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A Lipopolysaccharide (LPS)-Resistant Mutant Isolated from a Macrophagelike Cell Line, J774.1, Exhibits an Altered Activated-Macrophage Phenotype in Response to LPS

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A bacterial lipopolysaccharide (LPS)-resistant mutant was isolated from murine macrophagelike cell line J774.1. The mutant showed selective resistance to LPS and lipid A and was almost 10^5- to 10^6-fold more resistant than the parent; it grew even in the presence of 1 mg of *Escherichia coli* O55:B5 LPS per liter, whereas the parent did not grow with less than 10 ng of LPS per milliliter. We next examined the mutant for activation of various functions of macrophages on LPS treatment. This LPS-resistant mutant secreted interleukin-1 and tumor necrosis factor almost as effectively as the parent did. The mutant cells also changed transiently from a round to a spread form; however, they became round again afterwards. The mutant cells secreted less arachidonic acid in response to LPS. These results also suggest that this LPS-resistant mutant responds to LPS and shows activation of some macrophage functions. However, this mutant did not exhibit elevation of O_2^- generation or H_2O_2 generation after LPS treatment. Also, treatment of the mutant cells with murine recombinant gamma interferon was partly able to correct the defect in O_2^--generating activity in response to LPS, suggesting that this defect is probably due to some of the LPS signal pathways. This implies that there is some correlation between O_2^- metabolism in LPS-activated macrophages and decreases in cell growth and viability.

Macrophages are known to be activated by a series of natural products known as biological response modifiers (13) and acquire new functions important for immune system and defense mechanisms. Among these products, bacterial lipopolysaccharides (LPS) are some of the strongest activators of macrophages (4, 14). Treatment of macrophages with LPS directly elevates some macrophage functions, and this effect of LPS has been attributed to the lipid A portion of LPS (9, 18). Previously, we showed that murine macrophagelike cell line J774.1 is activated in vitro by LPS, lipid A, and monosaccharide precursors of lipid A, i.e., lipids X and Y (6, 7, 19, 23). However, the molecular mechanisms underlying macrophage activation by LPS and/or lipid A are little understood.

It is also well known that treatment of macrophages with higher concentrations of LPS leads to macrophage dysfunction and death (2, 21). In macrophages, expression of LPS binding due to lymphokines or gamma interferon (IFN-γ) seems to be closely correlated with the LPS-induced cytotoxicity of macrophages (1–3). However, little is known about the mechanisms underlying these cytotoxic effects of LPS on macrophages.

To elucidate the mechanisms underlying the LPS action on macrophages and to investigate the correlation between LPS activation of macrophages and LPS cytotoxicity toward macrophages, we tried to isolate LPS-resistant mutants from the J774.1 macrophagelike cell line with LPS-binding activity. Here we report that an LPS-resistant mutant, LPS1916, was isolated and that this mutant showed selective resistance to LPS and lipid A. As for LPS responses, this mutant released interleukin-1 (IL-1), tumor necrosis factor (TNF), and arachidonic acid. However, O_2^--generating activity was greatly reduced in LPS-treated mutant cells.

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MATERIALS AND METHODS

Materials. *Escherichia coli* O55:B5 LPS was obtained from Difco (Detroit, Mich.); LPS from the *E. coli* J5 (Rc) mutant and *Salmonella minnesota* R595 (Re) and fluorescein isothiocyanate-labeled LPS were from List Biological Laboratories (Campbell, Calif.); and synthetic *E. coli* lipid A (no. 506) was from Daiichi Pure Chemicals (Tokyo, Japan). In this report, LPS means that from *E. coli* O55:B5 throughout unless otherwise stated. Ham's F12 medium was purchased from Flow Laboratories (McLean, Va.), and fetal bovine serum (FBS) containing less than 60 pg of LPS per ml was obtained from GIBCO (Grand Island, N.Y.) and used throughout these experiments. Zymosan from *Saccharomyces cerevisiae*, calcium ionophore A23187, phorbol myristate acetate (PMA), cytochrome *c* from horse heart, superoxide dismutase (SOD) from bovine liver, horseradish peroxidase, scopoletin, and diisopropyl fluorophosphate were obtained from Sigma (St. Louis, Mo.); [1-^{14}C]arachidonic acid was purchased from New England Nuclear (Boston, Mass.); and [*methyl*-^{3}H]thymidine was from Amersham (Amersham, United Kingdom). Recombinant murine IFN-γ was a generous gift from K. Akagawa (1–3). All other reagents and chemicals were of the purest commercial grade available.

Cell culture and isolation of LPS-resistant mutants. Murine macrophagelike cell line J774.1 is composed of a heterogeneous mixture of cells with regard to morphology and response to LPS. To obtain a homogeneous cell population, we isolated a subline of J774.1, JA-4, which was cloned twice. JA-4 cells were cultured in Ham's F-12 medium containing 50 U of penicillin per ml, 50 μg of streptomycin (Flow Laboratories, Irvine, Scotland), and 10% (vol/vol) heat-inactivated FBS in a CO_2 incubator (5% CO_2–95% humidified air) at 37°C. For mutagenesis, cells were seeded at 5×10^5/10 ml in T-75 flasks (Corning/Iwaki Glass, Tokyo, Japan) and then incubated at 37°C overnight. The medium

