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Inhibition of *Legionella pneumophila* Growth by Gamma Interferon in Permissive A/J Mouse Macrophages: Role of Reactive Oxygen Species, Nitric Oxide, Tryptophan, and Iron(III)

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A/J mouse macrophages infected with *Legionella pneumophila* and treated with gamma interferon (IFN-γ) in vitro developed potent antimicrobial activity. This antilegionella activity was independent of the macrophage capacity to generate reactive oxygen intermediates, since the oxygen radical scavengers catalase, superoxide dismutase, mannitol, and thiourea had no effect on the antilegionella activity of IFN-γ-activated macrophages. Likewise, whereas the ability of IFN-γ-activated macrophages to synthesize reactive nitrogen intermediates was markedly inhibited by the L-arginine (Arg) analogs, N^G-monomethyl-L-arginine and L-aminoguanidine, as well as by incubation in L-Arg-free medium, their ability to inhibit the intracellular growth of *L. pneumophila* remained intact. The intracellular growth of *L. pneumophila* in A/J macrophages was inhibited by the iron(III) chelator desferrioxamine and reversed by Fe-transferrin as well as by ferric salts. Additionally, IFN-γ-activated macrophages incorporated 28% less ^{59}Fe(III) compared with nonactivated cells. Nonetheless, only partial blocking of growth restriction was observed when IFN-γ-stimulated macrophages were saturated with iron(III). Indole-propionic acid, which appears to inhibit the biosynthesis of L-tryptophan (L-Trp), was an L-Trp-reversible growth inhibitor of *L. pneumophila* in macrophages, implying that the intracellular replication of this pathogen is also L-Trp dependent. However, an excess of exogenous L-Trp did not reverse the growth inhibition due to IFN-γ, though a small synergistic effect was observed when the culture medium was supplemented with both iron(III) and L-Trp. We conclude that IFN-γ-activated macrophages inhibit the intracellular proliferation of *L. pneumophila* by reactive oxygen intermediate- and reactive nitrogen intermediate-independent mechanisms and just partially by nutritionally dependent mechanisms. We also suggest that additional mechanisms, still unclear, may be involved, since complete reversion was never obtained and since at higher concentrations of IFN-γ, iron(III) did not induce any significant reversion in the *L. pneumophila* growth inhibition.

Legionella pneumophila, first discovered in 1976 during the outbreak of Legionnaires' disease in Philadelphia, is a facultative intracellular pathogen that proliferates intracellularly in mononuclear phagocytes (25). The mechanisms of resistance to this bacterium suggest an important role for cell-mediated immunity in legionellosis (25, 26, 68). In vitro studies have shown that macrophages from a variety of sources, when activated with gamma interferon (IFN-γ), inhibit the intracellular growth of *L. pneumophila* (5, 7, 8, 27, 28, 31, 44, 57, 60) and of other intracellular pathogens (27).

Byrd and Horwitz showed that IFN-γ restricts the growth of *L. pneumophila* in human monocytes by reducing the intracellular availability of iron (7–9). They found a reduction of 73% in transferrin-receptor (TfR) expression and 82% in ferritin content. Hamilton et al. (24) also found a threefold reduction in TfR binding sites in thioglycolate-elicited murine macrophages treated with IFN-γ. Nonetheless, whereas some reports have shown that iron reverses the antimicrobial effect of IFN-γ in human and mouse mononuclear phagocytes (7, 8, 32, 33, 39), others have found that ferric salts or Fe-transferrin (Fe-Tf) does not have any effect (29, 42, 45).

Several laboratories have also shown that IFN-γ blocks the intracellular growth of *Toxoplasma gondii*, *Chlamydia psittaci*, *Chlamydia trachomatis*, and *Rickettsia conorii* by inducing the host cells to degrade L-tryptophan (L-Trp) (10, 38, 46, 53, 54). The same antimicrobial mechanism seemed to be involved, at least partially, in IFN-γ-stimulated macrophages infected with *Toxoplasma gondii*, *Chlamydia psittaci*, or *Leishmania donovani* (11, 43, 51). On the other hand, the intracellular proliferation of *Cryptococcus neoformans* (21, 22), *Leishmania* spp. (23, 34, 39), *Toxoplasma gondii* (1), *Trypanosoma cruzi* (41), *Naegleria fowleri* (16), *Mycobacterium* spp. (14, 17), *Listeria monocytogenes* (4), *Francisella tularensis* (2), and *Ehrlichia risticii* (45) were found to be inhibited in IFN-γ-stimulated mouse macrophages by an L-arginine (Arg)-dependent mechanism.

Inasmuch as the mechanisms whereby IFN-γ inhibits *L. pneumophila* growth in mouse macrophages are unknown, we examined the possible involvement of iron(III), L-Trp, reactive nitrogen intermediates (RNI), and reactive oxygen intermediates (ROI). We found that IFN-γ-activated macrophages inhibit the intracellular proliferation of this opportunistic bacterium by ROI- and RNI-independent mechanisms but partially by nutritionally dependent mechanisms.

