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Intracellular Killing of *Listeria monocytogenes* in the J774.1 Macrophage-Like Cell Line and the Lipopolysaccharide (LPS)-Resistant Mutant LPS1916 Cell Line Defective in the Generation of Reactive Oxygen Intermediates after LPS Treatment

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***Listeria monocytogenes* is a facultative intracellular pathogen and survives within phagocytic cells by escaping from phagosomes into the cytoplasm. It has been reported that, in vivo, *L. monocytogenes* is effectively eliminated through cell-mediated immunity, especially by macrophages which have been immunologically activated by cytokines such as gamma interferon (IFN-γ). However, this killing mechanism for *L. monocytogenes* and the role of macrophage activation in this bacterial killing are unclear. We demonstrated the listericidal effect of oxidative radicals induced by lipopolysaccharide (LPS) and IFN-γ, using a macrophage-like cell line, J774.1, and a mutant cell line, LPS1916. LPS1916 cells do not exhibit normal generation of O_2^- and H_2O_2 after treatment with 0.1 μg of LPS per ml, although J774.1 cells generate 100 times the normal level of oxidative radicals with the same LPS treatment. The growth of *L. monocytogenes* was strongly inhibited in J774.1 cells pretreated with 0.1 μg of LPS per ml or the combination of 0.1 μg of LPS per ml and 10 U of IFN-γ per ml. On the other hand, in LPS1916 cells, the growth of *L. monocytogenes* was not inhibited by treatment with LPS only, although LPS1916 cells pretreated with the combination of LPS and IFN-γ showed moderate inhibition of listerial growth. This killing was not influenced by treatment with N^G-monomethyl-L-arginine, which is a strong inhibitor of nitrite oxide generation. Interestingly, J774.1 cells treated with LPS did not show enhanced intraphagosomal killing of a nonhemolytic strain of avirulent *L. monocytogenes* that lacks the ability to escape from phagosomes, and this killing was not influenced by treatment with N^G-monomethyl-L-arginine either. These results suggest that the reactive oxygen radicals are more important than nitric oxide in the mechanism underlying the intracellular killing of virulent *L. monocytogenes* and that there seem to be different killing mechanisms for virulent and avirulent strains of *L. monocytogenes* in activated-macrophage cell lines.**

Listeria monocytogenes is classified as a facultative intracellular pathogen, and its survival and proliferation in macrophages, Kupffer cells, hepatocytes, and enterocytes have been reported (33, 37, 40, 41, 48). This intracellular persistence was studied by using primary and established cell lines, including macrophages, epithelial cells, and fibroblasts (16, 17, 22, 28, 32, 44, 46, 47, 49). These simple cellular infection systems together with genetic analyses of virulence genes on bacteria have facilitated the accumulation of knowledge of the processes by which *L. monocytogenes* infects and spreads in mammalian cells. The entry of *L. monocytogenes* into cells is mediated by the *inlAB* and *iap* genes, which produce internalin and protein p60, respectively (15, 31). The escape from endosomal compartments into the cytoplasm is associated with the production of a hemolysin, listeriolysin O, which is a major virulence factor of *L. monocytogenes* (8, 28). Bacteria that have escaped can proliferate in the cytoplasm and protrusively spread into neighboring cells by means of assembly of the actin bundles, a process which is encoded by the *actA* gene (11, 27, 34). Although *L. monocytogenes* can easily escape from and survive in phagocytic cells, it is completely eliminated from infected organs by macrophages and neutrophils through T-cell-mediated immunity (10, 29, 44, 47, 49, 50). Recent reports have shown that this protective immunity is enhanced by hemolysin as a T-cell-reactive antigen (4, 21) and by cytokines effective on phagocytes, such as interleukin-1 (IL-1) (25, 43), gamma interferon (IFN-γ), tumor necrosis factor (TNF), and colony-stimulating factors.

However, the crucial roles of macrophages and mechanisms by which they eliminate *L. monocytogenes* are still largely unknown. To elucidate the protective mechanisms of macrophages in listeriosis, we tried to use a mutant cell line isolated from the J774.1 macrophage-like cell line (2). This mutant cell line, originally isolated as a lipopolysaccharide (LPS)-resistant variant and designated LPS1916 cells, is characterized by a specific defect in the induction of reactive oxygen intermediates (ROI) (O_2^- and H_2O_2) after LPS treatment. In addition, this defect was partly overcome by the simultaneous addition of IFN-γ. Other activated-macrophage phenotypes of this mutant after LPS treatment, such as the release of IL-1, TNF, and arachidonic acid metabolites, showed no great difference from those of the parental cell line, providing some advantages in

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the use this mutant together with the parental cell line in the study of the role of ROI in listericidal activity in macrophages. We also tried to elucidate the mechanisms of killing in phagosomes, and both a virulent, hemolytic *L. monocytogenes* strain and an avirulent, nonhemolytic *L. monocytogenes* strain were examined with respect to growth in both cell lines with the combination of LPS and IFN-γ.

MATERIALS AND METHODS

Cells. JA-4 cells were subcloned from J774.1 cells to obtain a homogeneous cell population, because the conventional murine macrophage-like cell line J774.1 is composed of a heterogeneous mixture of cells. LPS1916 cells showed a high level of resistance to LPS (100 mg of *Escherichia coli* O55:B5 LPS per ml) and grew at the same rate as the JA-4 parent cells in the absence of LPS. Cells were cultured in Ham's F12 medium containing 50 U of penicillin per ml, 50 μg of streptomycin (Flow Laboratories, Irvine, Scotland), and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) in a CO_2 incubator (5% CO_2, 95% humidified air) at 37°C. The properties of JA-4 cells and LPS1916 cells were described in a previous report (2). All of the cells used in this experiment were maintained in a nonselective medium for at least 1 month and were periodically assayed for resistance to LPS, biological activity of TNF, and generation of O_2^- and H_2O_2 by the methods described in the previous report (2).

Activation of macrophage-like cell lines. Activation of macrophage-like cell lines and assays for O_2^- and H_2O_2 were performed as described previously (2). In brief, cells were seeded into a flat-bottom plate with 24 wells (Costar) at 2×10^5 cells per well in 0.5 ml of F12 plus 10% FBS and then were incubated overnight at 37°C. The medium was replaced with 0.5 ml of fresh F12 plus 10% FBS supplemented or not supplemented with 0.1 μg of LPS (Sigma; *E. coli* O55:B5) per ml and/or 10 U of murine recombinant IFN-γ (a generous gift from Toray Co., Tokyo, Japan) per ml, and then the cells were incubated at 37°C for 20 to 24 h. After this treatment, the JA-4 cells became spread or elongated, and they vacuolated after treatment with LPS, while LPS1916 cells showed fewer morphological changes. For biological assays, the medium was removed and the cells were processed for each assay, and for infection of bacteria, the medium was replaced with 0.5 ml of fresh F12 plus 2% FBS per well, unless otherwise noted.