was replaced with 10 ml of fresh medium containing 400 μg of ethyl methanesulfonate (Sigma), and the cells were incubated at 37°C for 18 h. The cells were then rinsed three times with medium and incubated in 10 ml of fresh medium per flask at 37°C for 4 days. For selection of LPS-resistant mutants, cells were detached by trypsinization, reseeded at 10^6/100-mm-diameter dish per 10 ml of medium, and then incubated at 37°C for 2 h to allow the cells to adhere to the dishes. *E. coli* O55:B5 LPS (100 μg/ml) was then added to the dishes, and the cells were incubated at 37°C for 7 to 10 days, until LPS-resistant colonies appeared. During this period, the medium was replaced every 3 to 4 days with fresh medium containing 100 μg of LPS per ml (selective medium). Finally, LPS-resistant colonies were picked up with steel rings and maintained in the selective medium. Fifty-nine colonies were formed from 5×10^6 cells, and the frequency of LPS resistance was about 1.5×10^{-5}. Twelve colonies were picked up, one of which, LPS19, showed high resistance to LPS. We recloned a subline from the LPS19 strain with 1 mg of LPS per ml, which we designated LPS1916. We used the latter mutant throughout this study, because it showed a stable LPS resistance phenotype, even in nonselective medium without LPS for more than 6 months, and it grew almost at the same rate as the parent, JA-4. All of the cells used in this experiment were maintained in nonselective medium for at least a month before assays.

Assays for LPS resistance and drug resistance. Resistance to LPS and other reagents was tested by three methods. Method 1 was the colony-forming assay, in which 400 cells were seeded in 5 ml of medium in plastic petri dishes and incubated at 37°C for 2 h. Various concentrations of LPS were then added, and the cells were incubated further at 37°C for 7 days in a CO_2 incubator. Finally, the medium was discarded and the colonies on the dishes were stained with 0.5% methylene blue in 50% ethanol as previously described (17). Method 2 was a spot test for rapid assaying in which 2×10^4 cells in 0.5 ml of medium were seeded into the wells of a 24-well clustered microplate (Costar, Cambridge, Mass.) and LPS and other reagents were added after 2 h of incubation at 37°C. The cells were incubated further for 3 days and then stained with 0.5% methylene blue in 50% ethanol as described above. Method 3 was a cell counting method in which 5×10^3 cells in 1 ml of medium were seeded into the wells of a 12-well clustered microplate (Costar) and incubated at 37°C for 4 h. LPS and other reagents were then added, and the cells were incubated further for 1 to 6 days. Usually after 5 days, the medium was removed and the cells were trypsinized and detached from the plates. The number of cells was determined with a Coulter Counter (Coulter Electronics, Hialeah, Fla.) as a suspension in Isoton II.

Assay of IL-1 and TNF. Cells were seeded at 10^6/ml of medium into the wells of a 12-well microplate and incubated at 37°C overnight. The medium was replaced with 1 ml of fresh medium containing 0 or 0.1 to 10 μg of LPS per ml, and the cells were further incubated at 37°C for 24 h. The culture supernatant was collected in a microcentrifuge tube and centrifuged at 10,000 rpm for 1 min at 4°C in an MRK-150 microcentrifuge (Tomy, Tokyo, Japan), and 0.5 ml of the supernatant was collected from the top and assayed for IL-1 and TNF. IL-1 was assayed by the method of Oppenheim et al. (20) with slight modifications. Four-week-old, specific-pathogen-free, male ICR mice (Nippon SLC, Hamamatsu, Japan) were sacrificed, and their thymuses were excised and minced in sterile ice-cold phosphate-buffered saline without divalent cations. The thymus cells were filtered through nylon mesh (400 mesh) and collected as a pellet by centrifugation (Partner-sp centrifuge; Tomy, Tokyo, Japan) at 1,000 rpm for 10 min at 4°C. The cells were suspended at 2×10^6/ml in RPMI–10% FBS. The assay mixture, comprising 2×10^5 thymus cells, 2.5 μg of concanavalin A (EY Laboratories, San Mateo, Calif.) per ml, and 20 μl of macrophage culture supernatant in 200 μl of RPMI–10% FBS, was poured into the wells of a 96-well clustered microplate (Costar). Twenty microliters of [^{3}H]thymidine (0.5 μCi per well in RPMI–10% FBS) was added, and the mixture was incubated at 37°C for 18 h. Radioactivity incorporated by thymus cells was recovered on a glass fiber filter with a Cell Harvester (Labo Science, Tokyo, Japan) and counted in a vial of Omnifluor (New England Nuclear)-toluene solution with a liquid scintillation counter (LS3801; Beckman). The biological activity of TNF was assayed against L929 cells as described previously (7). In brief, L929 cells were seeded at 3×10^6/10 ml of RPMI–10% FBS and then labeled with 50 μCi of [^{3}H]thymidine at 37°C for 18 h. The L929 cells were washed thoroughly with phosphate-buffered saline without divalent cations and detached by trypsinization. The cells were suspended in RPMI–10% FBS at 8×10^4/ml, and 100-μl aliquots of the cells were mixed with 100-μl aliquots of serially diluted macrophage culture supernatants in the wells of a 96-well clustered microplate. The plate was incubated at 37°C for 3 days, the remaining cells were collected on the glass fiber filter of a Cell Harvester, and the radioactivity of the cells was counted as described above. TNF activity was quantitated by means of titration curves, which crossed the 50% line of the control with no macrophage culture supernatant.

Morphologic observation. Cells were observed under a phase-contrast microscope (Diamat; Nikon, Tokyo, Japan), and photographs were taken on black and white film (Neopan F; Fuji Film, Tokyo, Japan) with a Nikon FE camera.