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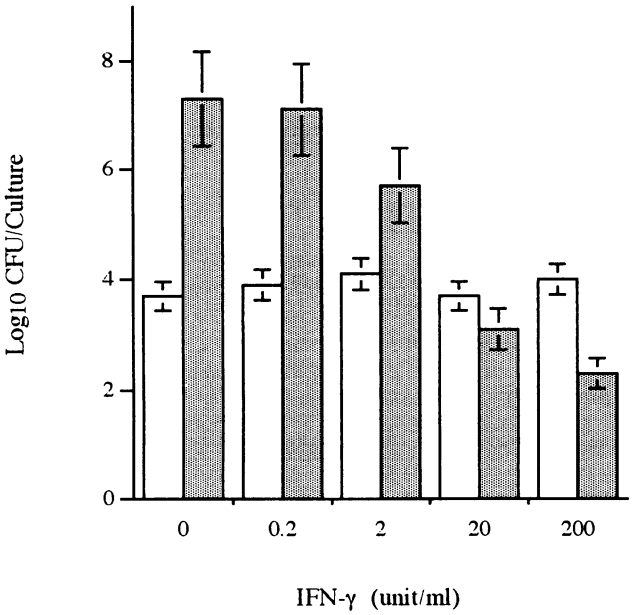


FIG. 1. Dose response of mouse IFN-γ for inhibition of *L. pneumophila* intracellular growth in mouse macrophages. Macrophages were incubated with various concentrations of IFN-γ for 24 h, infected with *L. pneumophila* for 30 min, and postincubated for 0 h (open bars) and 48 h (stippled bars) with medium. The data represent the mean ± standard deviation (SD) of three experiments.

We reported that whereas various inbred mouse strains tested in a number of laboratories are nonpermissive, i.e., resistant to infection by *L. pneumophila*, we found that A/J mice are relatively permissive for infection by this organism (69). This was confirmed by a number of other investigators. We also reported that peritoneal macrophages from A/J mice, unlike macrophages from other mouse strains, are permissive for growth of legionellae in vitro (69). However, activation of the macrophages, either in vitro or in vivo, by immunostimulants such as bacterial lipopolysaccharide and biologic response modifiers or cytokines like interferon converted the cells to nonpermissiveness for in vitro infection by legionellae. Thus, we believe it is of interest that the results of this study show that activation of macrophages from A/J mice by IFN-γ, which can abolish permissiveness of the macrophages for *Legionella* infection, is not completely reversed by metabolic mechanisms which have been reported to be involved in activation of macrophages to resist microbial infectious agents.

MATERIALS AND METHODS

Reagents. All chemicals and media were obtained from Sigma (St. Louis, Mo.) unless otherwise noted. Human Fe-Tf corresponded to the low-endotoxin lot. Ferric citrate (FeCit) was prepared by mixing in a 1:1 ratio trisodium citrate with ferric chloride and then adjusting the pH to neutrality with 1 M NaOH. ^{59}FeCit was prepared similarly by using ^{59}Fe-chloride which was purchased from Amersham (Arlington Heights, Ill.). Ferric nitrilotriacetate (FeNTA) was prepared in the same manner by mixing disodium nitrilotriacetate with ferric chloride as previously described (8). IFN-γ was purchased from Genzyme (Cambridge, Mass.).

Mice. Inbred female A/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) at approximately 6 weeks of age. They were kept in groups of 8 to 10 and fed commercial mouse chow and water ad libitum.

Bacteria. A virulent strain of *L. pneumophila*, serogroup 1, was obtained initially from a patient with legionellosis at Tampa General Hospital. The bacteria were maintained frozen at −70°C and, prior to use, grown on buffered charcoal yeast extract agar (Difco Laboratories, Detroit, Mich.) for 48 h.

Macrophages. Elicited macrophages, obtained by intraperitoneal injection of 3 ml of thioglycolate medium (Difco) 4 days prior to harvesting, were collected by peritoneal lavage with 5 ml of phosphate-buffered saline (PBS) with 2% fetal calf serum (HyClone, Logan, Utah). Cells were washed in PBS and resuspended in RPMI 1640 plus 10% fetal calf serum. Macrophage survival was determined by trypan blue exclusion. In all cases, viability was greater than 95%.

^{59}Fe uptake. Macrophages (10^5 cells per well) were made to adhere to 96-well tissue culture plates for 2 h at 37°C to remove the nonadherent cells. After the monolayer was washed, cells were preincubated with IFN-γ for 24 h. Thereafter, they were incubated with 100 μM ^{59}FeCit for 1 h and washed five times with cold Hanks' balanced salt solution. The cells were then harvested with 0.1% (wt/vol) saponin and counted in a Packard Cobra Gamma Counter (Sterling, Va.).

***L. pneumophila* CFU assay.** Macrophages (10^5 cells per well) were made to adhere to 96-well tissue culture plates (Costar, Cambridge, Mass.) for 2 h at 37°C to remove the nonadherent cells. After washing of the monolayer, cells were preincubated with IFN-γ for 24 h. Macrophages were then infected with *L. pneumophila* (10^6 bacteria per well) for 30 min at 37°C, and the nonphagocytosed *L. pneumophila* cells were removed by washing three times with Hanks' balanced salt solution. The cells were incubated for 48 h with fresh medium containing various compounds as indicated in every figure. At the end of the incubation period, aliquots of 50 μl from the supernatant were assayed to determine the concentration of nitrite (see below), and the monolayers were then washed and lysed with 100 μl of 0.1% (wt/vol) saponin per well. The resulting lysates were diluted in Hanks' balanced salt solution and plated on buffered charcoal yeast extract agar to determine the number of CFU as previously described (18).