O_2^- assay. For the assay of O_2^- generation, the medium was removed and the cells were washed three times with a modified Hanks balanced salt solution (containing [in grams per liter] NaCl, 8.0; KCl, 0.4; glucose, 1.0; $Na_2HPO_4 \cdot 2H_2O$, 0.06; KH_2PO_4, 0.06; and $NaHCO_3$, 0.35). The cells were incubated in 0.5 ml of a reaction mixture containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.625 mg of cytochrome *c* per ml, with or without 0.06 mg of superoxide dismutase (Sigma) per ml. 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 microplates were centrifuged at 2,000 rpm for 3 min at 4°C (Tomy Partner-sp centrifuge), and the A_{550}s of the supernatants were measured with a UV-160 photometer (Shimadz, Kyoto, Japan). The differences in A_{550} between the samples without and with superoxide dismutase were determined, and the amounts of O_2^- were calculated as the reduction of cytochrome *c* on the basis of the fact that 1 U of optical density at 550 nm corresponds to 47.2 nmol of O_2^-. The results were expressed as specific activities divided by the amounts of cellular proteins recovered in wells after the reaction mixture was washed off, with amounts of protein determined by the method of Lowry et al. (39).

NO_2^- assay. The activity of nitric oxide (NO·) production was estimated as the amounts of NO_2^- in the culture medium. After incubation of cells with or without LPS and/or IFN-γ at 37°C for 20 to 24 h, the culture supernatants were collected and then 100 μl of each of them was mixed with an equal volume of a 6-mg/ml concentration of Griess reagent (Wako Pharmaceutical Co., Tokyo, Japan) for the nitrite assay. Following mixing of the reaction mixture in a 96-well microplate, the A_{550} was monitored with a microplate reader (Titertek Multiscan Plus, model MKII), with background subtraction at A_{630}. Quantitative analysis was performed with known standard solutions of $NaNO_2$.

Bacteria. Virulent and avirulent strains of *L. monocytogenes* were used in this study. *L. monocytogenes* Y7, isolated from a patient's stomach (serovar 1/2b; 50% lethal dose = $10^{3.49}$ organisms per mouse) (from T. Enari, Joshi Idai, Tokyo, Japan), was used as the virulent strain. The type strain, *L. monocytogenes* ATCC 15313 (from M. Mitsuyama, Niigata University, Niigata, Japan), was used as the avirulent strain and was characterized as being nonhemolytic by means of the sheep erythrocyte agar and CAMP tests. *L. monocytogenes* that had proliferated up to 10^8 to 10^9/ml in brain heart infusion broth (Difco) was resuspended in F12 after being washed with phosphate-buffered saline (PBS) without calcium or magnesium [PBS(−)] and then was kept at −80°C until use. Numbers of viable bacteria were determined in each examination by seeding serial dilutions on soft agar plates. CFU were determined after incubation at 37°C for 18 to 24 h.

Infection and assaying of intracellular bacterial growth. A stock solution of frozen *L. monocytogenes* was thawed and precultured in F12 at 37°C for 30 min. After the cell surface was washed twice with PBS(−), the optimum dilution of thawed *L. monocytogenes* was inoculated into each well (100 μl per well), usually at the ratio of 100 bacteria per cell. The culture plates were incubated with the inoculum at 37°C for 15 min. After the cell surface was washed twice with PBS(−), the culture medium was replaced with Ham's F12 medium containing 10 μg of gentamicin (Wako Pharmaceutical Co., Tokyo, Japan) per ml to kill residual or adherent *L. monocytogenes* on the outside of the cells, and then the cells were incubated at 37°C. After the indicated times (see Results), the cell surface was washed with PBS(−) and the intracellular *L. monocytogenes* was released with 100 μl of 0.1% Triton X-100. The number of viable bacteria was determined by plating the lysate.

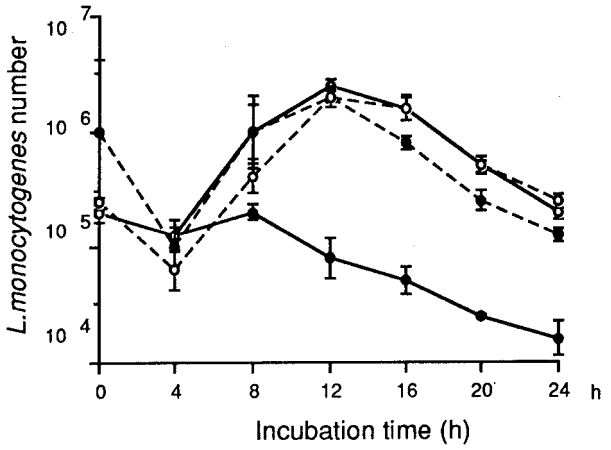

FIG. 1. Effect of treatment with 0.1 μg of LPS per ml on the intracellular growth of *L. monocytogenes* (Y7) in the macrophage-like cell lines JA-4 (——) and LPS1916 (– – –). ●, LPS added; ○, no LPS added. Data are presented as the means for triplicate cultures ± standard deviations.

Electron microscopy. Cells infected with *L. monocytogenes* were processed for observation under an electron microscope. After the cell surface was washed with PBS to remove extracellular bacteria, the cells were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 2 h at 4°C. The fixed cells were scraped off, collected, and then rinsed with 8% sucrose containing 0.02% NaN_3 in PB. The cells were postfixed with 1% osmium tetroxide and then stained with 1% uranyl acetate dissolved in Veronal buffer (pH 7.0). The dehydrated pellet was embedded in Epon, and then ultrathin sections were made with a Sovall MT-5000 ultramicrotome. Sections stained with uranyl acetate and lead citrate were observed under a JEM-100S electron microscope operating at 80 kV. More than 40 cells were examined in each sample, and typical cell morphologies are shown in the results given below (see Fig. 2 and 3).

Statistics. The data are expressed as arithmetic means ± standard deviations. The paired Student's *t* test was used to assess the significance of differences.

RESULTS

Growth curves for *L. monocytogenes* in JA-4 cells and LPS1916 cells. The growth curves for virulent *L. monocytogenes* Y7 in the two cell lines with and without LPS are presented in Fig. 1. All four groups showed a decrease in intracellular *L. monocytogenes* for the first 4 h at 37°C. However, the number of intracellular *L. monocytogenes* organisms increased during the next 8 h, except in JA-4 cells pretreated with 0.1 μg of LPS per ml, in which *L. monocytogenes* gradually decreased throughout the observation period (24 h). *L. monocytogenes* in the other three macrophage groups decreased between 12 and 24 h. These results suggest that *L. monocytogenes* cannot grow in JA-4 cells treated with LPS, while it can grow in LPS1916 cells irrespective of LPS treatment.