Arachidonic acid release. Cells were seeded at 2×10^5/0.5 ml of F12–10% FBS in the wells of a 24-well clustered microplate and then incubated at 37°C overnight. The medium was replaced with 0.5 ml of fresh medium containing 0.1 μCi of [1-^{14}C]arachidonic acid per ml, and the cells were incubated at 37°C for 4 h. The cells were rinsed with medium three times and incubated again in 0.5 ml of fresh medium containing 10 μg of LPS per ml, 0.5 mg of zymosan per ml, 25 μM calcium ionophore A23187 or 5 μg of PMA per ml at 37°C for 4 h. The culture supernatants were collected and centrifuged (Tomy MRK-150 microcentrifuge) in microcentrifuge tubes at 10,000 rpm for 1 min at 4°C, and 0.25-ml aliquots of the supernatants were mixed with ACS II (Amersham). Radioactivity was counted with a Beckman liquid scintillation counter as described above.

O_2^- generation. O_2^- generation was examined as described before (6). Cells were seeded at 2×10^5/0.5 ml of medium in the wells of a 24-well clustered microplate and then incubated at 37°C overnight. The medium was replaced with 0.5 ml of fresh medium containing 0 or 10^{-3} to 10 μg of LPS per ml, and the cells were incubated at 37°C for 24 h. The cells were washed three times with modified Hanks balanced salt solution containing 8.0 g of NaCl per liter, 0.4 g of KCl per liter, 1.0 g of glucose per liter, 0.06 g of $Na_2HPO_4 \cdot 2H_2O$ per liter, 0.06 g of KH_2PO_4 per liter, and 0.35 g of $NaHCO_3$ per liter and examined for O_2^- generation in 0.5 ml of a reaction mixture comprising 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.625 mg of cytochrome *c* per ml with or without 0.03 mg of SOD in 0.5 ml of Hanks balanced salt solution. The reaction was started by addition of 5 μg of PMA per ml, continued at 37°C for 90 min, and stopped by

sudden chilling on ice. The microplates were centrifuged (Tomy Partner-sp centrifuge) at 3,000 rpm for 5 min at 4°C, and the A_{550}s of the supernatants were measured in a UV-160 photometer (Shimadzu, Kyoto, Japan). The differences in A_{550} between the samples without and with SOD were determined, and the amounts of O_2^- generated 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 in wells recovered after washing off of the reaction mixture and quantitated by the method of Lowry et al. (16).

H_2O_2 generation. H_2O_2 generation was examined by the scopoletin fluorescence assay (22) with slight modifications. The cells were treated with LPS as described above for the O_2^- generation method, and the assay mixture for measurement of H_2O_2-generating activity was also similar to that for O_2^- generation, except that cytochrome *c* and SOD were not used but instead 2 μM scopoletin and 20 nM horseradish peroxidase were added. The reaction was started by addition of PMA as a stimulant and was performed at 37°C for 90 min in the microplates. Finally, the microplates were centrifuged (Tomy Partner-sp centrifuge) at 3,000 rpm for 5 min at 4°C, and fluorescence emission at 460 nm with an excitation wavelength of 350 nm was monitored in a Hitachi F-3000 fluorescence spectrophotometer. The amounts of H_2O_2 released from the cells were determined with a standard curve based on the emission decrease of scopoletin in the reaction mixture without cells by the known amounts of H_2O_2. The results were expressed as specific activities as described for the methods used to measure O_2^- generation.

RESULTS

LPS resistance of mutant cells. We examined the effect of LPS on cell growth by determining the cell number. As shown in Fig. 1A, JA-4 and LPS1916 cells grew at similar rates in the absence of LPS. However, growth of JA-4 cells was greatly inhibited by 0.1 μg of LPS per ml, while that of LPS1916 cells was not influenced by 10 μg of LPS per ml, with which JA-4 cells did not grow at all in a prolonged culture. By this method, we estimated the LPS resistance of these cells after 5 days of incubation. The 50% inhibitory doses were 7.9×10^{-4} μg/ml for JA-4 cells and 5.9×10^2 μg/ml for LPS1916 cells (Fig. 1B). These results suggest that LPS1916 cells are about 7×10^5-fold more resistant to LPS than are JA-4 cells and that this mutant can grow with high concentrations of LPS.

We next examined LPS resistance by means of the colony formation assay, which is one of the most stringent assays for resistance (17). As shown in Fig. 2, parental JA-4 cells formed a number of steady colonies in the absence of LPS; however, they formed only a few colonies in the presence of 10 μg of LPS per ml and no colonies at all in the presence of more than 300 μg of LPS per ml. On the other hand, LPS1916 cells formed rather small colonies in the absence and in the presence of 300 μg of LPS per ml. With 1 mg of LPS per ml, the mutant formed very tiny but steady colonies, although the number of colonies was reduced. Quantitative analysis of the relative cloning efficiency showed that the 50% inhibitory doses for LPS were less than 10 μg/ml and 4.6×10^2 μg/ml for JA-4 and LPS1916 cells, respectively (data not shown). These results suggest that LPS1916 cells are highly resistant to LPS and can even form colonies in the presence of high concentrations of LPS.

