INFECTION AND IMMUNITY, Sept. 1992, p. 3504–3508
0019-9567/92/093504-05$02.00/0
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Vol. 60, No. 9

L-Arginine-Dependent Killing of Intracellular *Ehrlichia risticii* by Macrophages Treated with Gamma Interferon

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Thioglycolate-induced murine peritoneal macrophages infected with *Ehrlichia risticii* and treated in vitro with gamma interferon (IFN-γ) developed antiehrlichial activity that eliminated the intracellular bacteria. This antiehrlichial activity was suppressed by N^G-monomethyl-L-arginine, a competitive inhibitor of nitric oxide synthesis from L-arginine, but not by L-tryptophan. Increased levels of nitrite, an oxidative product of nitric oxide, were measured in cultures of infected macrophages treated with IFN-γ. Sodium nitroprusside, which spontaneously releases nitric oxide, also showed the antiehrlichial activity. The antiehrlichial activity by reactive nitrogen intermediates was not mediated by elevation of the cellular concentration of cyclic GMP since the addition of 8-bromo-cyclic GMP itself had no influence on ehrlichial infection of macrophages. Addition of the intracellular iron chelator deferoxamine also inhibited *E. risticii* infection in vitro. These results suggest that intracellular *E. risticii* survival is iron dependent and that production of reactive nitrogen intermediates triggers iron loss from critical target enzymes of *E. risticii*, leading to lethal metabolic inhibition. However, addition of excess $FeSO_4$, ferric citrate, or iron-saturated transferrin did not counteract the antiehrlichial effect induced by IFN-γ.

Ehrlichia risticii, which causes Potomac horse fever, is an obligate intracellular bacterium in the family *Rickettsiaceae* (21). *E. risticii* organisms proliferate in phagosomes of macrophages (21). Gamma interferon (IFN-γ) treatment, but not tumor necrosis factor treatment, induces intracellular killing of *E. risticii* in murine peritoneal macrophages in vitro (20). The ehrlichiacidal mechanisms induced by IFN-γ are, however, unknown. Recent studies have shown that IFN-γ inhibits the growth of various classes of microorganisms in both macrophages and other types of cells by several different mechanisms. *Chlamydia psittaci* is inhibited in human uroepithelial cells (3) and macrophages by treatment with IFN-γ because of depletion of an essential amino acid, tryptophan, caused by oxygenative ring cleavage by indoleamine 2,3-dioxygenase activated by IFN-γ (4). Hence, the addition of L-tryptophan overrides the chlamydiacidal activity induced by IFN-γ (4). *C. trachomatis* killing in murine fibroblasts by IFN-γ is, however, not inhibited by the addition of L-tryptophan (6). Likewise, *Rickettsia prowazekii* killing in murine and human fibroblasts after IFN-γ treatment is not inhibited by the addition of tryptophan (23). The macrophage respiratory burst is not considered to be required for the killing of *R. prowazekii* in the macrophage-like cells by IFN-γ treatment since rickettsiae are killed in the variant cells that lack the ability to perform a respiratory burst as well as in the parent J774.16 cells (24).

Recently, an intracellular protozoan parasite, *Leishmania major* (12), and a facultative intracellular bacterium, *Mycobacterium bovis* (8), were shown to be killed in mouse macrophages by an L-arginine (L-Arg)-dependent mechanism. IFN-γ-activated macrophages synthesize reactive nitrogen intermediates (NO· and NO_2^-, etc.) from L-Arg which cause intracellular iron loss and inhibits the function of several critical iron-containing enzymes in the target organism. The enzymes NADH-ubiquinone oxidoreductase and succinate-ubiquinone oxidoreductase of the mitochondrial electron transport chain (9), aconitase of the citric acid cycle (7), and ribonucleotide reductase involved in DNA synthesis (18) have been identified as potential targets for the action of reactive nitrogen intermediates since they all contain catalytically active Fe-S centers (7, 9, 22). Hence, depletion of L-Arg by arginase or addition of a competitive inhibitor of nitric oxide synthase, N^G-monomethyl-L-arginine (N^GMMLA), suppresses the synthesis of these toxic molecules by IFN-γ, allowing intracellular parasites to multiply in the presence of IFN-γ. Cancer cells (14, 15) and larvae of the metazoan *Schistosoma mansoni* (16), which are extracellular, are also killed by murine macrophages activated by IFN-γ by the L-Arg-dependent mechanism.

