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Difference in *Legionella pneumophila* Growth Permissiveness between J774.1 Murine Macrophage-like JA-4 Cells and Lipopolysaccharide (LPS)-Resistant Mutant Cells, LPS1916, after Stimulation with LPS

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To elucidate the role of the oxidative burst in macrophage resistance to *Legionella* infection, we examined a murine macrophage-like cell line, J774.1, for permissiveness to *Legionella* growth, using a mutant that has a selective defect in the oxidative burst after lipopolysaccharide (LPS) stimulation. *Legionella pneumophila* serogroup 1 was infected into J774.1 monolayers, and then the extent of bacterial growth was estimated by a CFU assay. Both the parental cell line, JA-4, and the LPS-resistant mutant, LPS1916, were permissive for *Legionella* growth but became nonpermissive after pretreatment with gamma interferon. However, pretreatment of LPS1916 cells with LPS failed to inhibit bacterial growth, although LPS-treated JA-4 cells exhibited inhibited multiplication of the bacteria. The bacterial growth inhibition in JA-4 and mutant LPS1916 cells was correlated with the extent of the oxidative burst in the cells, as judged by cytochrome *c* reduction but not nitrite production. Neither transferrin receptor expression nor the iron content in JA-4 and LPS1916 cells, with or without LPS treatment, was correlated with suppression of *Legionella* growth. These results suggest that the restriction of *Legionella* growth in J774.1 cells is due to a bactericidal effect of the oxidative burst rather than reduction of the iron supply to the intracellular bacteria and that the effectors are reactive oxygen intermediates and not reactive nitrogen intermediates.

Legionella pneumophila is the causative agent of the human pneumonia called Legionnaires' disease. The bacteria can proliferate in monocytes/macrophages from humans and guinea pigs. In mice, however, only thioglycolate-elicited peritoneal (5, 17) and lung (5) macrophages from the A/J strain of inbred mice were reported to be permissive. Macrophages can be activated by many factors, especially gamma interferon (IFN-γ) and lipopolysaccharide (LPS), and the activated macrophages are antimicrobial to intracellular growing parasites. The number of transferrin receptors on the surface of human mononuclear phagocytes activated with IFN-γ was reported to decrease, and the iron supply to intracellular bacteria was reported to be inhibited (2, 3). However, the precise mechanisms by which host mononuclear phagocytes restrict bacterial growth after LPS stimulation remain to be determined. We previously reported that both JA-4, a subline of J774.1, and an LPS-resistant mutant line, LPS1916, can produce comparable amounts of interleukin-1, tumor necrosis factor, and arachidonic acid after stimulation with LPS but that only JA-4 cells can produce O_2^- and H_2O_2 upon LPS stimulation (1). *L. pneumophila* is susceptible to oxygen metabolites generated by both the myeloperoxidase-H_2O_2-halide (8, 11, 12) and xanthine oxidase cell-free (11, 12) systems. Therefore, we determined whether the O_2-generating activity of J774.1 cells is involved in the resistance of macrophages to *Legionella* infection.

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MATERIALS AND METHODS

Materials. *Escherichia coli* O55:B5 LPS was obtained from Difco (Detroit, Mich.). Fetal bovine serum (FBS) containing less than 60 pg of LPS per ml was obtained from GIBCO (Grand Island, N.Y.) and Biocell (Carson, Calif.). Cytochrome *c* from horse heart, superoxide dismutase (SOD) from bovine liver, and N^G-monomethyl-L-arginine acetate (N^GMMA) were obtained from Sigma (St. Louis, Mo.). Recombinant murine IFN-γ was a generous gift from Toray Industries, Inc. (Tokyo, Japan).

***Legionella* strain.** The virulent Nagasaki 80-045 strain of *L. pneumophila* serogroup 1 is a clinical isolate and was generously donated by Kazunori Tomono. The bacteria were passaged twice in guinea pigs and then stocked in 20% skim milk at −80°C. The organism was then inoculated onto buffered charcoal-yeast extract (BCYE) agar, incubated at 30°C for 3 days, and stored at 4°C for less than 2 weeks. The bacteria were passaged on another BCYE agar plate. Several colonies were scraped off the surface of BCYE agar and suspended in saline for infection.