Cross-resistance to various LPS, lipid A, and other toxic substances. The selectivity of the LPS resistance and the cross-resistance to various LPS and lipid A were examined in LPS1916 cells. First, we tested various LPS of different origins and structures and a chemically synthesized lipid A. In addition to the smooth-type LPS from *E. coli* O55;B5, which we used for isolation and selection of LPS-resistant

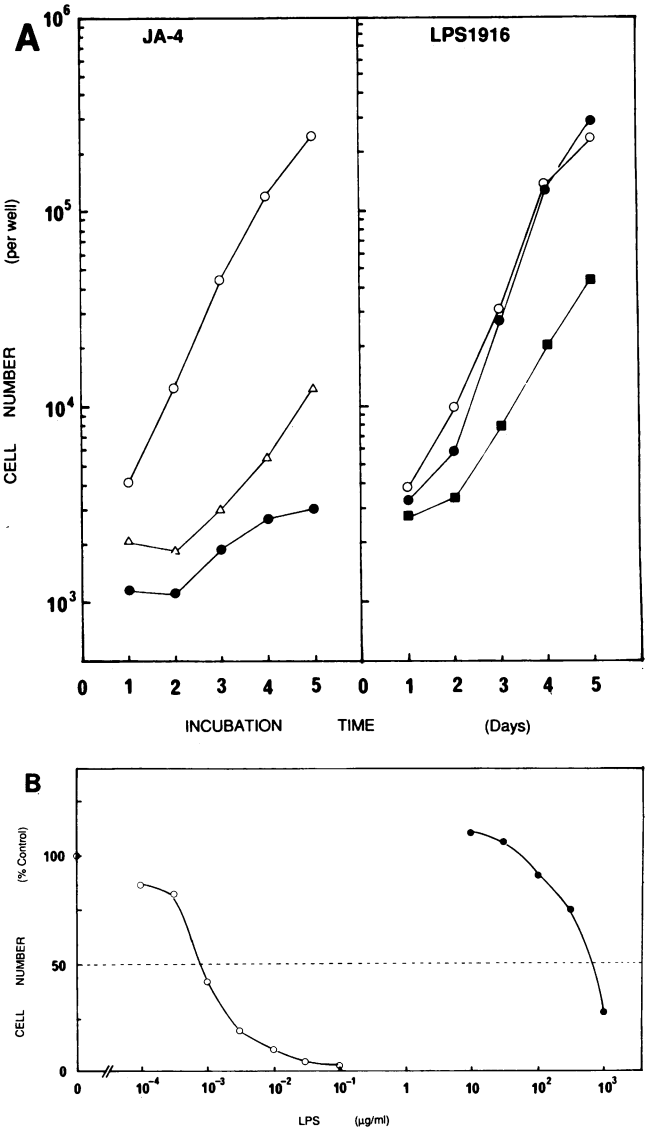

FIG. 1. Effects of LPS on cell growth. (A) Cell growth curves in the presence of LPS. Five thousand cells were seeded on day 0, and LPS was added 2 h later, at 0 (○), 0.1 (△), 10 (●) and 1,000 (■) μg/ml, followed by incubation at 37°C for 5 days. Numbers of adherent cells were determined every day after trypsinization of the cells as described in the text. The values are means for duplicate samples in a typical experiment. (B) LPS resistances of JA-4 and LPS1916 cells. JA-4 (○) and LPS1916 (●) cells were grown in the wells of 12-well clustered microplates at 37°C for 5 days in the absence or presence of LPS, the cells were detached from the culture plates by trypsinization, and cell numbers were determined as described for panel A. The values shown are relative to those of the controls without LPS, and the 50% lethal doses were obtained from the points where the curves crossed the 50% line. The results are means of two (JA-4) or four (LPS1916) independent experiments involving duplicate assays.

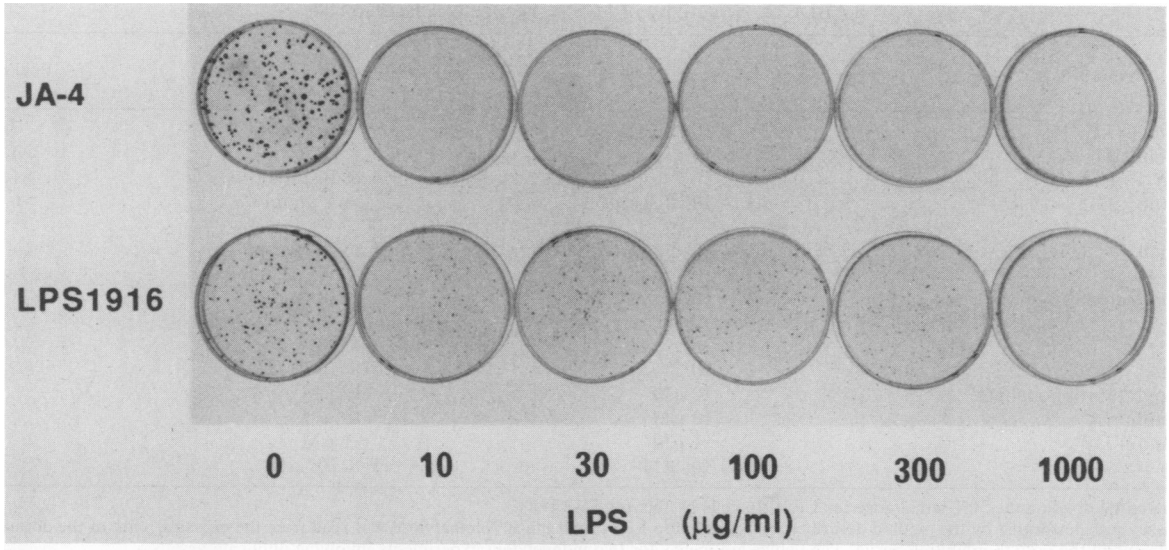

FIG. 2. Colony formation of JA-4 and LPS1916 cells in the presence of LPS. Four hundred cells were incubated in the presence of various concentrations of LPS at 37°C for 7 days and then stained with methylene blue as described in the text. This photograph was taken from the bottom of the dishes in a typical experiment. The numbers of colonies in the control dishes were 263 ± 14 for JA-4 cells and 308 + 2 for LPS1916 cells.