Another known IFN-γ-induced killing mechanism of intracellular microorganisms is the limitation of iron availability by down-regulation of transferrin receptors on the surface of macrophages (2, 19, 25). Hence, the addition of iron-saturated transferrin suppressed the IFN-γ-induced antimicrobial activity of macrophages (2, 19). In this report, we have focused our study on the antiehrlichial mechanism induced in murine macrophages by IFN-γ.

MATERIALS AND METHODS

Mice. Female Sprague-Dawley mice, 6 to 7 weeks old, were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.). All mice were fed antibiotic-free commercial laboratory chow and water ad libitum.

Mouse peritoneal macrophages. Mice were injected intraperitoneally with 2 ml of 5% thioglycolate broth (Difco Laboratories, Detroit, Mich.). Five days later, the mice were sacrificed by cervical dislocation and peritoneal cells were aspirated after intraperitoneal injection of 10 ml of sterile Dulbecco's phosphate-buffered saline (PBS; 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4). The collected cells were centrifuged at 500 × *g* for 5 min and resuspended in RPMI 1640 medium (GIBCO Laboratories,

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Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum (FBS; GIBCO), 1% 200 mM L-glutamine (L-Glu; GIBCO), and 1% antibiotic-antimycotic mixture (GIBCO) which contains 10^4 U of penicillin per ml, 1 mg of streptomycin per ml, and 25 U of amphotericin B per ml. The cell suspension was placed at a concentration of 0.6×10^6 cells per 0.4 ml in each chamber of a Lab-Tek eight-chamber slide (Nunc, Inc., Naperville, Ill.) and incubated at 37°C in 5% CO_2–95% air for 1 day. Nonadherent cells were then removed by washing vigorously with sterile PBS before infection with *E. risticii* as described previously (20).

E. risticii. *E. risticii* cells were cultured in the P388D1 murine macrophage cell line in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-Glu without antibiotics. When more than 90% of the P388D1 cells were infected as determined by Diff-Quik stain (Baxter Scientific Products, Obetz, Ohio), the infected cells were suspended in the RPMI 1640 medium at 10^6 cells per ml, sonicated at a power setting of 1.5 at 20 kHz for 5 s with an ultrasonic processor (model W-380; Heat System, Farmingdale, N.Y.), and centrifuged at 500 × *g* for 5 min. The supernatant containing cell-free *E. risticii* organisms was used to infect mouse peritoneal macrophages in the experiment which was terminated at 3 days postinfection. The supernatant was diluted to 1:1 with RPMI 1640 medium in the experiment which was terminated at 4 or 5 days postinfection.

Infection of macrophages. Mouse peritoneal macrophages adherent to the eight-chamber slide were inoculated with 0.15 ml of the cell-free *E. risticii* suspension per chamber and incubated at 37°C in a humidified environment of 5% CO_2–95% air for 3 h. A 0.25-ml volume of RPMI 1640 medium supplemented with 10% FBS and 2 mM L-Glu without antibiotics was then added to each chamber, and the incubation was continued for 3 or 5 days. The experimental conditions were set to ascertain that more than 90% of the cells were viable throughout the experiments. For each set of experiments, the time course of infection of the control culture was determined.

Evaluation of N^GMMLA and L-tryptophan on ehrlichiacidal activity of recombinant murine IFN-γ. The peritoneal macrophages on eight-chamber slides received 100 μM N^GMMLA (Calbiochem Corp., La Jolla, Calif.) or 1 mM L-tryptophan (L-Trp; Sigma Chemical Co., St. Louis, Mo.) at 0, 6, or 24 h postinfection and 50 U of recombinant murine IFN-γ (GIBCO Bethesda Research Laboratories, Gaithersburg, Md.) per ml at 3 h postinfection. The stock solution concentrations of N^GMMLA and L-Trp were 1 and 10 mM in PBS, respectively.

Effects of 8-Br-cGMP, sodium nitroprusside, and deferoxamine mesylate on *E. risticii* infection. 8-Bromo-cyclic GMP (8-Br-cGMP; Sigma) at 20 or 200 μM or sodium nitroprusside (Sigma) at 100 μM was added to peritoneal macrophages on the eight-chamber slide at −1 day, 0 h, 1 day, or 2 days postinfection. Deferoxamine mesylate (Sigma) at 15 or 50 μM was added 1 day before infection. The stock solution concentrations of 8-Br-cGMP, sodium nitroprusside, and deferoxamine mesylate were 2 mM in PBS, 10 mM in H_2O, and 1.5 mM in H_2O, respectively.