Nitrite determination. The nitrite concentration was measured colorimetrically after reaction with the Griess reagent (23). Briefly, 50-μl aliquots were mixed with equal volumes of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H_3PO_4), incubated at room temperature for 10 min, and quantified by measuring the optical density in an enzyme-linked immunosorbent assay reader (Molecular Devices, Menlo Park, Calif.) at 550 nm. NO_2^- concentration was determined by using $NaNO_2$ dissolved in RPMI (10% fetal calf serum) as a standard.

H_2O_2 assay. Production of hydrogen peroxide (H_2O_2) by macrophages was measured as previously described (66) by the colorimetric method by a standard procedure based on H_2O_2 conversion of phenol red to a product whose A_{610} is read. Mezerein was used as a nonspecific stimulator. H_2O_2 concentration was determined by using standards of known concentrations of H_2O_2.

Analysis of results. The Student's *t* test or analysis of variance was used to determine the statistical probability.

RESULTS

Inhibition of intracellular proliferation of *L. pneumophilab* in IFN-γ macrophages. Macrophages (10^5) were treated with

IFN-γ at varying concentrations for 24 h. Monolayers were then exposed to 10^6 *L. pneumophila* cells (bacteria/macrophage ratio of 10:1) for 30 min, and nonphagocytosed bacteria were removed by washing. The total number of viable bacteria was measured in the culture at 0 and 48 h postinfection (Fig. 1). The initial uptake of *L. pneumophila* did not show significant differences between IFN-γ-activated and nonactivated macrophages. At 48 h postinfection, IFN-γ inhibited in a dose-dependent manner the *L. pneumophila* proliferation, and the minimal effective dose was 2 U/ml. This restriction was specific since antibodies against IFN-γ blocked this effect (data not shown).

Effect of oxygen scavengers on the IFN-γ-induced inhibition of *L. pneumophila* growth. To test the role of the oxygen-dependent mechanism in the antilegionella activity of IFN-γ, various inhibitors or scavengers were added to macrophage cultures. Even though IFN-γ-activated macrophages produced 2.2-fold as much H_2O_2 as untreated cells in response to the nonspecific stimulator mezerein (Fig. 2a), neither catalase (which converts hydrogen peroxide to water and oxygen), superoxide dismutase (which converts superoxide to hydrogen peroxide), nor mannitol and thiourea (scavengers of hydroxyl radicals) reversed the inhibitory effect induced by IFN-γ (Fig. 2b), suggesting a lack of involvement of ROI.

Effect of RNI on the IFN-γ-induced inhibition of *L. pneumophila* growth. Because reactive nitric oxide has been recently shown to be involved in the antimicrobial activity of IFN-γ-activated macrophages in a number of intracellular pathogens (1, 2, 4, 14, 16, 17, 21–23, 34, 41), two inhibitors of L-Arg-dependent synthesis of nitrogen derivatives, N^G-monomethyl-L-arginine (N^GMMA) and L-aminoguanidine (AG), were tested for their capacity to block intracellular growth restriction. When infected macrophages treated with IFN-γ were exposed to these inhibitors, no reversion of growth restriction was observed, even though NO_2^- production was completely inhibited (Fig. 3). These two inhibitors had no direct effect on *Legionella* growth in broth cultures. Furthermore, when non-interferon-activated macrophages were incubated with NMMA or AG or in arginine-free medium, there were no differences in endogenous arginine content between the non-inactivated and interferon-activated macrophages, with or without infection with *Legionella* cells (nonpublished data). However, incubating infected macrophages in L-Arg-free medium blocked completely the production of nitric oxide by IFN-γ-activated macrophages, whereas the antilegionella activity remained intact (Fig. 3).

Effect of iron(III) on the IFN-γ-induced inhibition of *L. pneumophila* growth. In order to test whether the intracellular proliferation of *L. pneumophila* in macrophages is iron(III) dependent, cells were incubated with the siderophore desferrioxamine. This iron(III) chelator inhibited at 50 μM the replication of *L. pneumophila* in macrophages (Fig. 4). FeCit and FeNTA, as well as Fe-Tf, reversed such inhibition (Fig. 4), implying that the intracellular proliferation of *L. pneumophila* in macrophages is iron(III) dependent.

Since IFN-γ downregulates the expression of TfR in mouse macrophages (24), macrophages were pretreated with this cytokine for 24 h and then exposed to 100 μM ^{59}FeCit for 1 h. IFN-γ-activated macrophages in three separate experiments showed incorporation of an average of 28% more reactivity (6,350 ± 750 cpm/10^5 cells) compared with nonactivated cells (4,590 ± 330 cpm/10^5 cells). These differences were statistically significant ($P < 0.01$).