mutants, LPS1916 cells also showed high resistance to LPS from the *E. coli* J5 mutant (RcLPS), ReLPS from *S. minnesota*, and synthetic lipid A (no. 506) (Fig. 3). These results suggest that LPS1916 cells have similar resistances to various LPS and lipid A, although the resistance to lipid A seemed to be a little less than that to LPS (Table 1).

We next examined the cross-resistance to other toxic substances, with structures different from those of LPS and lipid A, which have been suggested to affect macrophages. Carageenan, PS-K, *Pseudomonas* exotoxin, colchicine, vincristine, diisopropyl fluorophosphate, prostaglandins E_2 and D_2, and H_2O_2 all showed similar toxicities for both JA-4 and LPS1916 cells (Table 1). These results show that LPS1916 cells show highly selective cross-resistance to various LPS and synthetic lipid A, suggesting that the structure of lipid A is involved in this selective resistance.

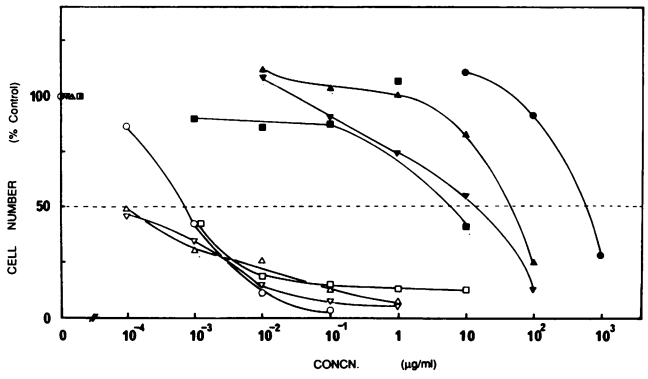

FIG. 3. Cross-resistance to various LPS and lipid A. JA-4 (○, △, ▽, and □) and LPS1916 (●, ▲, ▼, and ■) cells were grown in the presence or absence of LPS from *E. coli* O55:B5 (○ and ●), LPS from the *E. coli* J5 mutant (△ and ▲), ReLPS from *S. minnesota* (▽ and ▼), and chemically synthesized lipid A no. 506 (□ and ■) at 37°C for 5 days, and cell numbers were counted. Each point represents the mean for two independent experiments involving duplicate assays. The symbols ◑, ▽, △, and ◨ indicate the control values without addition of LPS or lipid A.

Morphological changes caused by LPS. To detect the LPS responses of mutant cells, morphological changes were observed under a phase-contrast microscope after LPS treatment (Fig. 4). JA-4 and LPS1916 control cells without LPS looked similar in shape and size. However, LPS at 10 μg/ml caused great differences in their sizes and shapes; while JA-4 cells became spread and/or elongated with numerous vacuoles and granules inside, LPS1916 cells did not become elongated or spread but mostly remained round with some protrusions. The latter cells seemed to become more adherent, with a few vacuoles, but their sizes were not so different from those of control cells without LPS. Even at higher concentrations of LPS, such as 0.3 to 1 mg/ml, LPS1916 cells did not become enlarged or elongated, as JA-4 cells did with 10 μg of LPS per ml. These results show that LPS1916 cells change slightly in shape and size in response to LPS, but the extents of these changes are far less than in the case of the parental JA-4 cells, suggesting that LPS1916 cells exhibit reduced morphological changes in response to LPS.

Secretion of IL-1 and TNF. We next examined the secretion of IL-1 and TNF by the mutant cells. Supernatants of macrophage cultures with or without 0.1 to 10 μg of LPS per ml were assayed for IL-1 and TNF as described in Materials and Methods. To test IL-1 secretion, mouse thymocytes were mixed with concanavalin A and the macrophage culture supernatants, and then incorporation of [^{3}H]thymidine was examined (Fig. 5). LPS1916 cells secreted increasing amounts of IL-1 with increasing concentrations of LPS, each amount being higher (1.5 to 1.8-fold) than the corresponding one with JA-4 cells.