Electron microscopic observation of *L. monocytogenes* Y7 in JA-4 cells and LPS1916 cells pretreated and not treated with LPS. Macrophages were observed at 0, 2, and 12 h postincubation after infection with *L. monocytogenes* following the first 15 min of the invasion phase. At 0 h, *L. monocytogenes* Y7 was packed in phagosomes, except for a few cells that were detectable in the cytoplasm. This phenomenon was observed in all groups with or without LPS treatment. After 2 h of incubation, *L. monocytogenes* was seen in the cytoplasm surrounded by a

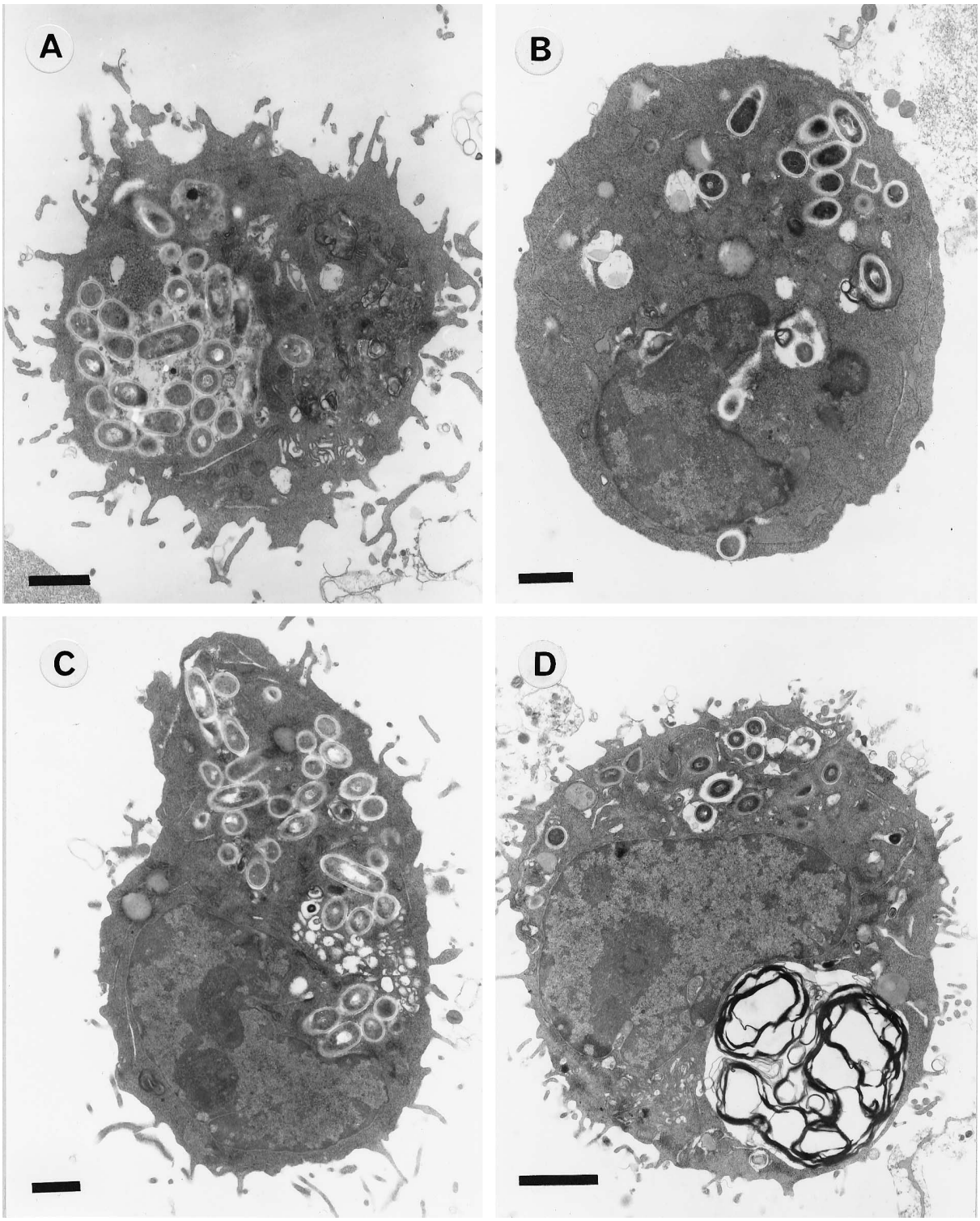

FIG. 2. Intracellular *L. monocytogenes* (Y7) phagocytosed by macrophage-like cell lines. Observation was at 2 h after infection. (A) JA-4 cells treated with 0.1 μg of LPS per ml. (B) JA-4 cells without LPS treatment. (C) LPS1916 cells treated with 0.1 μg of LPS per ml. (D) LPS1916 cells without LPS treatment. Each panel shows a typical cell morphology from 40 to 80 cells observed in each group. Organisms observed in the cytoplasm were surrounded by a translucent furrow and adjacent polymerized actin. Bars, 1 μm (A to C) or 2 μm (D).

translucent furrow and adjacent polymerized actin (Fig. 2). This polymerization of actin was commonly observed in all groups (detailed results not shown). *L. monocytogenes* in LPS-treated JA-4 cells was conglomerated, and the number of bacteria that had invaded the cytoplasm was less than that in other cells at this stage.

The number of intracellular *L. monocytogenes* Y7 bacteria after 12 h of incubation was about 10 per cell in JA-4 cells

without LPS treatment and in LPS1916 cells with or without LPS treatment. However, there were fewer intact bacteria in JA-4 cells pretreated with LPS; most *L. monocytogenes* Y7 organisms were destroyed in phagosomes, and the few cells that escaped into the cytoplasm were intact (Fig. 3A and B). The *L. monocytogenes* Y7 observed in LPS1916 cells pretreated with LPS and in both LPS1916 cells and JA-4 cells without LPS treatment (Fig. 3C and D) was intact not only in phagosomes but also in the cytoplasm. All of the *L. monocytogenes* Y7 in any group observed in the cytoplasm was surrounded by actin filaments (typical morphology is shown by arrowheads in Fig. 3B). These results suggest that the survival of *L. monocytogenes* in these macrophage cell lines is well correlated with the ability to escape from phagosomes into the cytoplasm and that only LPS-treated JA-4 cells seem to inhibit this process.

Effect of LPS and IFN-γ in combination on the intracellular growth of *L. monocytogenes*. The effect of IFN-γ on LPS-induced O_2^- generating activity was reported previously (2). The peak of O_2^- generation and the optimal dose of LPS changed after IFN-γ treatment of JA-4 cells and LPS1916 cells. We examined these effects of IFN-γ on the growth of *L. monocytogenes* after 8 h of incubation at a multiplicity of infection of 10 bacteria per cell (Fig. 4). Under these conditions, about 1 in 10 bacteria was phagocytosed by cells after the 15-min inoculation period. This ratio was not influenced by pretreatment with LPS and IFN-γ (data not shown). In JA-4 cells, the intracellular growth of *L. monocytogenes* was strongly inhibited by pretreatment with 0.1 μg of LPS per ml, and this inhibition was strongly enhanced by the combination of 0.1 μg of LPS per ml and 10 U of IFN-γ per ml. Treatment with 0.001 μg of LPS per ml was not effective for inhibition of intracellular growth. However, the simultaneous addition of 10 U of IFN-γ per ml and 0.001 μg of LPS per ml increased the killing activity significantly. On the other hand, treatment with 10 or 10^2 U of IFN-γ per ml caused inhibition of intracellular growth, with the effect being less than that of treatment with 0.1 μg of LPS per ml. Significant differences ($P < 0.05$) were observed between results with controls and with treatment with either 0.1 μg of LPS per ml or the combination of 0.1 μg of LPS per ml and 10 U of IFN-γ per ml. In LPS1916 cells, inhibition of intracellular growth was observed with the combination of 0.1 μg of LPS per ml and 10 U of IFN-γ per ml. IFN-γ alone at 10 or 100 U/ml also enabled this mutant cell line to kill *L. monocytogenes*, but treatment with 0.1 μg of LPS per ml was not effective in inhibiting the growth of *L. monocytogenes*. A significant difference ($P < 0.05$) was observed between results with the controls and the combination of 0.1 μg of LPS per ml and 10 U of IFN-γ per ml.