Macrophage-like cell lines. J774.1 macrophage-like cells were cultured on 100-mm-diameter dishes (Falcon 1001; Becton Dickinson, Lincoln Park, N.J.) in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). One-milliliter aliquots of cell suspensions (5×10^5 cells per ml) were allowed to adhere to 24-well tissue culture plates (Falcon 3047) in an incubator under 5% CO_2 and 95% humidified air at 37°C overnight. The resulting monolayers were incubated in 1 ml of RPMI 1640 medium containing 3% FBS, which prevented cell division. In some experiments, macrophage monolayers were

pretreated with murine recombinant IFN-γ or with LPS in RPMI 1640 medium containing 5% FBS before infection.

Infection of JA-4 and LPS1916 cells. The monolayers were infected with a bacterial suspension at a bacterium-to-cell ratio of 1, unless otherwise indicated, in RPMI 1640 medium containing 3% FBS without any antibiotics. The plates were centrifuged for 10 min at 450 × *g* at 4°C and then incubated for 1 h at 37°C. After this period, nonphagocytized bacteria were removed by three consecutive washes with prewarmed Hanks' balanced salt solution. One milliliter of fresh RPMI 1640 medium containing 3% FBS, without antibiotics, was added to each well, and then the plates were incubated for an additional 2 to 3 days. Finally, the cultures were treated with 0.1% Triton X-100, and the cell lysates were weakly sonicated for 24 s at 130 W with a Bioruptor UCD-200T (Cosmo Bio Co., Ltd., Tokyo, Japan). The number of viable bacteria in each lysate was determined by standard plate counting on BCYE plates after incubation at 35°C for 3 days and expressed as CFU.

Colorimetric assays. O_2^- generation was examined as described before in detail (1). Cells were seeded at 2×10^5 cells per 0.5 ml of medium in the wells of a 24-well tissue culture plate and then incubated at 37°C overnight. The medium was replaced with 0.5 ml of fresh medium with or without LPS or IFN-γ, and then the cells were incubated at 37°C for 24 h. The cells were washed three times with modified Hanks' balanced salt solution without phenol red, $CaCl_2$, and $MgCl_2$ and then examined for O_2^- generation in 0.5 ml of a reaction mixture comprising $CaCl_2$, $MgCl_2$, and cytochrome *c*, with or without SOD, in 0.5 ml of Hanks' balanced salt solution. The reaction was started by the addition of 5 μg of phorbol myristate acetate per ml, continued at 37°C for 90 min, and stopped by sudden chilling on ice. The differences in A_{550} of the supernatant between the samples without and with SOD were determined, and the amounts of O_2^- generated were calculated as the reduction of cytochrome *c*. The NO_2-concentration in the culture supernatants was determined spectrophotometrically at 550 nm after reaction with Griess reagents (15).

Examination of transferrin receptors. Transferrin receptors on these macrophage-like cell lines were examined by fluorescence-activated cell sorting (FACS) analysis of bound fluorescein isothiocyanate (FITC)-transferrin on the cell surface. FITC-transferrin was prepared by conjugation of FITC (isomer I; Sigma) and human transferrin (Calbiochem) in Na_2CO_3-$NaHCO_3$ buffer (pH 9.3), excess FITC being removed by gel filtration on a PD10 column (Pharmacia). JA-4 and LPS1916 cells were seeded at 2×10^6 cells per 5 ml of Ham's F12 medium–10% FBS, treated with or without 0.1 μg of LPS per ml at 37°C for 24 h, then washed three times with phosphate-buffered saline (PBS), and preincubated at 4°C for 15 min in 2 ml of Ham's F12 medium without serum but containing 0.02% bovine serum albumin. FITC-transferrin was added at 1 μg/ml and incubated with the cells on ice for 2.5 h. After being washed twice with PBS, the cells were detached by pipetting, filtered through 200-mesh nylon cloth, and then analyzed with a FACScan (Becton Dickinson). For selective analysis of viable cells, propiodium iodide-stained cells were removed from the points of analysis.