To test TNF secretion, macrophage culture supernatants were serially diluted with fresh medium and mixed with [^{3}H]thymidine-labeled L929 cells. Remaining radioactivity in L929 cells, which decreased on incubation with the macrophage culture supernatants containing TNF, was quantitated

TABLE 1. Selectivity of LPS resistance of LPS1916 cells

Reagent	50% lethal dose for:		Fold resistance[a]
	JA-4	LPS1916	
LPS (*E. coli* O55:B5)[b]	7.9×10^{-4} μg/ml	5.9×10^{2} μg/ml	7.5×10^{5}
LPS (*E. coli* J5)[b]	$<1 \times 10^{-4}$ μg/ml	4.3×10 μg/ml	$>4.3 \times 10^{5}$
ReLPS (*S. minnesota*)[b]	$<1 \times 10^{-4}$ μg/ml	1.4×10 μg/ml	$>1.4 \times 10^{5}$
Lipid A (no. 506)[b]	$<1 \times 10^{-3}$ μg/ml	6.4 μg/ml	$>6.4 \times 10^{3}$
Carageenan[c]	$\sim 1 \times 10^{2}$ μg/ml	$\sim 1 \times 10^{2}$ μg/ml	~1
PS-K[c]	$\sim 1 \times 10^{2}$ μg/ml	$\sim 1 \times 10^{3}$ μg/ml	~10
Pseudomonas exotoxin[c]	$\sim 1 \times 10^{2}$ μg/ml	$\sim 1 \times 10^{2}$ μg/ml	~1
Colchicine[c]	$\sim 1 \times 10^{-1}$ μM	$\sim 1 \times 10^{-1}$ μM	~1
Vincristine[c]	$\sim 5 \times 10^{-3}$ μM	$\sim 5 \times 10^{-3}$ μM	~1
Diisopropyl fluorophosphate[c]	~10 μM	~10 μM	~1
Prostaglandin E_2[c]	~10 μM	~10 μM	~1
Prostaglandin D_2[c]	~1 μM	~1 μM	~1
H_2O_2[c]	$\sim 1 \times 10^{3}$ μM	$\sim 1 \times 10^{3}$ μM	~1

[a] Calculated by dividing the 50% lethal dose for LPS1916 cells by that for JA-4 cells.

[b] This assay was performed by the method described in the legend to Fig. 1, and the 50% lethal dose was read from the crossing point of the dotted line (50% of the control) and the growth inhibition curve. The values are means for two independent experiments involving duplicate assays.

[c] This assay was performed by using the spot test as described in the text. The 50% lethal doses were determined from changes in cell density, with staining with methylene blue. Essentially the same results were obtained in spot tests and by the cell counting method, as shown in Fig. 1 and the upper protion of this table. The values are means for two independent experiments.

and plotted as a titration curve (Fig. 6A). The amounts of TNF, in arbitrary units, were determined as described in Materials and Methods and are plotted in Fig. 6B. LPS1916 cells secreted TNF, the amount of which increased with LPS treatment. Although the amounts of TNF secreted by LPS1916 cells were somewhat lower than those secreted by JA-4 cells (83 to 55%), LPS1916 cells secreted substantially more TNF in response to LPS than did control cells without LPS.

These results show that LPS1916 cells secrete both IL-1

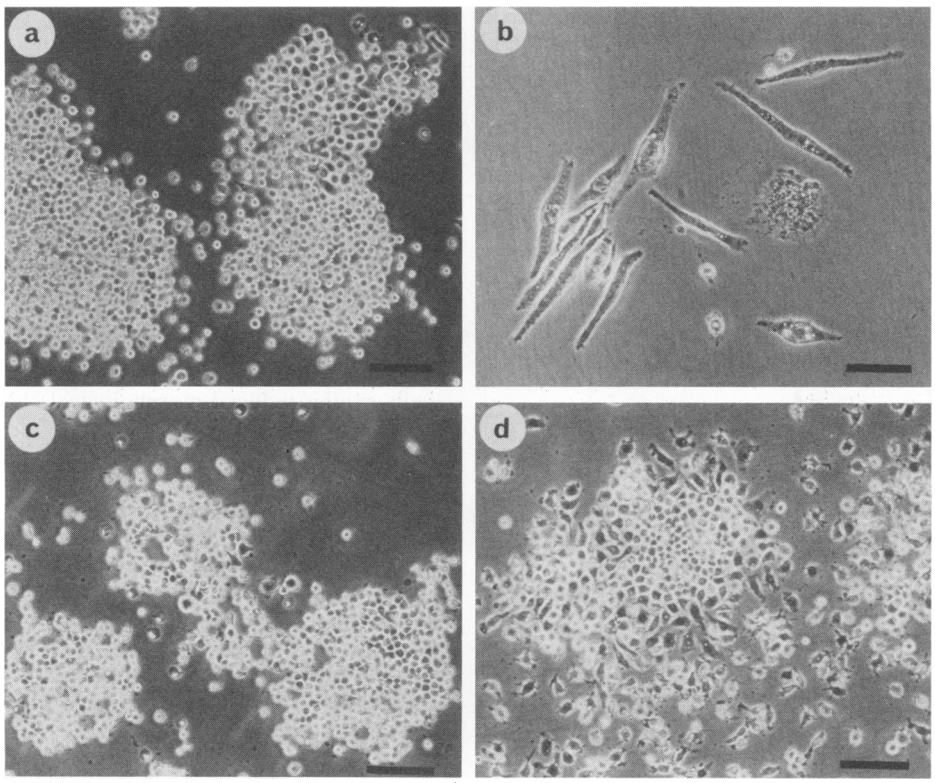

FIG. 4. Morphological changes of cells in response to LPS. Both JA-4 (a and b) and LPS1916 (c and d) cells were treated without (a and c) or with (b and d) 10 μg of LPS per ml for 5 days as described in the legend to Fig. 2. The photographs were taken as described in the text, and representative fields are shown here. Bar, 100 μm.