Effect of L-NMMA on the intracellular growth of *L. monocytogenes* in JA-4 cells and LPS1916 cells pretreated with LPS and IFN-γ. The effects of N^G-monomethyl-L-arginine (L-NMMA) on the intracellular growth of virulent *L. monocytogenes* Y7 and avirulent *L. monocytogenes* ATCC 15313 are shown in Fig. 5A and B, respectively. This effect on the growth of *L. monocytogenes* was observed after 12 h of incubation. The inoculated doses of virulent *L. monocytogenes* Y7 and avirulent *L. monocytogenes* ATCC 15313 were 1 and 10 bacteria per cell, respectively.

The intracellular growth of virulent *L. monocytogenes* Y7 was strongly inhibited in JA-4 cells pretreated with 0.1 μg of LPS per ml and the combination of 0.1 μg of LPS per ml and 10 U of IFN-γ per ml (Fig. 5A). This inhibition was significantly stronger with the combination than with LPS only ($P < 0.05$). In LPS1916 cells, the growth of *L. monocytogenes* Y7 was inhibited only in the case of pretreatment with LPS and IFN-γ ($P < 0.05$). However, treatment with 1 mM L-NMMA had no influence on the intracellular growth of *L. monocytogenes* Y7 in any cell line. These results suggest that nitric oxide is not critical for the intracellular killing of *L. monocytogenes*.

On the other hand, the number of intracellular avirulent *L. monocytogenes* ATCC 15313 was remarkably decreased in all groups of JA-4 cells and LPS1916 cells, and about 1 of 10 bacteria was phagocytosed after inoculation (Fig. 5B). This decrease in *L. monocytogenes* ATCC 15313 was significant in both cell lines pretreated with LPS and IFN-γ ($P < 0.05$). Treatment with L-NMMA did not influence the intracellular decrease of *L. monocytogenes* ATCC 15313 in any group, suggesting again that nitric oxide is not an important factor in *Listeria* killing.

Generation of O_2^- and NO_2^- in JA-4 cells and LPS1916 cells pretreated with LPS, IFN-γ, and L-NMMA. As shown in Fig. 6, the generation of O_2^- was strongly induced on treatment with 0.1 μg of LPS per ml and 10 U of IFN-γ per ml in JA-4 cells. The level of O_2^- generated with the combination of LPS and IFN-γ was 3 and 7 times those with only LPS and only IFN-γ, respectively. Treatment with L-NMMA had no significant effect on the generation of O_2^- in LPS-treated JA-4 cells; however, it significantly decreased the level of O_2^- generation in JA-4 cells treated with LPS and IFN-γ ($P < 0.001$).

In LPS1916 cells, O_2^- was generated on treatment with 0.1 μg of LPS per ml and 10 U of IFN-γ per ml, although the amount of O_2^- generated was only one-third of that in JA-4 cells. Treatment with L-NMMA did not influence the generation of O_2^- in LPS1916 cells treated with both LPS and IFN-γ, but L-NMMA slightly elevated the level of O_2^- generation in LPS1916 cells treated with LPS alone ($P < 0.025$).

The generation of NO_2^- was also assayed in both cell lines (Fig. 7). In JA-4 cells, NO_2^- was generated on treatment with LPS and IFN-γ. The amount of NO_2^- generated with the combination of LPS and IFN-γ was 3 times that with LPS. This generation of NO_2^- was strongly inhibited by treatment with 1 mM L-NMMA. On the other hand, generation of NO_2^- was not induced on treatment with LPS and IFN-γ in LPS1916 cells.

These results suggest that LPS alone induces both O_2^- and NO_2^- generation in JA-4 cells and that IFN-γ increases the LPS-induced generation of these molecules, while in LPS1916 cells, LPS alone had little effect on O_2^- or NO_2^- generation and only the O_2^--generating activity in LPS-treated cells was increased by IFN-γ. In addition, L-NMMA caused strong inhibition of nitric oxide production in these cell lines after treatment with LPS and/or IFN-γ, and this reagent also seems to have some modulating effect on the induction of O_2^--generating activity in JA-4 cells treated with LPS and IFN-γ and in LPS1916 cells treated with LPS alone. However, the mechanisms of these unexpected effects of L-NMMA on O_2^--generating activity remain to be elucidated.

DISCUSSION

In this report, we have described the intracellular killing of *L. monocytogenes* in the J774.1 macrophage-like cell line and in an LPS-resistant mutant cell line, LPS1916, selectively defective in the generation of ROI after LPS treatment. This mutant cell line showed activated-macrophage phenotypes, such as the release of IL-1, TNF, and arachidonic acid metabolites after treatment with LPS, that were similar to those of the parental JA-4 cell line (2). In addition, the defect in the induction of O_2^--generating activity in the LPS-treated mutant cells was partially restored by the simultaneous addition of low levels of IFN-γ. These unique characteristics of the activated-macrophage phenotypes of LPS1916 cells prompted us to examine