Effects of ferrous sulfate, ferric citrate, and transferrin on macrophage antiehrlichial activity induced by IFN-γ. The peritoneal macrophages received 50 μM ferrous sulfate (Sigma), 50 μM ferrous sulfate plus 50 μM sodium dithionite (Sigma), 500 μM ferric citrate (Sigma), or 6 mg of holo-form human transferrin (transferrin; Sigma) at 0 h postinfection. IFN-γ (50 U/ml) was added at 3 h postinfection. The stock solution concentrations of $FeSO_4$, sodium dithionite, ferric citrate, and transferrin were 5 mM in water, 5 mM in water, 50 mM in water, and 12 mg/ml in RPMI medium supplemented with 10% FBS and 1% L-Glu, respectively.

TABLE 1. Effect of N^GMMLA and L-tryptophan on macrophage antiehrlichial activities induced with IFN-γ

Reagent(s) and time when added[a]	% Infected cells[b]	Total no. of *E. risticii*/100 macrophages
None (control)	99 ± 1	7,860 ± 1,185
IFN-γ	2 ± 3	10 ± 15
IFN-γ + N^GMMLA (0 h)	85 ± 5	2,850 ± 890
IFN-γ + N^GMMLA (6 h)	75 ± 5	1,570 ± 325
IFN-γ + N^GMMLA (24 h)	14 ± 4	85 ± 50
IFN-γ + L-Trp (0 h)	1 ± 1	5 ± 5
IFN-γ + L-Trp (6 h)	2 ± 2	10 ± 10
IFN-γ + L-Trp (24 h)	2 ± 2	10 ± 10

[a] Recombinant murine IFN-γ (50 U/ml) was added at 3 h postinfection. N^GMMLA (100 μM) or L-Trp (1 mM) was added at 0, 6, or 24 h post-*E. risticii* infection, and infectivity was determined on day 4 postinfection.

[b] Data are percentages of total macrophages expressed as the means ± standard deviations of results from triplicate assays. Representative data from several experiments are shown.

Measurement of nitrite production. The nitrite (NO_2^-) concentration in the culture medium was measured by the Griess reaction (10). After placing 100 μl of test medium in a Linbro 96-well plate (Flow Laboratories, Inc., McLean, Va.), 100 μl of Griess reagent consisting of 1 part 2% sulfanilamide in 2.5% H_3PO_4 and 1 part 0.2% naphthylethylene diamine dihydrochloride in 2.5% H_3PO_4 was added to each well and the plate was incubated for 10 min at room temperature before the A_{580} was read in a V_{max} microplate reader (Molecular Devices Corp., Palo Alto, Calif.).

Evaluation of infectivity. To estimate infectivity, the cells on the eight-chamber slide were centrifuged at 350 × *g* for 5 min in a table-top centrifuge, and the medium and chamber part were removed. The cells attached to the slide were covered with a piece of Kimwipes paper (Kimberly-Clark Corp., Roswell, Ga.) that had been soaked in RPMI 1640 medium and centrifuged again at 250 × *g* for 2 min in a Cytospin 2 Cytocentrifuge (Shandon, Inc., Pittsburgh, Pa.). After centrifugation, the cells were stained with Diff-Quik stain (Baxter Scientific Products) and observed at ×1,000 magnification. The number of *E. risticii* organisms per cell was scored in 100 cells. Since *E. risticii* is a minute coccus and tends to grow in aggregates, it is impossible to accurately count individual organisms, especially when the cells are heavily infected. Thus, infected cells were grouped into categories of 0, 1 to 10, 11 to 50, 51 to 100, and over 100 *E. risticii* organisms per cell. The total numbers of *E. risticii* organisms per 100 macrophages were calculated by multiplying the mean number of *E. risticii* organisms per cell in each category (i.e., 0, 5, 25, 50, and 100 organisms per cell) with the number of macrophages in each category.