Because ferric salts and Fe-Tf completely restored the growth of *L. pneumophila* in IFN-γ-activated human monocytes (7, 8), FeCit and FeNTA, as well as Fe-Tf, were added to

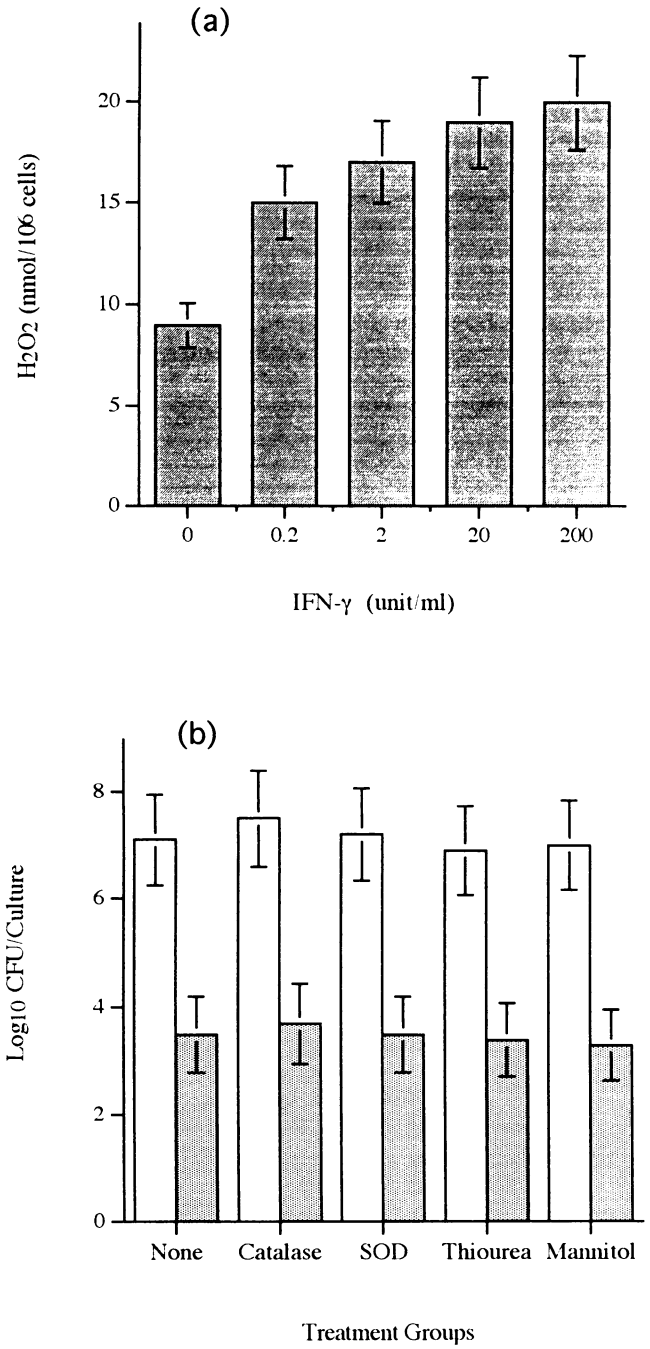
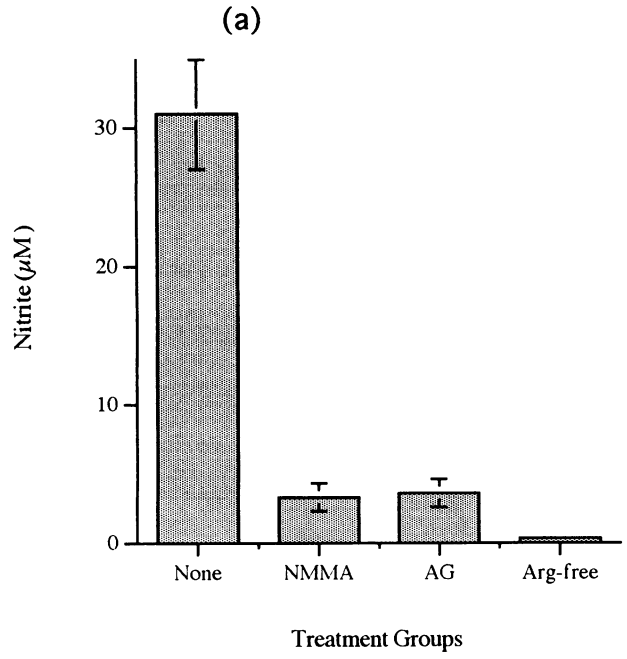


FIG. 2. Role of ROI in the *L. pneumophila* growth restriction of IFN-γ-activated macrophages. (a) Macrophages (10^5 cells per well) were incubated with various concentrations of IFN-γ for 24 h. H_2O_2 production was assayed by horseradish peroxidase-dependent oxidation of phenol red after stimulation with 100 μg of mezerein per ml. (b) Macrophages (10^5 cells per well) were treated with medium (open bars) or IFN-γ (20 U/ml) for 24 h (stippled bars), infected with *L. pneumophila* (10^6 bacteria per well) for 30 min, and postincubated with medium, catalase (2,500 U/ml), superoxide dismutase (SOD; 1,000 U/ml), thiourea (10 mM), and mannitol (10 mM) for 48 h. The data represent the mean ± SD of three experiments.