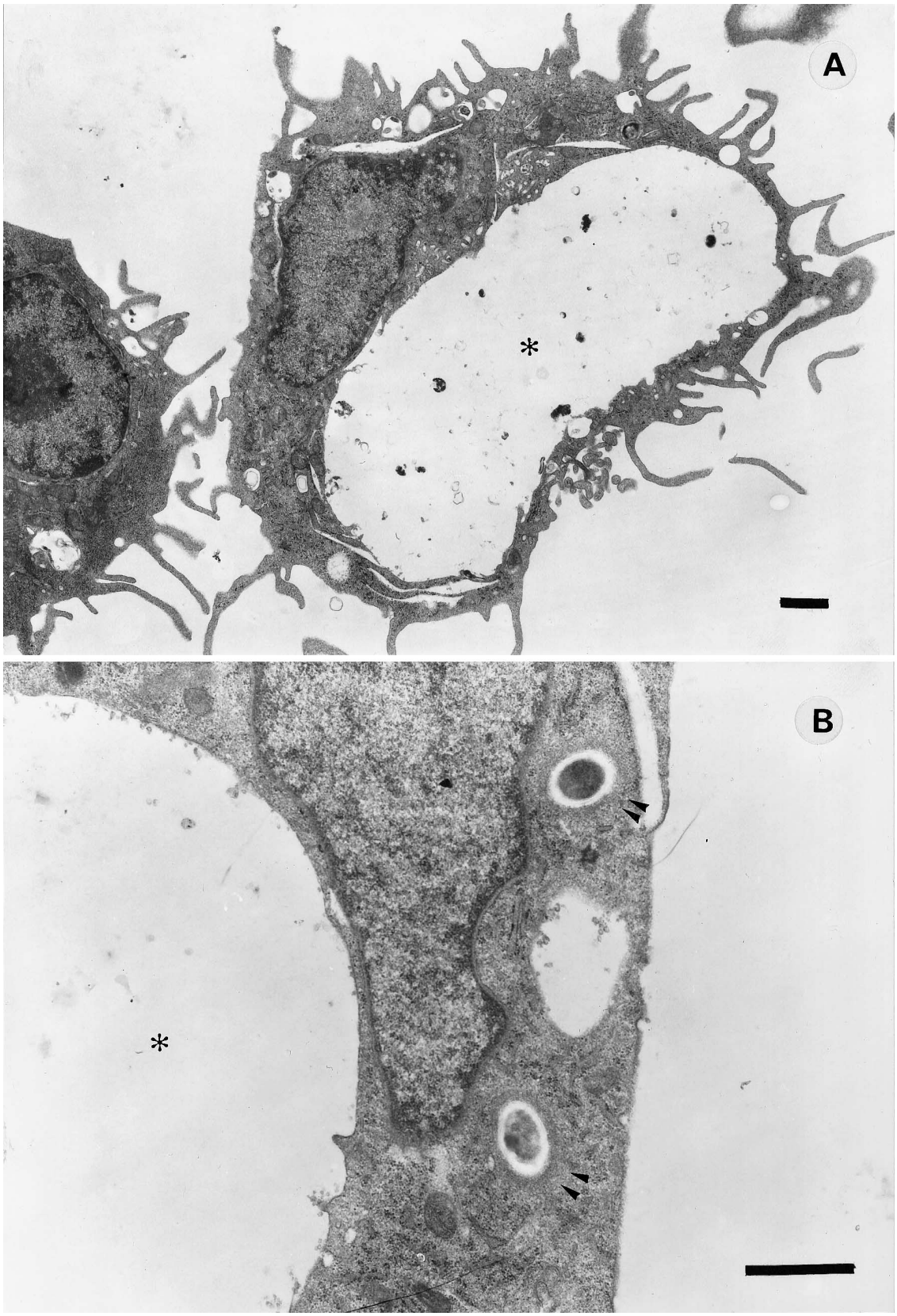

FIG. 3. Intracellular *L. monocytogenes* (Y7) phagocytosed by macrophage-like cell lines. Observation was at 12 h after infection. (A and B) JA-4 cells treated with 0.1 μg of LPS per ml. Intact organisms were not observed in phagosomes (asterisks), but a few organisms had escaped into the cytoplasm, surrounded by polymerized actin (arrowheads). (C and D) LPS1916 cells without LPS treatment. Organisms had escaped into the cytoplasm (arrowheads in panel C), and intact organisms were observed in secondary lysosomes (arrows in panel D). Bars, 1 μm.