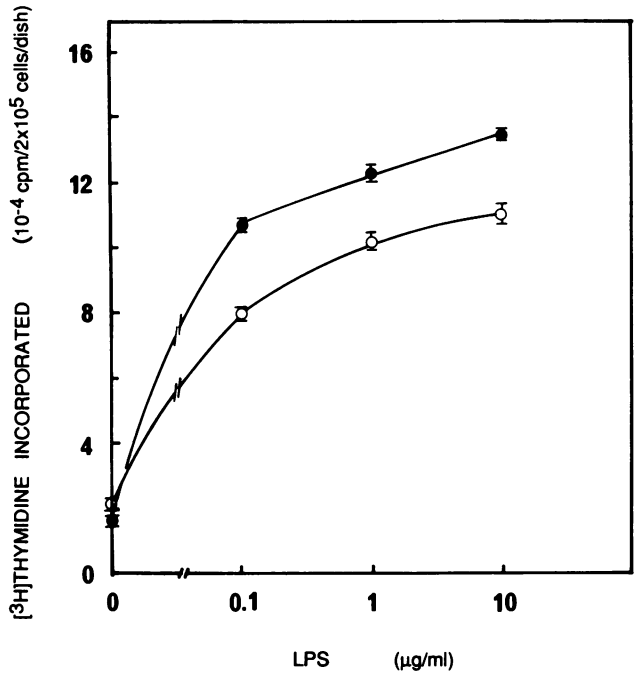

FIG. 5. Secretion of IL-1 by JA-4 and LPS1916 cells in response to LPS. Culture supernatants were obtained from JA-4 (○) and LPS1916 (●) cells and mixed with mouse thymocytes and concanavalin A at a final concentration of 10%. They were then mixed with 0.5 μCi of [^{3}H]thymidine per well, and each mixture was incubated at 37°C for 18 h. The radioactivity incorporated in thymocyte DNA was trapped on a glass fiber filter with a cell harvester and counted with a liquid scintillation counter as described in the text. The values are means ± standard errors for four independent experiments involving duplicate assays. The value for a negative control with no macrophage culture supernatant but with fresh medium was (1.15 ± 0.05) × 10^{-4} cpm/2 × 10^5 cells per 18 h.

and TNF on treatment with LPS and suggest that LPS1916 cells exhibit LPS responses.

Arachidonic acid release. Release of arachidonic acid and its metabolites was examined as a function of activated macrophages (24). More [^{14}C]arachidonic acid label was released by LPS-treated cells than by nontreated controls of both types of cells, but the extent of the release was greater in JA-4 cells than in LPS1916 cells (Fig. 7). Similar results were obtained on zymosan treatment, but little or no difference was seen on treatment of these cells with ionophore A23187 or PMA. These results suggest that LPS1916 cells exhibit an LPS response with respect to arachidonic acid release as well, but the extent of the release is somewhat decreased compared with the levels selectively released by JA-4 cells on LPS and zymosan treatment.

Induction of O_2^- and H_2O_2 generation by JA-4 and LPS1916 cells. O_2^- generating activity is one of the good criteria for detection of activated macrophages (6), and it was very low in both JA-4 and LPS1916 cells without LPS treatment (Fig. 8). Incubation of JA-4 cells with LPS at 37°C for 24 h induced O_2^--generating activity in a dose-dependent manner, with maximal activity at 0.1 μg/ml. However, O_2^- generation by LPS1916 cells was not noticeably induced by LPS in the range of LPS concentrations shown in Fig. 8. Similar results were obtained in another set of experiments in which zymosan was used as an inducer of the respiratory burst instead of the PMA used in this experiment. To determine whether the defect in O_2^- generating activity in LPS-treated mutant cells was caused by a block in the single-electron reduction of O_2, we assayed PMA-stimulated H_2O_2 release from both JA-4 and LPS1916 cells treated with various amounts of LPS (Fig. 9). As with O_2^- generation (Fig. 8), H_2O_2- generating activity was remarkably elevated in LPS-treated JA-4 cells but not in LPS-treated LPS1916 cells. These results suggest that induction of H_2O_2-generating activity in LPS1916 cells by LPS is also defective and