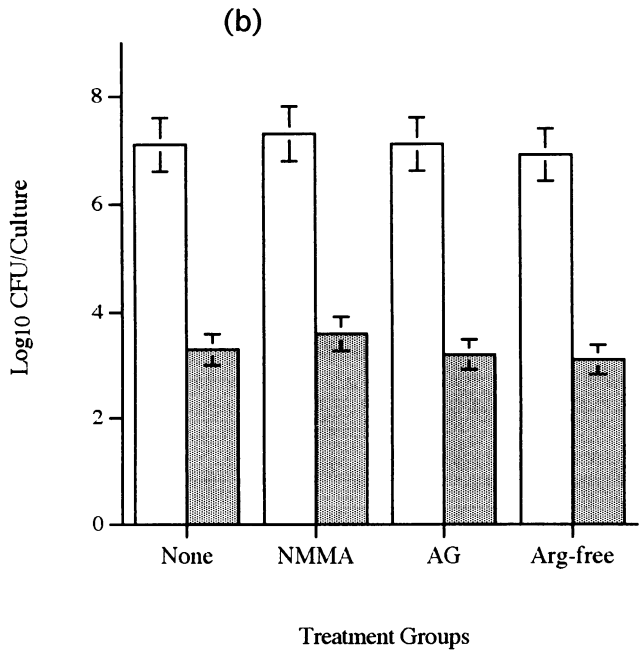


FIG. 3. Role of RNI in the *L. pneumophila* growth restriction of IFN-γ-activated macrophages. Macrophages (10^5 cells per well) were treated with medium (open bars) or IFN-γ (20 U/ml) for 24 h (stippled bars), infected with *L. pneumophila* (10^6 bacteria per well) for 30 min, and postincubated (stippled bars) with medium, NMMA (2 mM), AG (1 mM), and L-arginine (Arg)-free medium. Nitrite concentration was assayed by the Griess reagent (a), and the number of CFU (b) was determined after 48 h postinfection. The data represent the mean ± SD of three experiments.

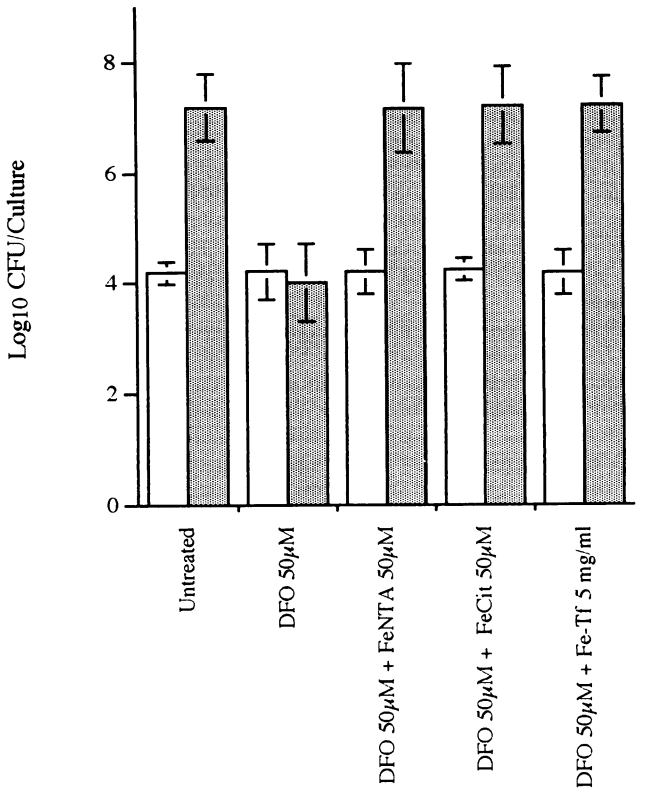


FIG. 4. The intracellular proliferation of *L. pneumophila* in macrophages is iron dependent. Macrophages (10^5 cells per well) were incubated with desferrioxamine (DFO) and various ferric solutions as indicated in the figure for 24 h, infected with *L. pneumophila* (10^6 bacteria per well) for 30 min, and postincubated with the same solutions of iron for 0 (open bars) and 48 (stippled bars) h. The data represent the mean ± SD of three experiments.

macrophages treated with 20 U of IFN-γ per ml. FeCit restored only partially the IFN-γ-induced growth restriction of *L. pneumophila* (Fig. 5a), the reversion being significant ($P < 0.001$) and dose dependent from 25 μM (0.8 log out of 3.7 log), reaching a maximum at 100 μM (1.5 log out of 3.7 log). Similarly, FeNTA partially blocked the inhibition induced by IFN-γ (1.6 log out of 3.7 log). However, Fe-Tf did not show any effect. At higher concentrations of IFN-γ (200 U/ml), neither ferric salts nor Fe-Tf induced significant reversion (Fig. 5b).

Effect of L-Trp on the IFN-γ-induced inhibition of *L. pneumophila* growth. To examine whether the intracellular proliferation of *L. pneumophila* in macrophages is L-Trp dependent, macrophage cultures were incubated with L-indole-propionic acid (IPA), a compound which inhibits the synthesis of L-Trp (37). As is shown in Fig. 6, IPA at 25 μM significantly inhibited the replication of *L. pneumophila* in mouse macrophages ($P < 0.003$), and the effect was restored by adding an excess of L-Trp. This suppression was not due to an effect on the viability of macrophages as determined by the MTT reduction assay (data not shown).