Iron content. The iron contents of the macrophages were determined by inductively coupled plasma emission spectrometry analysis. Five million cells were treated with or without 0.1 μg of LPS per ml at 37°C for 24 h, washed twice with PBS and then once with saline, and finally harvested with a cell scraper. The cell pellets were treated with 3 ml of concentrated HNO_3, digested at room temperature overnight, and then heated at 110°C for 3 h on a hot plate. After cooling of the samples, the resultant residues were resuspended to a volume of 4.5 ml in 0.1 N ultrapure HNO_3. One milliliter of each sample was analyzed for atomic contents with an inductively coupled plasma emission spectrometer (model ICAP61; Thermal Jarrel Ash, Walton, Mass.).

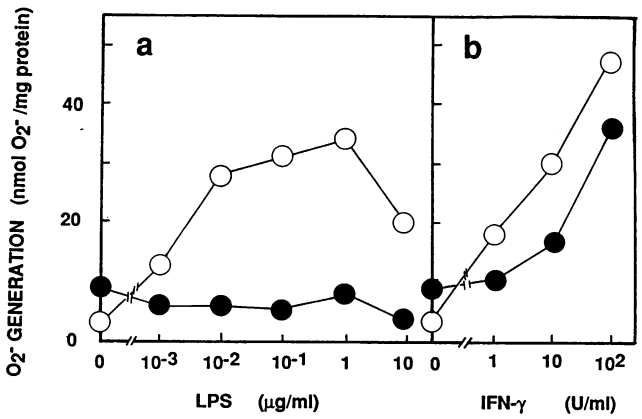

FIG. 1. Induction of O_2-generating activity in JA-4 (○) and LPS1916 (●) cells by LPS (a) or IFN-γ (b). Both types of cells were treated with each reagent at 37°C for 24 h. The values are means for duplicate cultures in a representative experiment.

RESULTS

Induction of O_2^--generating activity in JA-4 and LPS1916 cells by LPS or IFN-γ. Incubation of JA-4 cells with LPS at 37°C for 24 h induced O_2^--generating activity in an LPS dose-dependent manner (Fig. 1a). However, LPS1916 cells did not show the response to LPS in a wide range of concentrations. On the other hand, IFN-γ induced O_2^--generating activity in both types of cells in a dose-dependent manner (Fig. 1b), although LPS1916 cells were somewhat refractory to an increase in the IFN-γ dose.

Effect of LPS on the susceptibility of JA-4 and LPS1916 cells to infection with *L. pneumophila*. To determine whether O_2^- generation is involved in macrophage resistance to legionellae, monolayers of both types of cells were infected with legionellae and then the extent of bacterial growth was estimated by a CFU assay. On the day of infection, similar numbers of bacteria were phagocytized, and by 2 days the bacteria had multiplied 250- to 430-fold in both the parent and mutant cells without LPS treatment (Fig. 2). On day 2, some of these cells were swollen and appeared necrotic under a light microscope. The bacteria destroyed their host cells, became distributed around a nucleus, parasitized other cells, and finally occupied the substratum of the cultures. When the cells were treated with 0.1 μg of LPS per ml for 24 h before infection, which is one of the optimal conditions for JA-4 activation of the oxidative burst (1), only JA-4 cells spread and showed restricted *Legionella* growth within 2 days, LPS1916 mutant cells remaining round and permissive for *Legionella* growth.

