. After incubation of the cells for at least an additional 24 h, the culture media were collected and centrifuged, and the supernatant fluids were assayed for nitrite (a degradation product of NO) as described previously (28). The amount of nitrite detected in the LK therein was subtracted from the amount detected in the corresponding supernatant fluids collected from treated L929 cell cultures. After removal of the media, the L929 cells were washed once and incubated for 1 h at 34°C with rickettsiae diluted in Hanks’ balanced salt solution supplemented with 0.1% gelatin and 5 mM monopotassium glutamate (HBSSGG) (approximately 8×104 viable rickettsiae per ml, 500 μl per well). After being washed three times with MS, the cells were dried, fixed, and stained for rickettsiae (10). The rickettsial infections were evaluated as previously described (23).

RAW264.7 cells (4×104 cells per well) in a volume of 400 μl, unless otherwise noted) were planted in 24-well plates in MS alone or MS that contained IFN-γ (final concentration of 25 U/ml) and other additions (LPS, 80 ng/ml; aminoguanidine, 0.3 mM) as required. Coverslips were placed in some of the wells so that the supernatant fluids containing 2 μl of IFN-γ and IFN-γ (23). The number of viable R. prowazekii in each well was determined by the method of Murphy and Noack (14) (NO) (99%) was purchased from Gulf States Airgas, Inc. (Theodore, Ala.).
Nitrite production and NOS pathway-dependent suppression of the initial rickettsial infection in cytokine-treated L929 cells. To determine if suppression of the initial rickettsial infection in cytokine-treated L929 cells was dependent on the NOS pathway, NOS inhibitors were used and nitrite was assayed in the culture media collected from untreated and treated L929 cell cultures (Table 1). Substantial amounts of nitrite were produced in L929 cell cultures treated for 24 h with high concentrations of LK (8.3 or 25%) and in cultures treated for 24 h with IFN-γ plus TNF-α but not in untreated cultures or cultures treated with IFN-γ alone or TNF-α alone (Table 1). Suppression of the initial rickettsial infection was observed only in cultures in which substantial amounts of nitrite had been produced. Addition of an NOS inhibitor (aminoguanidine or Nω-ωmethyl-L-arginine) prevented the production of nitrite and alleviated the suppression of the initial rickettsial infection in cytokine-treated cultures in treated with LK and cultures treated with IFN-γ plus TNF-α (Table 1). We also established that neutralization of the IFN-γ in the LK prevented the LK from inducing nitrite production in L929 cells (Fig. 1), and we confirmed that such neutralization of the IFN-γ alleviated the ability of the LK to cause suppression of the initial rickettsial infection in L929 cells. Thus, suppression of the initial rickettsial infection in cytokine-treated L929 cell cultures was correlated with the production of substantial amounts of nitrite, and both nitrite production and suppression of the initial rickettsial infection were prevented by NOS inhibitors.

NOS pathway-dependent suppression of the initial rickettsial infection in RAW264.7 cell cultures treated with IFN-γ plus LPS. We previously determined that substantial concentrations of nitrite (averaging about 50 μM) are present in culture media collected from RAW264.7 cell cultures treated for 24 h with IFN-γ (25 U/ml) plus LPS (80 ng/ml), whereas much lower concentrations are detected in media collected from RAW264.7 cell cultures treated with IFN-γ alone or LPS alone (29). To determine if NOS pathway-dependent suppression of the initial rickettsial infection occurs in macrophage-like RAW264.7 cell cultures treated with IFN-γ plus LPS (and to begin evaluating the hypothesis that suppression of the rapid killing of the RAW264.7 cells occurs in these cultures [29] because the rickettsiae are prevented from infecting the RAW264.7 cells), the initial rickettsial infections in untreated RAW264.7 cell cultures, cultures treated with IFN-γ plus LPS, and cultures treated with IFN-γ plus LPS plus aminoguanidine were compared (Fig. 2). The initial rickettsial infection was significantly lower in IFN-γ plus LPS-treated RAW264.7 cell cultures than in untreated control cultures (P < 0.01), and the addition of aminoguanidine abrogated the suppression of the initial rickettsial infection (Fig. 2). Thus, NOS-pathway-dependent suppression of the initial rickettsial infection does occur in IFN-γ plus LPS-treated RAW264.7 cells, and such suppression explains (at least in part) the protection of IFN-γ plus LPS-treated RAW264.7 cells from R. prowazekii-mediated, rapid killing.
TABLE 1. Effect of treatment of L929 cells with cytokines and NOS inhibitors on nitrite production and the initial rickettsial infection of the cells with *R. prowazekii*.