However, supplementation of the culture medium with an excess of exogenous L-Trp did not protect against the inhibi-

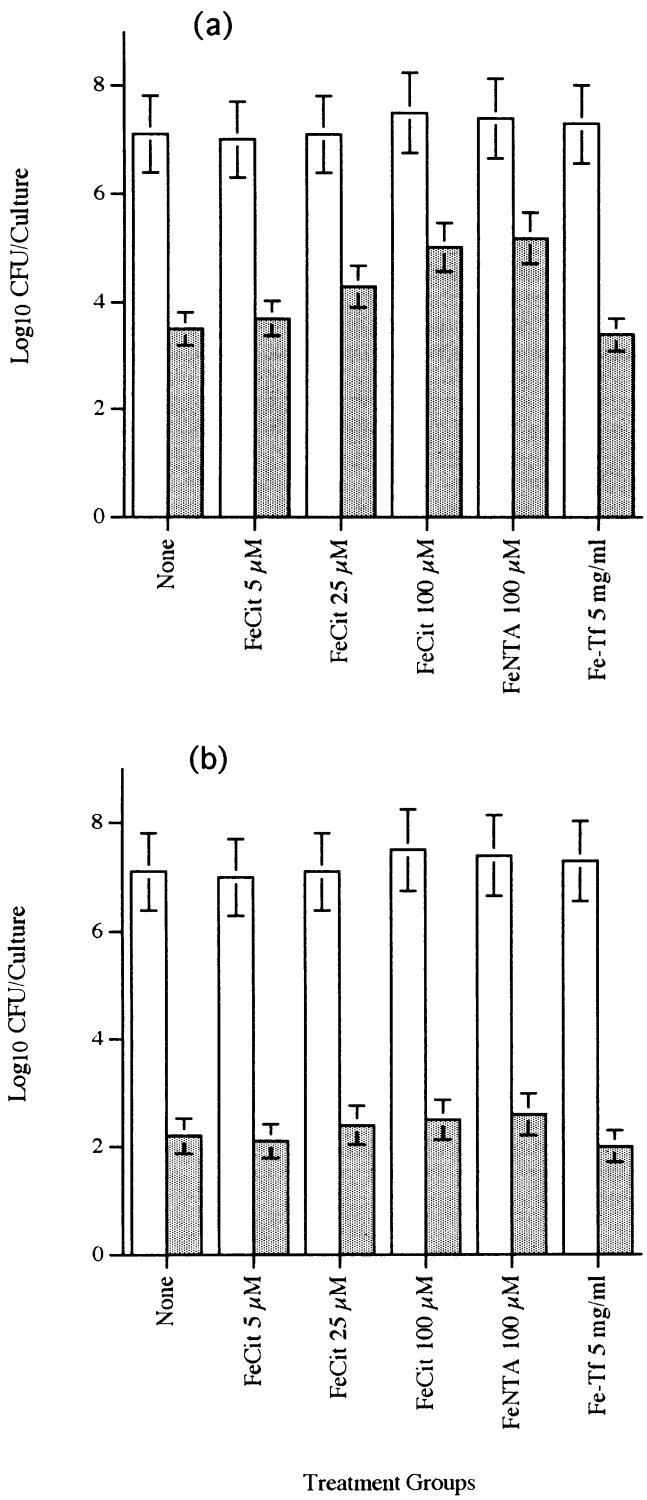


FIG. 5. Role of iron in the *L. pneumophila* growth restriction of IFN-γ-activated macrophages. Macrophages (10^5 cells per well) were treated for 24 h with medium (open bars) and two concentrations of IFN-γ (stippled bars) (20 U/ml [a] and 200 U/ml [b]), infected with *L. pneumophila* (10^6 bacteria per well) for 30 min, and postincubated with medium and various iron solutions as indicated in the figure for 48 h. The data represent the mean ± SD of three experiments.

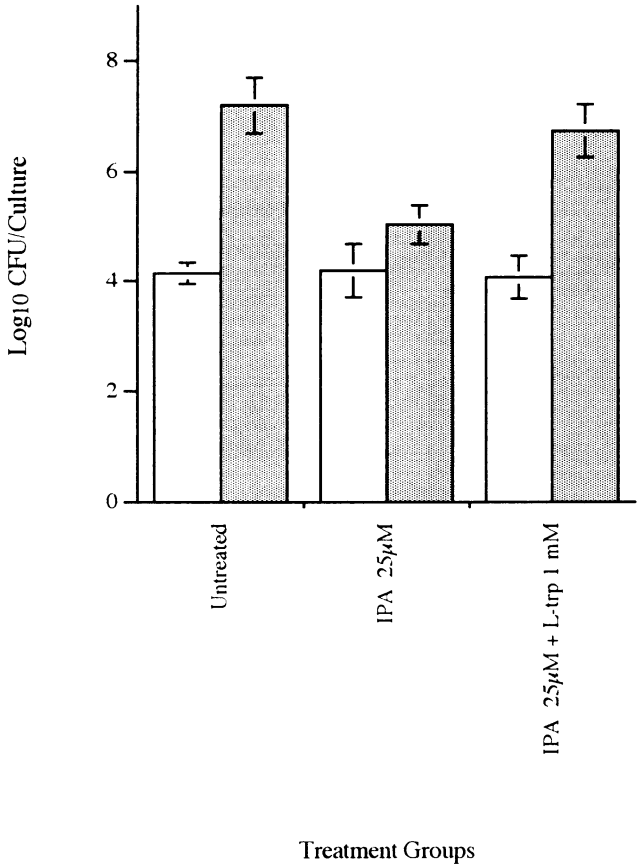


FIG. 6. The intracellular proliferation of *L. pneumophila* in macrophages is L-tryptophan (L-Trp) dependent. Macrophages (10^5 cells per well) were incubated with IPA and L-Trp as indicated in the figure for 24 h, infected with *L. pneumophila* (10^6 bacteria per well) for 30 min, and postincubated with the same solutions for 0 (open bars) and 48 (stippled bars) h. The data represent the mean ± SD of three experiments.

tory effect induced by IFN-γ (Fig. 7), although adding FeCit and L-Trp together to the culture medium produced a modest synergistic effect (2.2 log out of 3.6 log). It is noteworthy that adding FeCit plus L-Trp together to nonactivated macrophages did not further increase the intracellular proliferation of *L. pneumophila* (Fig. 7).