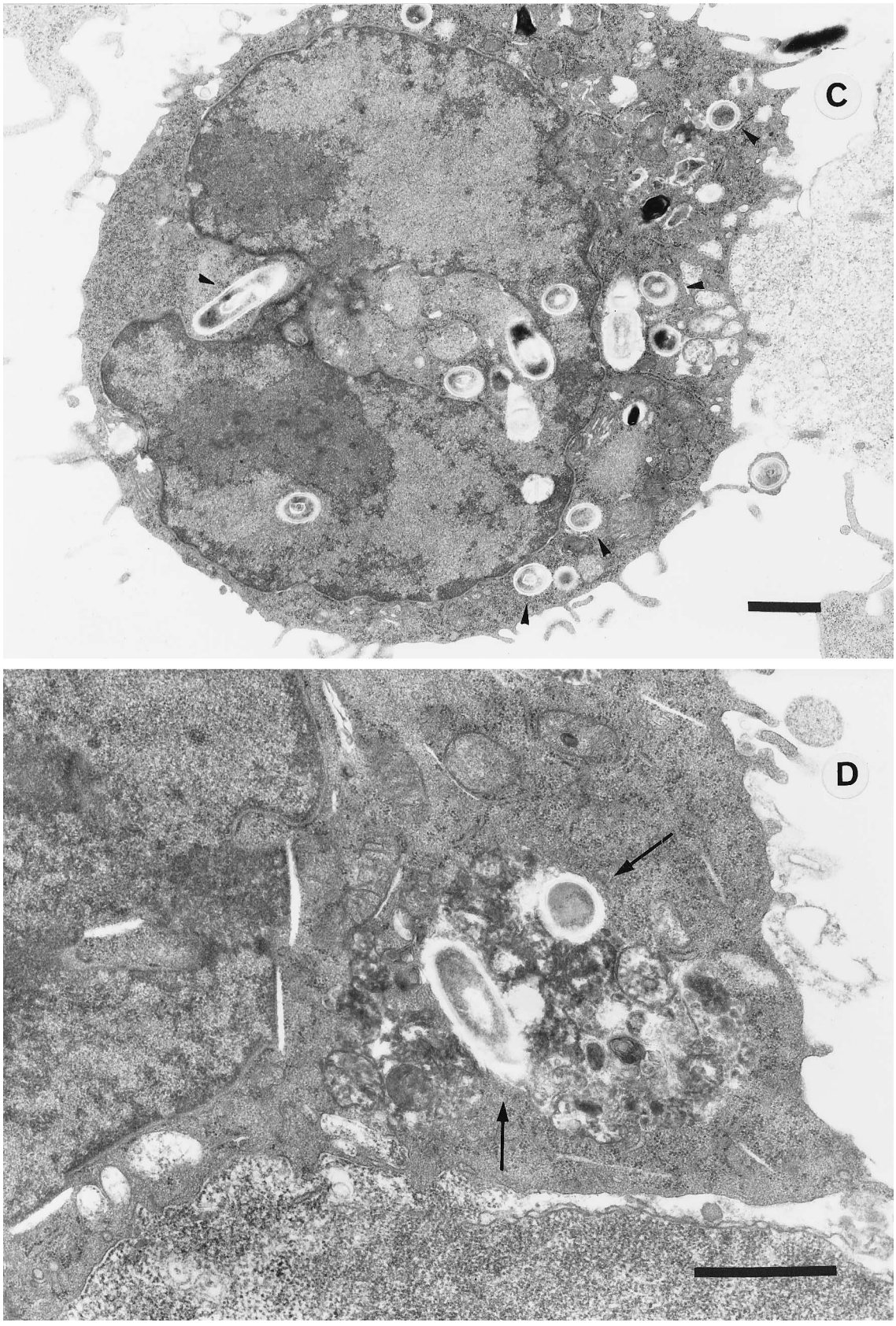

FIG. 3—*Continued.*

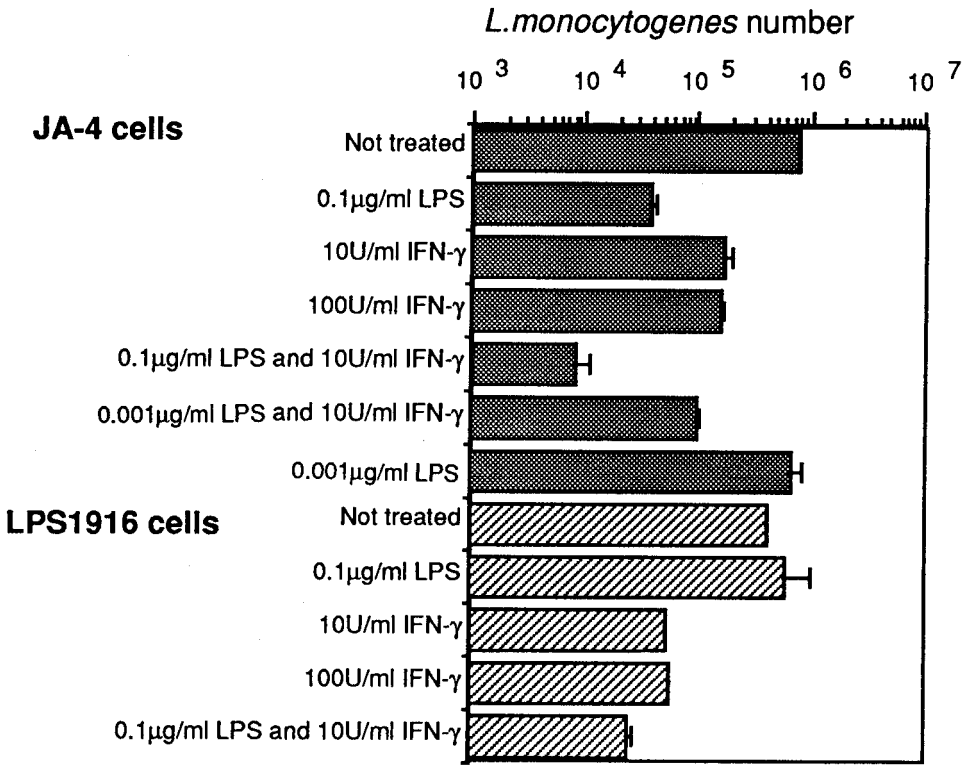

FIG. 4. Effect of treatment with LPS and IFN-γ on the intracellular growth of *L. monocytogenes* (Y7) in the macrophage-like cell lines JA-4 and LPS1916. Observation was at 8 h after *Listeria* infection. Data are presented as the means for triplicate cultures ± standard deviations.

the involvement of ROI in microbicidal activity (35). The production of hydrogen peroxide, superoxide, and the hydroxyl radical is correlated with the killing of a wide range of intracellular pathogens (36, 54). The importance of these oxygen metabolites for antilisterial activity has been suggested by the case of catalase-negative *Listeria* strains, which are less virulent in vivo than most catalase-positive strains, and also by the high levels of antilisterial activity in polymorphonuclear leukocytes and immune macrophages, which produce more oxidative radicals than monocytes or nonimmune macrophages (6, 12, 18, 54). However, it has been argued that nonoxidative mechanisms are also important, since inhibitors of oxidative antimicrobial systems fail to completely inhibit the killing of *Listeria* spp. (6, 9, 18). In our experiments, catalase and superoxide dismutase, which decrease H_2O_2 and O_2^-, respectively, showed no effect on listerial growth in these cell lines (data not shown), suggesting that these catabolic enzymes are poorly able to reach the sites of reactive oxygen production.

As shown in Fig. 1, *L. monocytogenes* could proliferate in LPS-pretreated LPS1916 cells, although in JA-4 cells pretreated with LPS the intracellular proliferation of *L. monocytogenes* was strongly inhibited. A mutant cell line, LPS1916, was characterized by a defect in O_2^- generation in response to LPS (2). Electron micrographs of intracellular *L. monocytogenes* infecting JA-4 cells and LPS1916 cells are shown in Fig. 2 and 3. *L. monocytogenes* was rarely observed in LPS-treated JA-4 cells, with a few intact *L. monocytogenes* cells in the cytoplasm. In contrast, in LPS1916 cells, many intact *L. monocytogenes* cells were observed, not only in the cytoplasm but also in phagosomes. LPS treatment did not influence the ability of *L. monocytogenes* to polymerize actin filaments, which is related to the intracellular movement of *L. monocytogenes* (11, 34). These results suggest that the major listericidal function of JA-4 with LPS is due to the generation of O_2^- in phagosomes of macrophages and, as a result, to the inhibition of intracellular movement of *Listeria* bacteria under these conditions.

In previous reports, we also showed that the level of O_2^- generation was influenced by the dose and presence of IFN-γ in JA-4 cells and LPS1916 cells (2, 35). Strong inhibition of the intracellular growth of *L. monocytogenes* was observed in JA-4 cells treated with LPS (0.1 μg/ml) and in LPS1916 cells treated with both LPS (0.1 μg/ml) and IFN-γ. These results also suggest that the major listericidal function of macrophages was due to the generation of O_2^- in the macrophages. The simultaneous addition of LPS (0.1 μg/ml) and IFN-γ (10 U/ml) to JA-4 cells caused greater suppression of the growth of intracellular *L. monocytogenes* than either LPS or IFN-γ alone (Fig. 4), which also correlated with the elevated level of O_2^- generation in these cells (Fig. 6). In spite of the close correlation between listericidal activity and O_2^--generating activity of these macrophage cell lines after treatment with LPS and/or IFN-γ, other killing factors involved in listericidal activity remain to be elucidated, because our experimental conditions could not entirely rule out the possibility that LPS and/or IFN-γ induced killing factors in macrophages, such as lysosomal enzymes (30) or recently reported microbicidal proteins (23). Peck reported that monocytes treated with IFN-γ kill *L. monocytogenes* through oxygen-dependent listericidal activity (45), and Portnoy et al. reported that the role of IFN-γ was to prevent access of *L. monocytogenes* to the macrophage cytoplasm, which would prevent cell-to-cell spreading of the bacterium (47). Our data suggest that the role of IFN-γ in *Listeria* killing is well correlated with the generation of O_2^-. It is not