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>No. of expts</th>
<th>Rickettsial infection (% of control value)</th>
<th>Nitrite (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%R</td>
<td>RI</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>100 [88 ± 3]</td>
<td>100 [3.6 ± 0.3]</td>
</tr>
<tr>
<td>AG* (0.5 mM)</td>
<td>5</td>
<td>100 ± 1</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>NGMA* (1 mM)</td>
<td>2</td>
<td>104 ± 0</td>
<td>105 ± 8</td>
</tr>
<tr>
<td>LK (2.5%)</td>
<td>2</td>
<td>98 ± 3</td>
<td>91 ± 14</td>
</tr>
<tr>
<td>LK (8.3%)</td>
<td>2</td>
<td>91 ± 2</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>LK (25%)</td>
<td>13</td>
<td>68 ± 3</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>LK (2.5%) + AG (0.5 mM)</td>
<td>2</td>
<td>99 ± 2</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>LK (8.3%) + AG (0.5 mM)</td>
<td>2</td>
<td>99 ± 2</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>LK (25%) + AG (0.5 mM)</td>
<td>7</td>
<td>98 ± 1</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>LK (25%) + NGMA (1 mM)</td>
<td>5</td>
<td>99 ± 2</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>IFN-γ (100 U/ml) + TNF-α (100 U/ml)</td>
<td>7</td>
<td>50 ± 7</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>IFN-γ (100 U/ml) + TNF-α (100 U/ml) + AG (0.5 mM)</td>
<td>3</td>
<td>100 ± 1</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>IFN-γ (100 U/ml) + TNF-α (100 U/ml) + NGMA (1 mM)</td>
<td>3</td>
<td>99 ± 1</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>IFN-γ (100 U/ml)</td>
<td>4</td>
<td>103 ± 2</td>
<td>121 ± 8</td>
</tr>
<tr>
<td>IFN-γ (420 U/ml)</td>
<td>2</td>
<td>103 ± 3</td>
<td>116 ± 10</td>
</tr>
<tr>
<td>IFN-γ (3,000 U/ml)</td>
<td>3</td>
<td>103 ± 2</td>
<td>122 ± 7</td>
</tr>
<tr>
<td>TNF-α (100 U/ml)</td>
<td>3</td>
<td>98 ± 3</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>IFN-γ (100 U/ml) + AG (0.5 mM)</td>
<td>1</td>
<td>104 ± 1</td>
<td>109 ± 1</td>
</tr>
<tr>
<td>TNF-α (100 U/ml) + AG (0.5 mM)</td>
<td>2</td>
<td>101 ± 0</td>
<td>99 ± 1</td>
</tr>
</tbody>
</table>

* After treatment of the L929 cells for at least 24 h, the culture media were removed and assayed for nitrite and the L929 cells were incubated with *R. prowazekii* for 1 h and washed.

† Each value represents the mean ± standard error, except that each value for cultures treated with IFN-γ (100 U/ml) plus AG (0.5 mM) represents the mean ± standard deviation for duplicate cultures in a single experiment.

‡ R, percentage of cells infected; RI, rickettsiae per infected cell; NR, rickettsiae per cell. The actual values for %R, RI, and NR in the untreated control cultures are given in brackets. The percent control values for NR were used for statistical analysis. Means that were significantly different from 100 as determined by a one-sample *t* test are indicated as follows: *, *P* < 0.05; ***, *P* < 0.01; ††, *P* < 0.001. Values for cytokine-plus-NGMA-treated cultures and cytokine-plus-AG-treated cultures which differed significantly from those for the corresponding cultures treated with cytokine alone (as determined by the two-sample *t* test) are indicated as follows: †, *P* < 0.05; ††, *P* < 0.001.

* AG, aminoguanidine.

†† NGMA, N^6^-methyl-L-arginine.