DISCUSSION

Our study shows that IFN-γ-activated A/J macrophages inhibit the intracellular proliferation of *L. pneumophila* by ROI- and RNI-independent mechanisms, and just partially by nutritionally dependent mechanisms and by other mechanisms that still remain to be elucidated. There are several potential mechanisms whereby the inhibition of replication of *L. pneumophila* in the macrophages may have been effected. We first investigated the relevance of a toxic oxygen mechanism. Although IFN-γ-activated macrophages produced higher levels of hydrogen peroxide in response to stimulation with mezerein compared with nonactivated cells, high concentrations of several ROI scavengers (i.e., catalase, superoxide dismutase, mannitol, and thiourea) failed to overcome this effect. These results suggest that ROI do not appear to constitute the

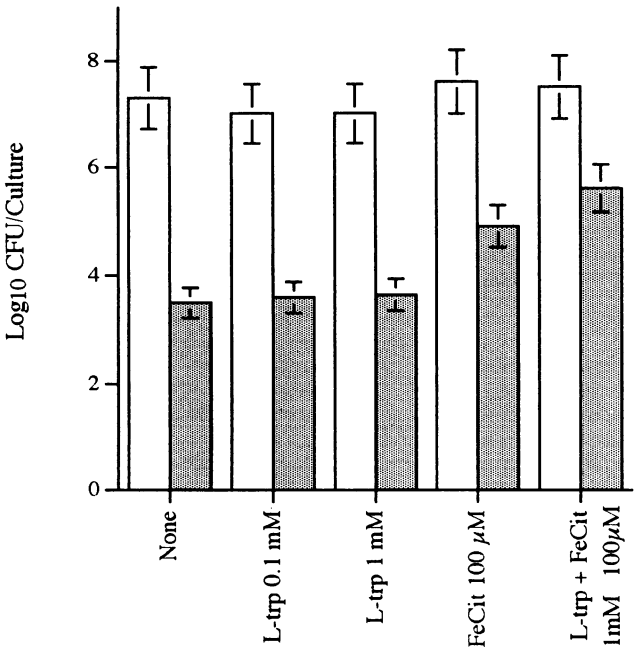


FIG. 7. Role of tryptophan (L-Trp) in the *L. pneumophila* growth restriction of IFN-γ-activated macrophages. Macrophages (10^5 cells per well) were treated for 24 h with medium (open bars) and 20 U of IFN-γ per ml (stippled bars), infected with *L. pneumophila* (10^6 bacteria per well) for 30 min, and postincubated with medium and various tryptophan solutions as indicated in the figure for 48 h. The data represent the mean ± SD of three experiments.

mechanism of inhibition of *L. pneumophila* growth in IFN-γ-activated macrophages, which is consistent with observations in alveolar exudate macrophages for which resistance to *L. pneumophila* was found to be not dependent on augmented respiratory burst (55, 64).

The second potential mechanism investigated was the L-Arg-dependent production of nitric oxide and subsequent conversion to nitrite and nitrate. Drapier and Hibbs (15) found that activated macrophages show L-Arg-dependent metabolic inhibition and speculated that such changes might affect the replication of intracellular pathogens. Indeed, a number of reports have later shown that activated macrophages inhibit the intracellular proliferation of fungi (21, 22), parasites (1, 16, 23, 34, 39, 41), and bacteria (4, 14, 17, 45) by an L-Arg-dependent mechanism. In our system, IFN-γ-activated macrophages exhibited increased production of NO_2^-, which was markedly inhibited by the L-Arg analogs N^GMMA and AG. Conversely, neither N^GMMA nor AG was able to overcome the *L. pneumophila* growth inhibition caused by IFN-γ. Furthermore, IFN-γ-activated macrophages incubated in L-Arg-free medium could strongly restrict the intracellular proliferation of *L. pneumophila*, whereas their ability to synthesize NO_2^- was completely blocked. In contrast to our findings, Summersgill et al. (57) reported that killing of *L. pneumophila* in IFN-γ-activated RAW 264.7 cells, a murine macrophage cell line, is mediated by nitric oxide. This discrepancy may be due to differences between both host cells. Actually, we also found that sodium nitroprusside (1 to 10 mM), a generator of RNI in aerobic conditions, impairs the extracellular proliferation of *L. pneumophila* in broth (data not shown). However, electron micrographs of infected thioglycolate-elicited A/J macrophages revealed that the bacterium was contained within a membrane-bound cytoplasmic vesicle which was studded with ribosomes (66). Thus, we believe that *L. pneumophila* is located in an intracellular compartment which is somehow protected from the deleterious effect of RNI. However, this possibility remains to be demonstrated. In agreement with our results, Saito et al. (50) found that the L-Arg-dependent mechanism of activated murine thioglycolate-elicited macrophages was ineffective in restricting the intracellular growth of the following bacteria: *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*. Bermudez (4) also found that neither competitive inhibition with N^GMMA nor depletion of L-Arg by arginase had any effect on the inhibition of intracellular growth of *Mycobacterium avium* by IFN-γ-activated murine peritoneal macrophages. Bermudez conversely found that the antimicrobial activity of the same host cells infected with *Listeria monocytogenes* was significantly inhibited by the addition of N^GMMA or arginase. Appelberg and Orme (3) also found that the IFN-γ-induced bacteriostatic effect of murine macrophages infected with *Mycobacterium avium* was mediated by an RNI-independent mechanism. Therefore, our data support the results of our recent study in which nitric oxide appeared to show an immunoregulatory role for cytokine production rather than for antilegionella activity in IFN-γ-activated macrophages (67).