Effect of IFN-γ on the susceptibility of JA-4 and LPS1916 cells to *Legionella* infection. Different from LPS, murine recombinant IFN-γ effectively restricted *Legionella* growth after pretreatment of both JA-4 and LPS1916 cells (Fig. 3). IFN-γ at 1 U/ml was ineffective, but 10 U/ml was enough to restrict *Legionella* growth within 3 days. LPS1916 cells showed restricted bacterial growth at a lower dose of IFN-γ than JA-4 cells. However, LPS1916 cells generated less O_2^- than JA-4 cells upon pretreatment with various concentrations of IFN-γ (Fig. 1b). This may be due to the combined effect of IFN-γ and

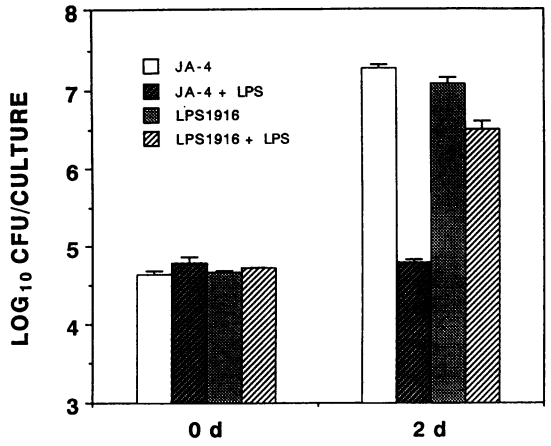

FIG. 2. Effect of LPS on the susceptibility of JA-4 and LPS1916 cells to infection with *L. pneumophila*. Cells were treated with 0.1 μg of LPS per ml at 37°C for 24 h before the challenge with *L. pneumophila*. CFU values on the day (d) of infection and on day 2 are shown. Each column and vertical bar represents the mean for three separate wells ± standard error in a representative experiment.

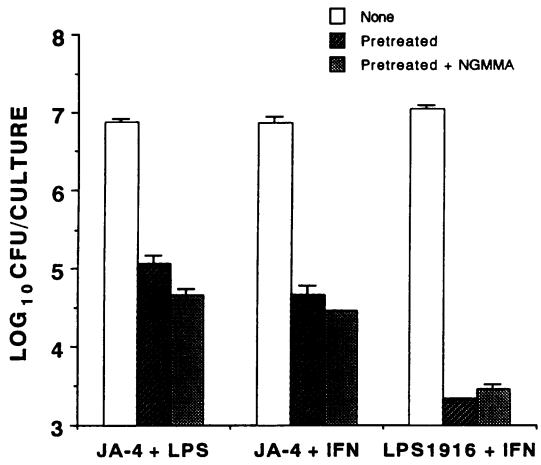

FIG. 4. Effect of N^GMMA on *Legionella* growth in activated JA-4 or LPS1916 cell cultures. One millimolar N^GMMA was added to precultures of the cells with or without LPS or IFN-γ and also added to the culture medium of the cells with bacteria throughout the experiment. Bacterial growth was estimated at 2 days after infection. Other conditions are the same as in the legend to Fig. 2.

the endogenous LPS of *L. pneumophila* itself because LPS1916 cells generate more O_2^- than JA-4 cells upon pretreatment with 10^{-2} to 10 mg of LPS per ml with 10 U of IFN-γ per ml (1). We confirmed the state of the bacteria in J774.1 cells by electron microscopy. Twenty-four hours after infection, many bacteria resided in vacuoles side by side or head to head in nontreated JA-4 cells and LPS1916 cells. IFN-γ-treated JA-4 cells and LPS1916 cells exhibited similar morphology, with few bacteria in vacuoles but with much electron-dense material and membranous debris. LPS-treated JA-4 cells also showed morphology similar to that of IFN-γ-treated JA-4 cells and LPS1916 cells. However, in LPS-treated LPS1916 cells, a considerable number of intact bacteria were observed in vacuoles. *Legionella* growth in vacuoles of these macrophage-like cell lines was suggested to be allowed only under the condition that reactive oxygen intermediates were not generated, which corresponded well with the bacterial colony-forming activity shown in Fig. 2 and 3.