Elimination of NOS pathway-dependent suppression of the initial rickettsial infection in appropriately treated L929 cells and RAW264.7 cells by addition of oxyhemoglobin during the rickettsial infection. Although the data indicated that suppression of the initial rickettsial infection in appropriately treated L929 cells and RAW264.7 cells was NOS pathway dependent, the experiments with NOS inhibitors did not allow evaluation of the relative importance of intracellular and extracellular NO and/or NO products in mediating suppression of the initial rickettsial infection. To address this question, oxyhemoglobin, a scavenger of extracellular NO, was added during incubation of the cells with the rickettsiae (Fig. 3 and 4). Addition of 3 µM oxyhemoglobin during incubation with the rickettsiae eliminated the suppression of the initial rickettsial infection in L929 cell cultures treated with LK or with IFN-γ plus TNF-α (Fig. 3). Similarly, the inhibition of the initial rickettsial infection in RAW264.7 cell cultures treated with IFN-γ plus LPS was relieved in a dose-dependent manner as the concentration of oxyhemoglobin was increased to 75 µM (Fig. 4). With both cell lines, the control hemoglobin preparation that contained primarily methemoglobin was relatively ineffective. The data suggest that NO (or some product of NO) released into the extracellular space is required for suppression of the initial rickettsial infection in LK-treated L929 cells, IFN-γ-plus-TNF-α-treated L929 cells, and IFN-γ-plus-LPS-treated RAW264.7 cells.

Elimination of NOS pathway-dependent suppression of *R. prowazekii*-mediated, rapid killing of treated RAW264.7 cells by addition of oxyhemoglobin during the rickettsial infection. Oxyhemoglobin was also used to evaluate the role of extracellular NO in protecting IFN-γ-plus-LPS-treated RAW264.7 cells from *R. prowazekii*-mediated, rapid killing (Fig. 5). More than 70% of the RAW264.7 cells were rapidly killed in IFN-γ-treated cultures that were incubated with rickettsiae. However, as expected, less than 5% of the RAW264.7 cells were killed in IFN-γ-plus-NGMA-treated cultures that were incubated with rickettsiae. Whereas oxyhemoglobin at a concentration of 25 µM restored the *R. prowazekii*-mediated killing of the IFN-γ-plus-LPS-treated RAW264.7 cells, the hemoglobin preparation that contained primarily methemoglobin was relatively ineffective at restoring the killing (Fig. 5). These data suggest that NO (or some product of NO) released into the extracellular space protects the RAW264.7 cells from being killed by the rickettsiae in cultures treated with IFN-γ plus LPS. Thus, extracellular NO is important for both suppression of the initial rickettsial infection and suppression of *R. prowazekii*-mediated killing of the RAW264.7 cells in cultures treated with IFN-γ plus LPS.

Ability of NO treatment of rickettsiae to inhibit their capacity to infect cultured cells and to kill IFN-γ-treated RAW264.7 cells. To evaluate the ability of NO and/or NO products to kill rickettsiae, isolated *R. prowazekii* organisms were treated with...
NO and then added to cultured cells. Exposure of isolated rickettsiae to various concentrations of NO (2.8 to 25 μM) for 10 min significantly inhibited their ability to infect L929 cells (NO versus N2, P < 0.01), and the degree of inhibition was directly related to the concentration of NO (Fig. 6). Similarly, exposure of isolated rickettsiae to these concentrations of NO for 20 min significantly impaired both their abilities to infect and to cause cytotoxicity in IFN-γ-treated RAW264.7 cells (NO versus N2, P < 0.01); the extent of impairment of both activities was directly related to the concentration of NO (Fig. 7). Furthermore, when isolated rickettsiae were incubated with 5 μM NO for various periods of time, increased exposure times were associated with increased impairments in the ability of the rickettsiae to initially infect L929 cells (Fig. 8). Likewise, when isolated rickettsiae were incubated with 8.3 μM NO for various periods of time, inhibition of their ability to initially infect IFN-γ-treated RAW264.7 cells and suppression of their ability to cause cytotoxic effects in IFN-γ-treated RAW264.7 cells were both directly related to the duration of the exposure of the rickettsiae to NO (Fig. 9). Thus, the extent of impairment of these rickettsial activities was directly related to the concentration of NO used and the time of exposure. In no instance did the N2-treated rickettsiae differ significantly from the rickettsiae in the air controls (P > 0.1) (Fig. 6 through 9).