Several laboratories have reported that the extracellular and intracellular growth of *L. pneumophila* are significantly inhibited by restricting iron availability by adding either different iron chelators, lactoferrin, or serum transferrin (7–9, 19, 47). Particularly, IFN-γ-activated human monocytes restrained the intracellular proliferation of *L. pneumophila* mainly by down-regulating ferritin and TfR expression and, as a consequence, by restricting the intracellular availability of iron (7–9). Because IFN-γ-activated murine thioglycolate-elicited macrophages also exhibit downshift in TfR (24), we proceeded to study the involvement of iron. First, we found that the intracellular proliferation of *L. pneumophila* in thioglycolate-elicited macrophages was inhibited by desferrioxamine, a siderophore which directly chelates and removes iron(III) from the cytosol of macrophages (30, 35, 49, 62). Such inhibition was completely restored by adding Fe-Tf or ferric salts, indicating that the intracellular proliferation of this bacterium in these cells is iron dependent. Second, the uptake of ^{59}Fe(III) by IFN-γ-treated macrophages was reduced by 28% compared with untreated macrophages, which might be explained by the downshift in TfR expression. Third, the growth restriction induced by activation with IFN-γ was only partially reversed with FeCit and FeNTA, but not with Fe-Tf. It should be noted that even higher concentrations of FeCit and FeNTA, such as 200 or 500 μM, which approached the toxic level for mouse macrophages, only slightly reversed activation of the macrophages by interferon. It seems possible that the lack of effective Fe-Tf could be due to the downshift in TfR expression that limits the incorporation of iron(III) into the cells. Besides, the rate of iron uptake by macrophages is 50 to 300 times greater from iron salts than from Fe-Tf (36). That is, the exact mechanism of incorporation of iron chelators such as FeCit or FeNTA is not known, but it is independent of the TfR system and seems to be mediated either by fluid endocytosis or by an unknown Tf-independent pathway (36, 48, 56, 62).

Using the Prussian blue stain assay, we found that IFN-γ-activated macrophages treated with ferric salts for 24 h exhibited high levels of Fe^{3+} in the cytoplasm (data not shown). Thus, whereas iron(III)-treated activated macrophages showed no iron(III) limitation, the *L. pneumophila* growth restriction induced by IFN-γ was only partially restored. Furthermore, at a higher concentration of IFN-γ (200 U/ml),

neither ferric salts nor Fe-Tf showed significant reversion, suggesting that additional mechanisms become more important, which might explain why in some reports iron does not overcome the growth inhibition induced by IFN-γ (29, 42, 45).

L. pneumophila is also a nutritionally fastidious microorganism that requires amino acids, including L-Trp, as a source of energy and carbon (20, 58). Mandelbaum-Shavit et al. (37) reported that the replication of *L. pneumophila* cultivated extracellularly in a chemically defined medium and intracellularly in human monocytes is inhibited by IPA, a compound with structural resemblance to L-Trp and the precursors of its biosynthetic pathway. We also found that IPA is an efficient L-Trp-reversible growth inhibitor of *L. pneumophila* in murine macrophages, implying that the intracellular proliferation of this pathogen is L-Trp dependent. On the other hand, it is known that a variety of human and mouse cells, including monocytes and macrophages, are activated by IFN-γ to initiate the catabolism of L-Trp along the kynurenine pathway (6, 12, 13, 61, 70–72). Particularly, the activity of indoleamine 2,3-dioxygenase, the interferon-induced protein that metabolizes L-Trp, is sevenfold as high in IFN-γ-treated macrophages as in monocytes (13). This mechanism of inhibition was first demonstrated by Pfefferkorn (46) in human fibroblasts infected with *Toxoplasma gondii* and later confirmed in other intracellular microorganisms (10, 11, 38, 43, 51, 53, 54). We found that adding an excess of L-Trp to IFN-γ-activated macrophages did not affect the intracellular growth restriction of *L. pneumophila*, but a small synergism was observed when the culture medium was supplemented with both L-Trp and FeCit. The involvement of various mechanisms might explain why in some instances L-Trp partially blocks the inhibition (11, 38, 43, 51) and in others shows no reversion (40, 52, 59, 63, 65). Therefore, we believe that several antimicrobial mechanisms are acting in conjunction, some of which become effective only in certain situations depending on the intracellular pathogen, the type of host cell, and/or the class and degree of cell activation.

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