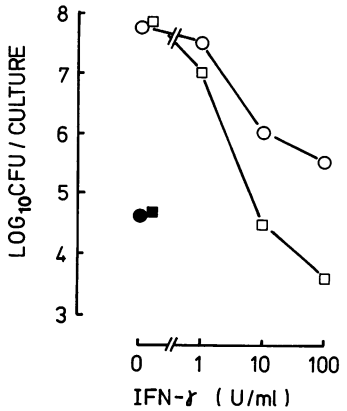

FIG. 3. Effect of IFN-γ on the susceptibility of JA-4 (○) and LPS1916 (□) cells to *Legionella* infection. Cells were pretreated with various concentrations of IFN-γ at 37°C for 24 h before infection. CFU values on the day of infection (closed symbols) and on day 3 (open symbols) are shown. Each symbol and vertical bar represents the mean for three separate wells ± standard error in a representative experiment.

Effect of N^GMMA on *Legionella* growth in activated JA-4 or LPS1916 cell cultures. We next examined the possibility of the involvement of reactive nitrogen intermediates as effector molecules for the antibacterial activity of macrophages. The presence of N^GMMA, an inhibitor of the synthesis of nitrogen oxide, in cultures did not abrogate the ability of the three types of activated cells to inhibit *Legionella* growth (Fig. 4). This reagent certainly inhibited NO_2^- production by JA-4 cells, as shown by the finding that the concentrations of NO_2^- in the culture supernatants of nontreated, LPS-treated, and 1 mM N^GMMA-plus-LPS-treated groups were 2.0 ± 0.1, 5.4 ± 0.1, and 1.3 ± 0.1 μM, respectively, in triplicate cultures before infection. Therefore, nitrogen-reactive intermediates do not seem to be of primary importance as antibacterial effector molecules in the activated macrophages in our system.

Distribution of transferrin receptors on the surface of JA-4 and LPS1916 cells. The distribution of transferrin receptors on the surface of macrophage-like cell lines after treatment with or without LPS was analyzed by FACS, using FITC-transferrin as a ligand (Fig. 5). No significant difference was observed between JA-4 and LPS1916 cells in the pattern of FITC-transferrin binding, although a slight decrease in the number of transferrin receptor-positive LPS-treated JA-4 cells was seen in the peak fraction. However, such a decrease does not seem to be correlated well with the nonpermissiveness for *Legionella* growth in these cells (Fig. 2).

Iron contents of the macrophage-like cell lines with or without LPS treatment. The iron contents of these macrophage-like cell lines were not significantly different between cells treated with and without LPS, although there were significant differences between the iron contents of JA-4 and LPS1916 cells (Table 1). These results obviously suggest that the decrease in iron content is not due to the differences in nonpermissiveness for *Legionella* growth in our experimental system, because JA-4 cells after LPS treatment showed the highest iron contents although only these cells show restricted *Legionella* growth.

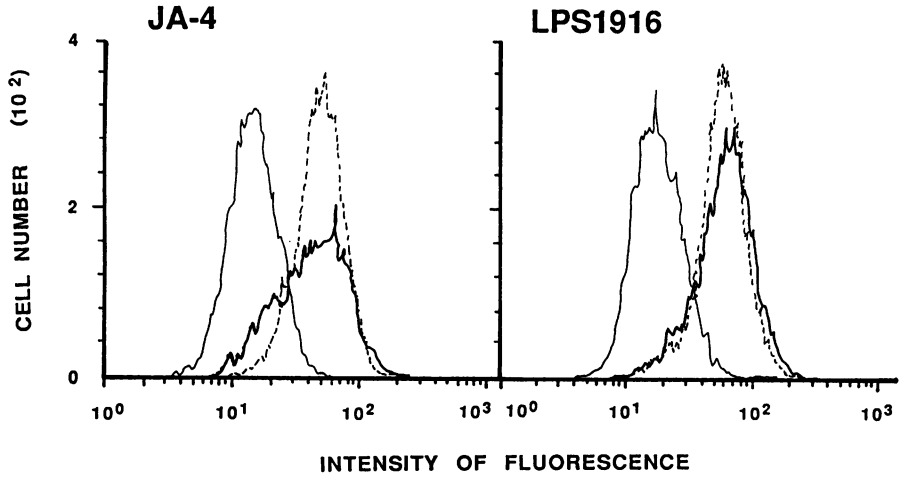

FIG. 5. Distribution of transferrin receptors on the surface of JA-4 and LPS1916 cells after treatment with (heavy line) or without (dashed line) 0.1 μg of LPS per ml. FITC-transferrin (1 μg/ml) was incubated with each type of cell at 4°C for 2.5 h, and the cells were analyzed with a FACScan. The abscissa shows the intensity of fluorescence, and the ordinate shows the number of cells. Light line, control (no FITC-transferrin).