**Lack of effect of residual NO or degradation products of NO on L929 cells.** In order to eliminate the possibility that the observed suppressive effects of treating isolated rickettsiae with NO might be due to some action(s) of residual NO and/or its degradation products (remaining in the rickettsial suspensions at the time of addition of the cultured cells) on the cultured cells, a 25 μM solution of NO in deoxygenated SPG-Mg and a solution of deoxygenated SPG-Mg equilibrated with N2 (control) were exposed to air, diluted, and used to treat L929 cells before the cells were infected with untreated rickettsiae. The results indicated that the average values for the initial rickettsial infection parameters in L929 cells that had been pretreated with NO test solution (25 μM NO diluted in HBSSGG in air) did not differ significantly from the respective values in the control L929 cells (P > 0.1). Specifically, in

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**FIG. 1.** Reduction of both suppression of the initial rickettsial infection and nitrite production in cultures of L929 cells treated with LK plus antibody against murine IFN-γ. Each bar represents the mean ± standard error of the mean for the number of rickettsiae per cell (expressed as a percentage of the value in the control cultures) in the following numbers of experiments: control, 2; 25% LK (IFN, 420 U/ml), 4; antibody against murine IFN-γ (105 U/ml), 2; and LK plus antibody, 5. In the control cultures, the percentage of cells infected was 89% ± 2%, the number of rickettsiae per infected cell was 2.9 ± 0.2, and the number of rickettsiae per cell was 2.6 ± 0.2.

**FIG. 2.** NOS-dependent suppression of the initial rickettsial infection in RAW264.7 cells treated with IFN-γ (25 U/ml) plus LPS (80 ng/ml). Each bar represents the mean ± standard error of the mean for the number of rickettsiae per cell (expressed as a percentage of the value in the control cultures) in 10 experiments. In the control cultures, the percentage of cells infected was 79% ± 8%, the number of rickettsiae per infected cell was 4.4 ± 1.0, and the number of rickettsiae per cell was 4.0 ± 1.1. AG, aminoguanidine.

**FIG. 3.** Elimination of suppression of the initial rickettsial infection in L929 cell cultures by addition of oxyhemoglobin during the rickettsial infection. Each point represents the mean for the number of rickettsiae per cell (expressed as a percentage of the value in the control cultures) in at least two experiments, except for points representing cultures with 1 μM methemoglobin. The latter points represent the means of duplicate or triplicate determinations in a single experiment. The standard errors and standard deviations averaged 14% ± 2% of the mean values. In the control cultures, the percentage of cells infected was 86% ± 2%, the number of rickettsiae per infected cell was 3.3 ± 0.3, and the number of rickettsiae per cell was 2.9 ± 0.4. CONC, concentration; C, control; IT, IFN-γ (100 U/ml); TNF-α (100 U/ml); MET, methemoglobin; OXY, oxyhemoglobin. LK was at 25%.
the L929 cells that had been pretreated with the NO test solution, the initial percentage of cells infected and the initial number of rickettsiae per cell were 103% ± 6% and 107% ± 62% (respectively) of the corresponding values observed in the control L929 cells (88% ± 1% and 13.3 ± 1.0) (means ± standard deviations for two experiments). These data indicate that the suppression of the initial rickettsial infection that is observed after NO-treated rickettsiae are incubated with L929 cells does not result from the action of residual NO and/or its degradation products on the L929 cells.

Lack of effect of degradation products of NO on the ability of rickettsiae to infect L929 cells. To determine whether exposure of isolated rickettsiae to either nitrite or nitrate (degradation products of NO) could impair their ability to infect L929 cells, samples of isolated rickettsiae were incubated in SPG-Mg alone (untreated control), SPG-Mg plus 4 mM NaNO2 or SPG-Mg plus 4 mM NaNO3 for 2 h at room temperature before being added to suspensions of L929 cells. Neither rickettsial treatment notably altered the ability of the rickettsiae to initially infect L929 cells. In L929 cells infected with these treated rickettsiae, the percentage of cells infected and the number of rickettsiae per cell were (respectively) 92% ± 8% and 95% ± 18% of the corresponding values observed in the controls (L929 cell suspensions infected with rickettsiae that had been incubated for 10 min with deoxygenated SPG-Mg equilibrated with air) (70% ± 13% and 4.6 ± 1.4). In contrast (as expected), when samples of the NO solutions (25 μM) were incubated with isolated rickettsiae for 10 min and then the mixtures were bubbled with O2 and added to L929 cells, the initial infection in the L929 cells was dramatically and significantly reduced (P < 0.01). The percentage of cells infected and the number of rickettsiae per cell in L929 cell suspensions infected with such rickettsiae were (respectively) 28% ± 8% and 17% ± 5% of the corresponding values observed in the controls (62% ± 20% and 3.7 ± 1.9) (means ± standard deviations for four experiments). These data indicate that any degradation products of NO formed in the presence of oxygen are unlikely to have inhibited the ability of the rickettsiae to initially infect L929 cells under the experimental conditions used in this study.