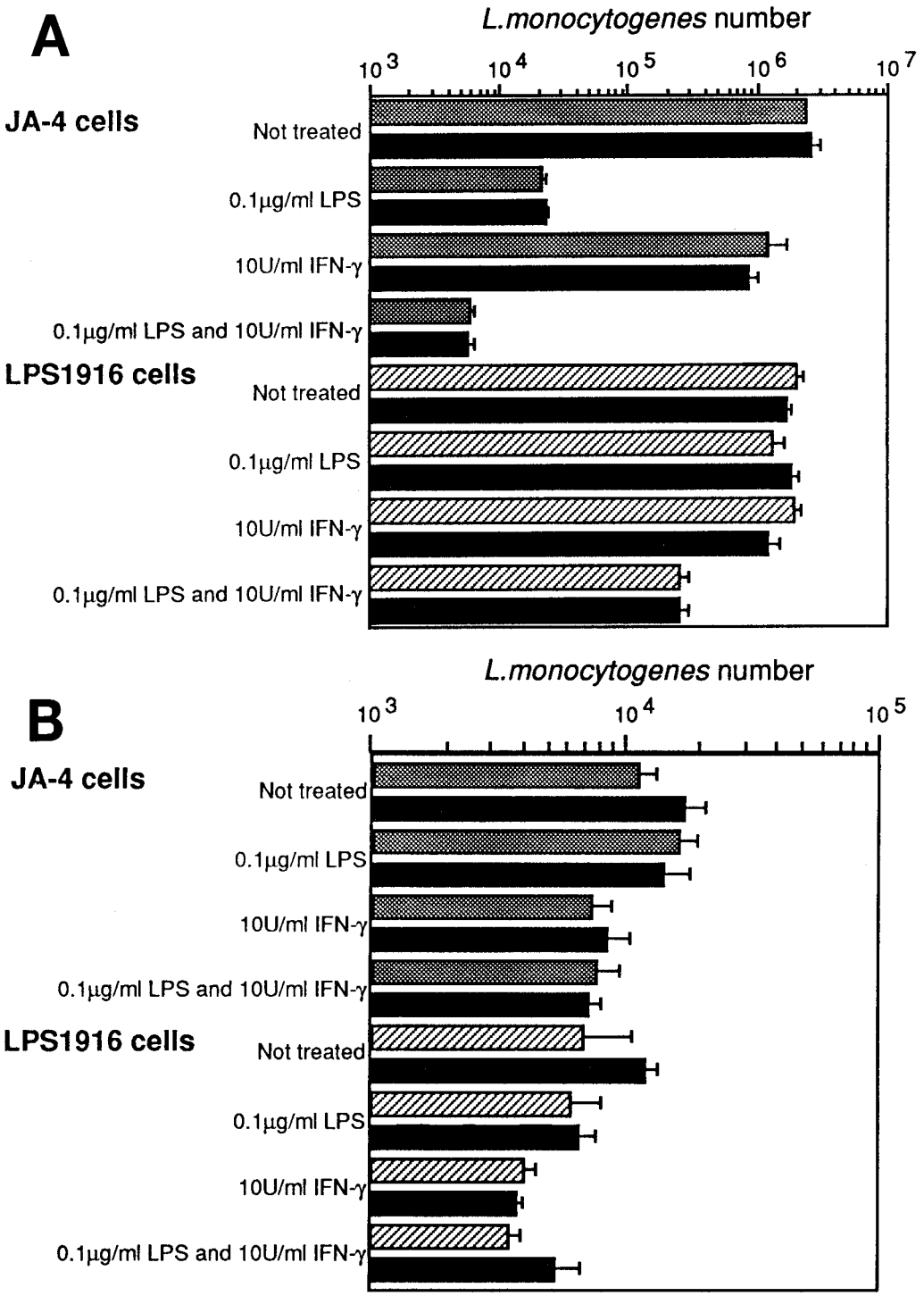

FIG. 5. Effect of L-NMMA on the intracellular growth of virulent *L. monocytogenes* (Y7) (A) or avirulent *L. monocytogenes* (ATCC 15313) (B) in the macrophage-like cell lines JA-4 and LPS1916 treated with LPS and/or IFN-γ. Observation was at 12 h after *Listeria* infection (2×10^5 cells for strain Y7 and 5×10^6 cells for strain ATCC 15313). ■, L-NMMA present; ▨ and ▧, L-NMMA absent. Data are presented as the means for triplicate cultures ± standard deviations.

definitely known whether ROI themselves are actually able to prevent the escape of listeriae from phagosomes to the cytoplasm by mechanisms other than the direct killing of listeriae in phagosomes, but IFN-γ, either alone or in combination with LPS, was shown to be an effective inducer of listericidal activity in these macrophage-like cell lines in this study. On the other hand, van Dissel et al. reported that IFN-γ, as a single activating stimulus, is not capable of activating the antilisterial effector function of peritoneal macrophages (51). Campbell et al. reported that IFN-γ causes a single population of fetal calf serum-elicited mouse macrophages to express tumoricidal but not listericidal activity (7). In our case, JA-4 cells, a macro-

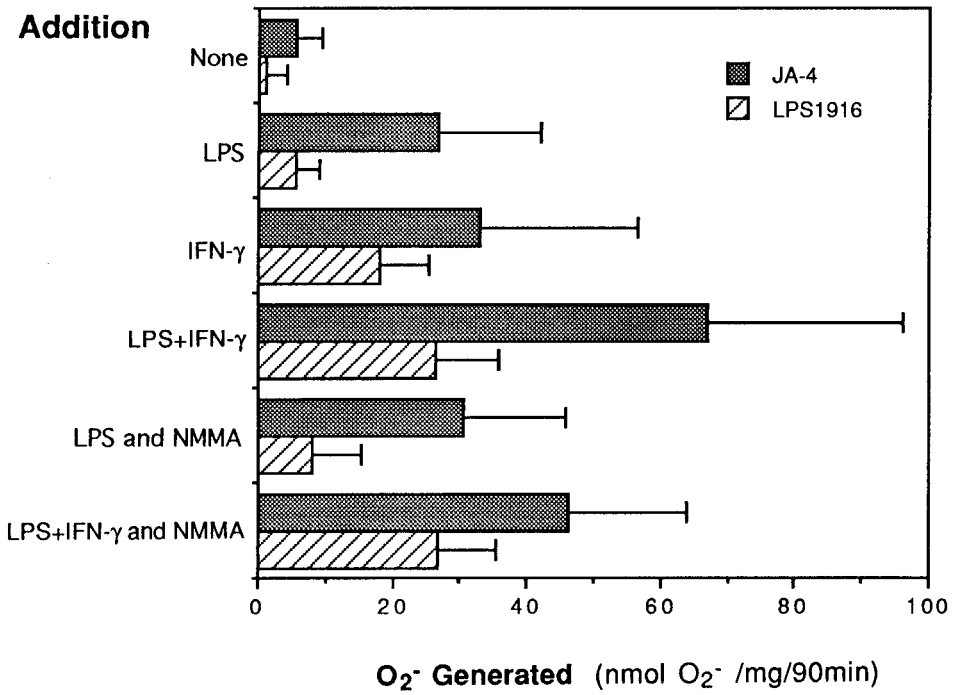

FIG. 6. O_2^- generation from JA-4 and LPS1916 cells treated with LPS and/or IFN-γ in the presence or absence of L-NMMA. Cells were precultured and then treated with 0.1 μg of LPS per ml and/or 10 U of IFN-γ per ml in the presence ■ or absence (▨ and ▧) of 1 mM L-NMMA at 37°C for 20 to 24 h. The assay for O_2^- generation was performed as described in the text. Values are means ± standard deviations for nine independent experiments.

phage-like cell line, treated with IFN-γ showed listericidal activity correlated with O_2^- generation.

As shown in Fig. 4 and 5, the inhibition of the intracellular growth of *L. monocytogenes* in these macrophage-like cell lines pretreated with only IFN-γ was not as strong as when they were pretreated with IFN-γ and LPS. The inhibition induced in LPS1916 cells with the combination of LPS and IFN-γ was about 1/10 of that in JA-4 cells. These results are well correlated with the levels of production of O_2^- in JA-4 cells and LPS1916 cells treated with LPS and IFN-γ (Fig. 6). Higginbotham et al. reported that IFN-γ plus LPS increased the ability of proteose-elicited macrophages to kill *L. monocytogenes*, probably by enhancing phagocytosis (24). However, no such elevation of phagocytosis was observed in our study after treatment of the cells with LPS and IFN-γ. Instead, our observations suggest that the listericidal effect after treatment with IFN-γ was enhanced in cells which were simultaneously stimulated by another signal, such as LPS, and this enhancement of the antilisterial activity seems to show some correlation with a mutated gene linked to the pathway of induction of O_2^- generation in LPS1916. In order to show definitively that reactive oxygen species are involved in the listericidal activity of these macrophage-like cell lines, we performed some preliminary experiments with *Listeria* infection as described for Fig. 1 but under anaerobic conditions with a sealed chamber gassed with nitrogen and with modified F12 medium supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-NaOH buffer (pH 7.5) and 2% FBS. After incubation at 37°C for 8 h, these conditions did not allow listeriae to multiply sufficiently even in control macrophages of both cell lines pretreated with medium alone (data not shown). These results suggest that oxygen-dependent metabolic pathways in macrophages are necessary for listerial invasion processes such as entry from phagosomes to cytoplasm or polymerization of actin. It was difficult, but still seems to be important, to show that ROI are definitively involved in the listericidal activity of macrophages by using these cell lines.