DISCUSSION

Various approaches have been used in other laboratories to clarify the role of the oxidative burst in the prevention of microbial infection of macrophages. The addition to cultures of scavengers such as SOD and catalase is one approach. The killing of primate alveolar macrophage-associated legionellae was reported to be inhibited by the addition to cultures of the hydroxyl radical scavenger mannitol or a combination of SOD and catalase (7). However, it is unclear whether such scavengers added to cultures can gain access to the phagosomes of cultured macrophages when they have no effect (10). Another approach is comparison of the abilities to secrete H_2O_2 and O_2^- between leukocytes permissive and nonpermissive for *Legionella* growth in vitro. Nonpermissive macrophages from BDF1 mice produce larger amounts of H_2O_2 and O_2^- than permissive macrophages from guinea pigs and A/J mice when stimulated with zymosan (16). Polymorphonuclear leukocytes, which are active with respect to the oxidative burst, have not been reported to be permissive in vitro and can damage intracellular bacteria in vivo (4, 9). However, this approach is not optimal because the genetic backgrounds of these leukocytes are heterogeneous.

In this study, we used a somatic cell mutant with an altered activated-macrophage phenotype with respect to the oxidative burst in response to LPS and IFN-γ. With the mutant, we found that the oxidative burst rather than the nitric oxide reaction is important in the anti-*Legionella* effector mechanism. Goldberg et al. showed that a similar approach with other somatic cell mutants was useful for studying the regulatory effect of IFN-γ on viral infection (6). We also found, for the first time, that J774.1 is a macrophage-like cell line of mouse origin that is susceptible to *Legionella* growth even though it is derived from a female BALB/c mouse whose macrophages were naturally resistant to *Legionella* infection. Why J774.1 cells became permissive as to *Legionella* growth is unknown.

Reduction of the iron supply to intracellular legionellae was reported to be a mechanism restricting *Legionella* growth in human monocytes stimulated with IFN-γ (2, 3). However, transferrin receptor expression on LPS1916 cells was not significantly different from that on JA-4 cells after LPS stimulation, and the iron content was highest in JA-4 cells after LPS treatment, so the iron supply may not be critical for growth restriction of legionellae in our experimental system. We also showed that the inhibition of nitrite production had no effect on bacterial growth inhibition in J774.1 cells. Similarly, human HL-60 cells treated with IFN-γ were reported to remain bacteriostatic in spite of the presence of N^GMMA (14). JA-4 cells and LPS1916 cells can produce comparable amounts of tumor necrosis factor and interleukin-1 when stimulated with LPS (1), but only LPS1916 cells remain susceptible to *Legionella* infection. These findings suggest that the monokines are not the effector molecules directly involved in the resistance of macrophages to *Legionella* infection in the case of J774.1, although tumor necrosis factor can increase the anti-*Legionella* activity of human monocytes (13). In conclusion, our results suggest that the acquired resistance of macrophages to *Legionella* infection is due, at least in part, to the capability for O_2^- production and that the use of the LPS1916 mutant cell line will help us to clarify the host-parasite relationships in other bacterial infections.

TABLE 1. Iron contents of JA-4 and LPS1916 cells treated with or without LPS

Cells	LPS	Iron content (μg/5 × 10^6 cells)[a]
JA-4	−	1.42 ± 0.44
	+	1.82 ± 0.49
LPS1916	−	0.81 ± 0.13
	+	0.97 ± 0.14

[a] Mean ± standard error for three independent samples.

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