RESULTS

Dependence of macrophage ehrlichiacidal activity on L-Arg. As shown by others by using a similar system with *Schistosoma* larva (16) or *L. major* (12), 100 μM N^GMMLA, a competitive inhibitor of nitric oxide synthase, suppressed the antiehrlichial activity of thioglycolate-induced mouse peritoneal macrophages treated with IFN-γ (Table 1). N^GM MLA added at 0 h postinfection suppressed the antiehrlichial activity of IFN-γ added at 3 h postinfection. N^GMMLA

TABLE 2. Production of NO_2^- by IFN-γ or sodium nitroprusside-treated peritoneal macrophages

Treatment[a]	NO_2^- production (nmol/10^6 macrophages/72 h)
Expt 1	
E. risticii infection	6.2 ± 1.0
E. risticii infection + N^GMMLA	7.0 ± 0.3
E. risticii infection + IFN-γ	33.6 ± 2.7
E. risticii infection + IFN-γ + N^GMMLA	8.2 ± 1.5
Expt 2	
E. risticii infection	8.0 ± 1.3
E. risticii infection + sodium nitroprusside	25.0 ± 1.6
Expt 3	
No infection	1.5 ± 0.9
No infection + IFN-γ	12.6 ± 1.2
E. risticii infection	4.2 ± 0.7
E. risticii infection + IFN-γ	30.2 ± 2.5
E. risticii infection + sodium nitroprusside	29.3 ± 3.5

[a] Macrophages were cultured as described in the text, and supernatant fluids were removed at 3 days postinfection for assay by the Griess reaction. IFN-γ (50 U/ml), N^GMMLA (100 μM), and sodium nitroprusside (100 μM) were added at 3, 0, and 0 h postinfection, respectively. Results are the means ± standard deviations for triplicate assays.

TABLE 3. Antiehrlichial activity of sodium nitroprusside, deferoxamine mesylate, and 8-Br-cGMP

Treatment[a]	% Infected cells[b]	Total no. of *E. risticii*/100 macrophages
Expt 1		
Control	60 ± 6	1,390 ± 270
IFN-γ	0	0
Sodium nitroprusside	15 ± 3	235 ± 205
15 μM Deferoxamine mesylate	36 ± 4	545 ± 235
50 μM Deferoxamine mesylate	4 ± 3	20 ± 15
Expt 2		
Control	77 ± 4	2,050 ± 350
20 μM 8-Br-cGMP	78 ± 3	2,530 ± 390
Expt 3		
Control	91 ± 3	3,330 ± 470
200 μM 8-Br-cGMP	97 ± 2	2,445 ± 615

[a] Sodium nitroprusside (100 μM) and 8-Br-cGMP (20 or 200 μM) were added at 0 h postinfection. IFN-γ (50 U/ml) was added at 3 h postinfection. Deferoxamine mesylate (15 μM or 50 μM) was added at 1 day preinfection. Infectivities were scored on day 3 postinfection.

[b] Data are percentage of total macrophages expressed as the mean ± standard deviation of results from triplicate experiments.

added at 6 h postinfection was still effective, indicating that *E. risticii* was still viable at 3 h after IFN-γ treatment. However, when it was added at 24 h postinfection, N^GM MLA was ineffective, suggesting that most *E. risticii* were killed by 21 h of treatment with IFN-γ. N^GMMLA alone had no effect on *E. risticii* proliferation (data not shown).

L-tryptophan at 1 mM, added at 0, 6, or 24 h postinfection, had no effect on the IFN-γ-induced ehrlichiacidal mechanism (Table 1).

Nitrite production by *E. risticii*-infected macrophages treated with IFN-γ. Nitric oxide has been postulated to participate in arginine-dependent tumor cytotoxicity by activated macrophages (14). Nitric oxide has an ultrashort half-life (probably less than 5 s) (5, 13) and is rapidly oxidized to NO_2^- and NO_3^-, which accumulate in macrophage culture supernatants at a ratio of 3:2 (9). Supernatant fluids from mouse peritoneal macrophage were assessed for the presence of nitrite in a standard Griess reaction. Since reactive nitrogen intermediates are small molecules which diffuse through cell membranes, we assume that a steady state was achieved on the day of the measurement (day 3). As shown in Table 2, supernatant fluids from IFN-γ-treated macrophages infected with *E. risticii* contained significant levels of NO_2^- after 3 days of culture. The addition of N^GMMLA at 0 h suppressed NO_2^- production (Table 2).

Effect of sodium nitroprusside and 8-Br-cGMP. Nitroprusside is known to spontaneously release nitric oxide (1). Nitroprusside was as effective as IFN-γ in killing *E. risticii* in macrophages (Table 3) as well as in generating nitrite production (Table 2). Since nitric oxide activates guanyl cyclase that increases intracellular cGMP in macrophages (1) and cGMP is known to act as a second messenger to alter various intracellular activities, the direct effect of 8-Br-cGMP on ehrlichial proliferation in the macrophages was examined. 8-Br-cGMP, an analog of cGMP, is not hydrolyzed by intracellular phosphodiesterases. At 20 or 200 μM, regardless of the time at which it was added, 8-Br-cGMP had no effect on ehrlichial infectivity (Table 3; data resulting from additions at 0 h are shown).