FIG. 4. Elimination of suppression of the initial rickettsial infection in treated RAW264.7 cell cultures by addition of oxyhemoglobin during the rickettsial infection. Each point represents the mean for the number of rickettsiae per cell (expressed as a percentage of the value in the control cultures) in at least two experiments. The standard errors averaged 12% ± 1% of the mean values. In the control cultures, the percentage of cells infected was 99% ± 1%, the number of rickettsiae per infected cell was 6.3 ± 0.2, and the number of rickettsiae per cell was 6.2 ± 0.2. CONC, concentration; CTRL, control; IFN, IFN-γ (100 U/ml); MET, methemoglobin; OXY, oxyhemoglobin. LPS was at 80 ng/ml.

FIG. 5. Restoration of R. prowazekii-mediated killing of RAW264.7 cells in cultures treated with IFN-γ plus LPS by addition of oxyhemoglobin during the rickettsial infection. Each point represents the mean for the percentage of RAW264.7 cells killed in at least two experiments. The standard errors averaged 8% ± 11% of the mean values. CONC, concentration; CTRL, control; IFN, IFN-γ (100 U/ml); MET, methemoglobin; OXY, oxyhemoglobin. LPS was at 80 ng/ml.
SPG-Mg equilibrated with N2, or various concentrations of NO (diluted in deoxygenated SPG-Mg equilibrated with air, deoxygenated to L929 cells, samples of isolated rickettsiae were incubated at room temperature for 10 min with deoxygenated SPG-Mg equilibrated with air, deoxygenated SPG-Mg equilibrated with N2, or various concentrations of NO (diluted in deoxygenated SPG-Mg equilibrated with N2). Data are expressed as percentages of the corresponding values observed for rickettsial samples incubated with deoxygenated SPG-Mg equilibrated with air (air controls). In the L929 cell suspensions infected with these air control rickettsiae, the percentage of cells infected was 71% ± 9% and the number of rickettsiae per cell was 3.3 ± 1.3. Each point represents the mean ± standard deviation for at least three experiments.

**DISCUSSION**

Inhibition of the initial infection of activated macrophages by obligate or facultative intracellular microorganisms has been observed by many researchers working with different microorganisms (reviewed in references 5 and 16). The first studies to demonstrate suppression of the initial infection of activated macrophages by a *Rickettsia* (*Orientialia*) species were studies with LK-treated mouse macrophages and *Rickettsia tsutsugamushi* (15). Studies with *R. prowazekii* have indicated that the initial rickettsial infection is suppressed in macrophagelike RAW264.7 cells pretreated with high concentrations of LK (23), with high concentrations of IFN-γ (23), or with IFN-γ (at a low concentration) plus LPS (Fig. 2). In all three instances, suppression of the initial rickettsial infection is associated with protection of the treated RAW264.7 cells from *R. prowazekii*-mediated, rapid killing. It was further demonstrated that high concentrations of LK (but not high concentrations of IFN-γ) suppress the initial *R. prowazekii* infection in fibroblast L929 cells and that neutralization of the IFN-γ in the LK eliminates its ability to cause suppression of the initial rickettsial infection in L929 cells (24, 26). In the present study we found that the initial rickettsial infection is suppressed in IFN-γ plus-TNF-α-treated L929 cells (Table 1). Although TNF activity was not detected in the LK used in the present study, it seems likely that IFN-γ and some other cytokine in the LK cooperate in inducing suppression of the initial rickettsial infection in L929 cells.