Listeriolysin O, an extracellular SH-activated hemolysin, is a major virulence factor in *L. monocytogenes*. Genetic studies and cell biological examinations demonstrated that the production of listeriolysin O would allow the escape of listeriae into the cytoplasm from phagosomes and support the survival of Listeriae in phagocytes (8, 28, 33). Listeriolysin O has also been reported to be a cytolytic as well as a granulolytic factor in *Listeria* infection, a major factor for expression of macrophage Ia, and an inducer of IL-1 (30, 42, 55). These reports suggest that listeriolysin O has diverse roles in *Listeria* infection. To examine the role of listeriolysin O in *Listeria* killing in macrophages, we observed the activity of JA-4 cells and LPS1916 cells in regard to the intraphagosomal killing of nonhemolytic, avirulent *L. monocytogenes* lacking the ability to escape from phagosomes. Nonhemolytic, avirulent *L. monocytogenes* ATCC 15313 could not proliferate in these macrophage cell lines, and the number of viable bacteria decreased in LPS1916 cells as well as in JA-4 cells (Fig. 5B). This complete killing was also observed in both cell lines infected with *Listeria innocua*, which is also a nonhemolytic, avirulent *Listeria* sp. (data not shown). However, LPS treatment did not enhance the listericidal activity of either cell line infected with nonhemolytic, avirulent *L. monocytogenes*, as was the case for the cells infected with hemolytic, virulent *L. monocytogenes* (Fig. 5). In addition, interestingly, the listericidal activity enhanced by IFN-γ treatment was stronger in LPS1916 cells than in JA-4 cells (Fig. 5B). These data suggest that the remarkable listericidal activity observed in JA-4 cells pretreated with IFN-γ and LPS is characteristic of infection by hemolytic, virulent *L. monocytogenes.*

Another oxidative effector molecule, the reactive nitrogen

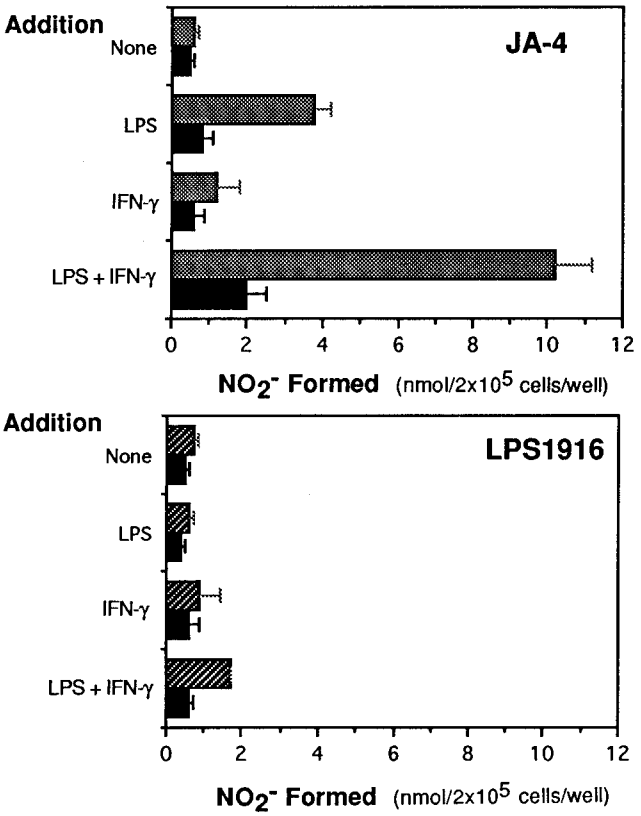

FIG. 7. NO_2^- formation by JA-4 and LPS1916 cells treated with LPS and/or IFN-γ in the presence or absence of L-NMMA. Cells were precultured and then treated with 0.1 μg of LPS per ml and/or 10 U of IFN-γ per ml in the presence or absence of 1 mM L-NMMA at 37°C for 20 to 24 h. The assay for NO_2^- recovered in the culture supernatants was performed as described in the text. Values are means ± standard deviations for three independent experiments.

oxide intermediate(s), exhibits antimicrobial activity in vitro against a variety pathogens, such as *Cryptococcus neoformans* (19), *Leishmania major* (38), *Schistosoma mansoni* (26), *Toxoplasma gondii* (1), *Trypanosoma musculi* (52), *Mycobacterium bovis* (13), and *Francisella tularensis* (3). For *L. monocytogenes*, in recent reports it was concluded that nitric oxide was a critical effector molecule in the killing of this bacterium by IFN-γ-activated macrophages (5, 14). However, our data in Fig. 5 show no effect on the killing of intracellular *L. monocytogenes* in both JA-4 and LPS1916 cells treated with L-NMMA, a competitive inhibitor of the L-arginine-dependent production of nitric oxide radicals by nitric oxide synthase in macrophages. As shown in Fig. 7, L-NMMA inhibited the production of NO_2^- in JA-4 cells treated with LPS and IFN-γ, although the production of O_2^- in JA-4 cells and LPS1916 cells was somewhat inhibited or slightly stimulated by L-NMMA after treatment with LPS and with IFN-γ or LPS, respectively (Fig. 6). Also, LPS1916 cells did not produce as much NO_2^- as JA-4 cells after treatment with LPS and IFN-γ (Fig. 7), although these cells killed listeriae significantly (Fig. 4). These results also suggest that nitrogen oxide is not a crucial effector molecule for *Listeria* killing. Higginbotham et al. reported no correlation between the production of reactive nitrogen intermediates and the listericidal activity expressed by elicited peritoneal macrophages in vitro (24). Gregory et al. also reported that reactive nitric oxide would inhibit the metabolic activity of immune cell populations as well, resulting in the suppression of the host defense to infection (20). In our study, reactive nitric oxide had no primary effect on the killing of intracellular *L. monocytogenes* in macrophage-like cell lines in vitro.

We have reported here that the reactive oxygen radicals are much more important than nitric oxide in the killing of intracellular *L. monocytogenes* and that listeriolysin O may play an important role in *Listeria* infection processes and in regulatory mechanisms for listericidal activity in macrophage cell lines. In this study, we showed some advantages of the use of a macrophage mutant which lacks the pathway for anti-*Listeria* infection after LPS treatment. By use of this mutant in combination with LPS and/or IFN-γ and also with listerial mutants with some definite genetic disorder in virulence, the mechanisms will be clarified more precisely in further studies on the intracellular killing of *L. monocytogenes* in macrophages together with the protective effect against listeriolysin O.

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