Inhibition of ehrlichial proliferation in macrophages by deferoxamine. *E. risticii* proliferation in mouse peritoneal macrophages was significantly inhibited by an intracellular iron chelator deferoxamine mesylate at 50 μM (Table 3), indicating that iron is essential for ehrlichial growth. Because reactive nitrogen intermediates cause intracellular iron loss, thereby inhibiting various iron-dependent enzymes (7, 9), the effect of excess exogenous irons on antiehrlichial activity of IFN-γ-treated macrophages was examined. The addition of 50 μM $FeSO_4$, 50 μM $FeSO_4$ plus sodium dithionite, 500 μM ferric citrate (17), or 6 mg of iron-saturated transferrin per ml to cultures of IFN-γ-treated mouse peritoneal macrophages did not override the antiehrlichial effects induced by IFN-γ (data not shown).

DISCUSSION

The results of this study demonstrated that *E. risticii* is killed in IFN-γ-activated mouse peritoneal macrophages by an L-Arg-dependent mechanism. Both NO_2^- production and ehrlichial killing were substantially reduced when cells were treated with IFN-γ in the presence of a structural analog, N^GMMLA, suggesting that ehrlichial killing relates to the production of nitrogen-containing effector molecules. The inhibition of *E. risticii* infection by nitroprusside, a spontaneous nitric oxide generator, supports this interpretation. Although it has not been demonstrated whether or not *E. risticii* has enzymes which have Fe-S-reactive centers, inhibition of *E. risticii* infection by deferoxamine mesylate and the ability of the microorganism to produce ATP from L-glutamine (26) suggest that it has such an enzyme.

Our data on nitrite synthesis and release by activated macrophages indicate that secretion of NO_2^- at levels sufficient to affect the viability of intracellular *E. risticii* organisms after stimulation with IFN-γ alone does not occur in uninfected macrophages. As shown in the study using *L. major* (11), it is possible that an intracellular bacterium such as *E. risticii* may also function as a cosignal in the induction

of inorganic nitric oxide synthesis from L-Arg by activated macrophages. The generation of nitric oxide by activated macrophages also induces the heme-dependent activation of guanylate cyclase, with a subsequent increase of the secondary messenger, cGMP (1). This is, however, not the antiehrlichial mechanism induced by nitric oxide, since 8-Br-cGMP alone had no effect on ehrlichial infectivity.

IFN-γ induces iron loss in activated macrophages by at least two mechanisms, namely, reduced iron uptake by down-regulation of transferrin receptor (2, 19, 25) and removal of iron from iron-dependent enzymes by nitric oxide generation (7, 9, 22). None of the studies examined both mechanisms of iron depletion in the same system. It is not clear whether the organism is sensitive to only one mechanism or requires both mechanisms for inhibition or whether only one mechanism is in operation, since suppression of either of the inhibitory mechanisms alone allows normal growth of the particular microorganisms studied. The study with an intracellular iron chelator, deferoxamine, showed that *E. risticii* infection and proliferation are dependent on intracellular iron. However, in contrast to findings with an extracellular organism, *S. mansoni* (16), exogenous iron did not counteract macrophage antiehrlichial activity induced by IFN-γ. In other studies, the multiplication of *Histoplasma capsulatum* (19) and *Legionella pneumophila* (2) in phagosomes was inhibited by IFN-γ. The antimicrobial effect was overridden by the addition of iron-saturated transferrin. Whether these intracellular microorganisms are also susceptible to L-Arg-dependent iron depletion was, however, not reported. In our study, the antiehrlichial activity induced by IFN-γ was not inhibited by the addition of iron-saturated transferrin. Thus, in contrast to reports on *S. mansoni* (16), *Histoplasma* spp. (19), and *L. pneumophila* (2), this study suggests that exogenous ferrous or ferric ions or irons bound to transferrin are less effectively or not at all incorporated into intracellular *E. risticii* in the macrophage, which suggests that *E. risticii* organisms may have an iron acquisition mechanism for their survival that is different from that of the microorganisms previously reported. Another possibility may be that ehrlichial DNA is directly damaged by nitric oxide, as shown by Wink et al. (27).

ACKNOWLEDGMENT

This research was supported by grant R01 AI300010 from the National Institutes of Health.

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