Our results demonstrate that suppression of the initial *R. prowazekii* infection in appropriately treated L929 cells (Table 1) and RAW264.7 cells (Fig. 2) and protection of IFN-γ plus-LPS-treated RAW264.7 cells from *R. prowazekii*-mediated, rapid killing (29) are both dependent on the NOS pathway. It is likely that another cytokine in the LK acts together with IFN-γ to induce NOS in L929 cells. Amber et al. (1) showed that interleukin-1 as well as TNF-α, when given in combination with IFN-γ, could induce NOS activity in murine EMT-6 mammary adenocarcinoma cells. The actions of multiple cytokines in the LK and/or the production of additional cytokines by the RAW264.7 cells after treatment with LK may explain the ability of the LK to suppress the initial rickettsial infection in RAW264.7 cells and to protect the RAW264.7 cells from being rapidly killed by *R. prowazekii* (23, 24, 26). Treatment of RAW264.7 cells with high concentrations of recombinant IFN-γ may have resulted in suppression of the initial rickettsial infection (23, 24, 26) because the RAW264.7 cells produced (cooperating) cytokines that are not produced by RAW264.7 cells treated with low concentrations of IFN-γ. Alternatively, it may be that the IFN-γ used contained very small traces of LPS which were sufficient to stimulate the RAW264.7 cells to produce large amounts of NO when high concentrations of IFN-γ were used.

Suppression of the ability of *R. prowazekii* organisms to initially infect appropriately treated L929 cells and RAW264.7 cells was abrogated by the addition of oxyhemoglobin during the rickettsial infection. Because oxyhemoglobin would not be expected to enter the L929 cells and RAW264.7 cells in appreciable amounts, these results implicate an extracellular agent in mediating the suppression. The ability of oxyhemoglobin to scavenge NO (14) is consistent with the hypothesis that NO and/or a product(s) of NO is responsible for the observed inhibition of the initial rickettsial infection.

It is interesting that different concentrations of oxyhemoglobin were effective in alleviating the inhibition of the initial rickettsial infection in L929 cells and RAW264.7 cells. A concentration of 3 μM oxyhemoglobin eliminated suppression of the initial rickettsial infection in treated L929 cells (Fig. 3), whereas 50 to 75 μM oxyhemoglobin was needed to eliminate suppression of the initial rickettsial infection in treated RAW264.7 cells (Fig. 4). The concentration of oxyhemoglobin used contained very small traces of LPS which were sufficient to stimulate the RAW264.7 cells to produce large amounts of NO when high concentrations of IFN-γ were used.

**FIG. 6.** Inhibition of the initial rickettsial infection in L929 cells after exposure of isolated rickettsiae to various concentrations of NO. Before being added to L929 cells, samples of isolated rickettsiae were incubated at room temperature for 10 min with deoxygenated SPG-Mg equilibrated with air, deoxygenated SPG-Mg equilibrated with N2, or various concentrations of NO (diluted in deoxygenated SPG-Mg equilibrated with N2). Data are expressed as percentages of the corresponding values observed for rickettsial samples incubated with deoxygenated SPG-Mg equilibrated with air (air controls). In the L929 cell suspensions infected with these air control rickettsiae, the percentage of cells infected was 71% ± 9% and the number of rickettsiae per cell was 3.3 ± 1.3. Each point represents the mean ± standard deviation for at least three experiments.

**FIG. 7.** Inhibition of the abilities of the rickettsiae to initially infect IFN-γ-treated RAW264.7 cells and to cause cytotoxicity in IFN-γ-treated RAW264.7 cells after exposure of isolated rickettsiae to various concentrations of NO. Before being added to RAW264.7 cells (pretreated with IFN-γ plus aminoaudi-nidine), samples of isolated rickettsiae were incubated at room temperature for 20 min with deoxygenated SPG-Mg equilibrated with air, deoxygenated SPG-Mg equilibrated with N2, or various concentrations of NO (diluted in deoxygenated SPG-Mg equilibrated with N2). Data are expressed as percentages of the corresponding values observed for rickettsial samples incubated with deoxygenated SPG-Mg equilibrated with air (air controls). In RAW264.7 cell cultures infected with these air control rickettsiae, the percentage of cells infected was 90% ± 6%. Each point represents the mean ± standard deviation for at least three experiments.
required correlated with NO production; the concentrations of nitrite detected in media collected from RAW264.7 cell cultures treated for 21 h with IFN-γ plus LPS were about three times higher (29) than those detected in media collected from L929 cell cultures treated for 21 to 24 h with LK or with IFN-γ plus TNF-α (Table 1). The reasons for the variability exhibited by the control groups and the IFN-γ-treated groups in Fig. 4 are unknown.

Two lines of evidence suggest an important role for NOS-independent mechanisms in influencing R. prowazekii infections in human cells. First, cytokine-treated human macrophages restrict the growth of R. prowazekii (38); however, evidence that human macrophages produce large amounts of NO is lacking. Second, R. prowazekii strains resistant to cytokine-induced, NOS-independent inhibition of their growth in mouse L929 cells are also resistant to IFN-γ-induced inhibition of their growth in human fibroblasts (30). The possible role of the NOS pathway in altering interactions between R. prowazekii organisms and human host cells has not been adequately evaluated, and (to our knowledge) cytokine-induced suppression of the initial R. prowazekii infection in human cells has not been reported. Although some human cells, such as macrophages, apparently do not produce large amounts of NO (18, 35), human hepatocytes (17) and human epithelial cells (2, 12) are capable of producing substantial amounts of NO. Hepatocytes (in addition to endothelial cells) are of interest, since liver involvement has been documented in infections of humans with Rickettsia typhi (19), the causative agent of endemic or murine typhus. It is possible that the ability (or inability) to produce substantial amounts of NO influences the susceptibility of various types of human cells to R. prowazekii infection in vivo.

Inhibition of the initial R. prowazekii infection in appropriately treated L929 cells and RAW264.7 cells and suppression of the R. prowazekii-mediated, rapid killing of treated RAW264.7 cells appear to be due to NO-mediated damage to the rickettsiae. R. prowazekii organisms appear to be very sensitive to NO in comparison with certain other bacteria. In the present study, most of the rickettsiae were killed (as judged by their inability to infect host cells) within 10 to 20 min of exposure to NO at concentrations of less than 10 μM, and the effectiveness of 3 μM oxyhemoglobin in preventing suppression of the initial rickettsial infection in cytokine-treated L929 cells is consistent with this marked sensitivity of the rickettsiae to NO. On the other hand, Brunelli et al. (4) found that E. coli organisms are not killed by exposure to 1 mM NO for 1 h and that E. coli organisms are much more sensitive to peroxynitrite (a product of the reaction of NO and superoxide) than to NO (4). Similarly, DeGroote et al. (6) showed that Salmonella typhimurium is killed by a peroxynitrite-generating compound, is inhibited by S-nitrosoglutathione, and is not killed or inhibited by a compound that donates NO. Finally, Kaplan et al. (13) observed that Staphylococcus aureus bacteria are not killed by 2 h of exposure to NO; however, many of the bacteria are killed after 24 h of exposure. Determination of the reason for the greater sensitivity of R. prowazekii to NO and exactly how the NO kills the rickettsiae awaits further investigation.

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REFERENCES


FIG. 8. Inhibition of the initial rickettsial infection in L929 cells after exposure of isolated rickettsiae to NO for various periods of time. Before being added to L929 cells, samples of isolated rickettsiae were incubated at room temperature for various time periods with deoxygenated SPG-Mg equilibrated with air, deoxygenated SPG-Mg equilibrated with N2, or 5 μM NO in deoxygenated SPG-Mg equilibrated with N2. Data are expressed as percentages of the corresponding values observed for rickettsial samples incubated with deoxygenated SPG-Mg equilibrated with air (air controls). In L929 cell suspensions infected with these air control rickettsiae, the average percentages of cells infected ranged from 52% to 71%, and the average numbers of rickettsiae per cell ranged from 3.3 to 4.9. Each point represents the mean ± standard deviation for at least two experiments.

FIG. 9. Inhibition of the abilities of the rickettsiae to initially infect IFN-γ-treated RAW264.7 cells and to cause cytotoxicity in IFN-γ-treated RAW264.7 cells after exposure of isolated rickettsiae to NO for various periods of time. Before being added to RAW264.7 cells (pretreated with IFN-γ plus aminoguanidine), samples of isolated rickettsiae were incubated at room temperature for various time periods with deoxygenated SPG-Mg equilibrated with air, deoxygenated SPG-Mg equilibrated with N2, or 8.3 μM NO in deoxygenated SPG-Mg equilibrated with N2. Data are expressed as percentages of the corresponding values observed for rickettsial samples incubated with deoxygenated SPG-Mg equilibrated with air (air controls). In RAW264.7 cell cultures infected with these air control rickettsiae, the average percentages of cells infected ranged from 90% to 92%, the average numbers of rickettsiae per cell ranged from 11.1 to 11.9, and the average percentages of RAW264.7 cells killed ranged from 94% to 99%. Each point represents the mean ± standard deviation for at least two experiments.