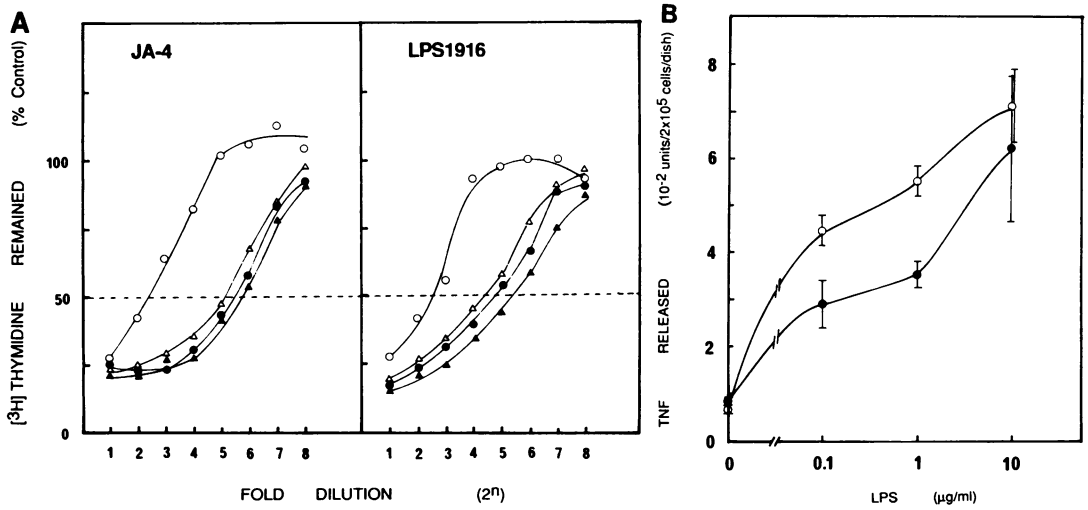

FIG. 6. Secretion of TNF by JA-4 and LPS1916 cells in response to LPS. (A) Titration curves for TNF. Macrophage culture supernatants were prepared after treatment of cells with 0 (○), 0.1 (△), 1 (●), or 10 (▲) μg of LPS per ml as described in the text. In each experiment, lane 1 finally contained 20% of the culture supernatant and lane 2 finally contained 10% of the supernatant. The other lanes contained culture supernatants serially diluted with fresh medium. One unit of TNF activity was taken as the amount of the supernatant corresponding to the crossing point between each titration curve and the 50% line of the control without culture supernatant. The values are means for three independent experiments involving duplicate assays. (B) TNF release from parent and mutant macrophages. As determined from the titration curves for TNF in panel A, the total amounts of TNF recovered in the culture supernatants of JA-4 (○) and LPS1916 (●) cells are shown here. The abscissa shows the concentrations of LPS used for macrophage activation. The values are means ± standard errors for three independent experiments.

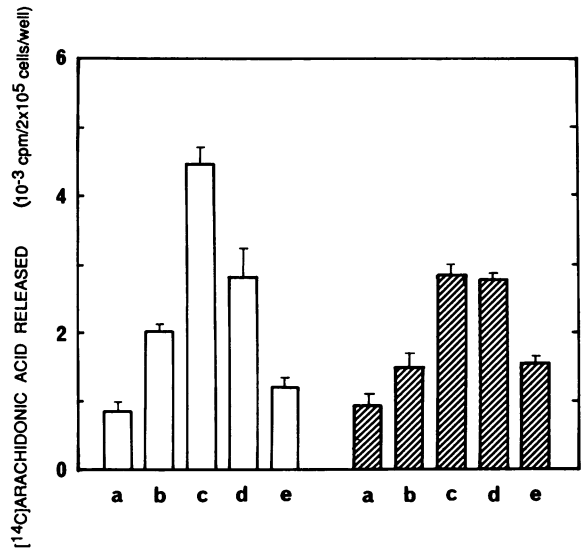

FIG. 7. Arachidonic acid release by JA-4 and LPS1916 cells. JA-4 (open bars) and LPS1916 (hatched bars) cells were labeled with [^{14}C]arachidonic acid at 37°C for 4 h, washed, and incubated further at 37°C for 4 h alone (a) or in the presence of 10 μg of LPS per ml (b), 0.5 mg of zymosan per ml (c), 25 μM A23187 (d), or 5 μg of PMA per ml (e). The released radioactivity was counted as described in the text. The values are means ± standard errors for three independent experiments involving duplicate assays. The total amounts of radioactivity incorporated by JA-4 and LPS1916 cells did not significantly differ between experiments (data not shown).

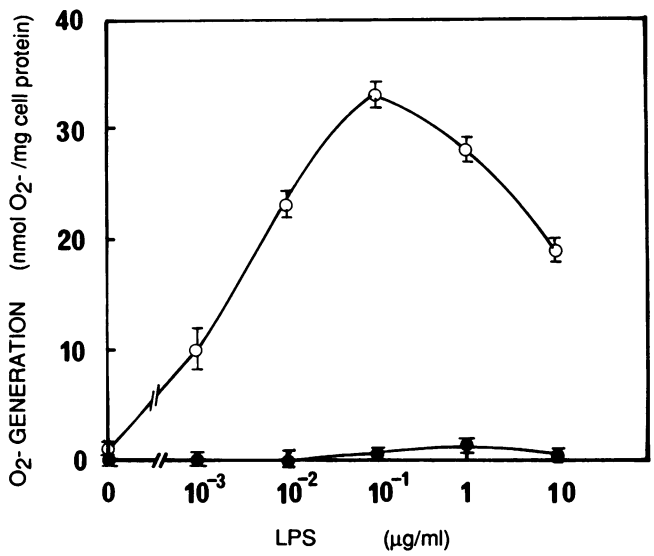

FIG. 8. Induction of O_2^- generating activity by LPS. JA-4 (○) and LPS1916 (●) cells were treated with various concentrations of LPS, as shown on the abscissa, at 37°C for 24 h as described in the text. O_2^- generation was examined in modified Hanks balanced salt solution as SOD-sensitive cytochrome *c* reduction at 37°C for 90 min. The results are expressed as specific activities of the cells. The values are means ± standard deviations for two independent experiments involving duplicate assays.

that the lack of induction of O_2^- generating activity in LPS1916 cells by LPS is due not to the decrease in the steady state of O_2^- by SOD but rather the block in the initial reaction of O_2^- metabolism, namely, single-electron reduction of O_2.

We next tested the effect of IFN-γ on LPS-induced O_2^- generating activity. Addition of 10 U of IFN-γ per ml, which had only a little effect on induction of O_2^- generating activity, to cultures of JA-4 and LPS1916 cells simultaneously with LPS showed significant effects on both cell lines (Fig. 10). In JA-4 cells, the peak of O_2^- generation became sharper and higher, and the optimal dose of LPS was shifted from 0.1 to 0.001 μg/ml by IFN-γ treatment. In LPS1916 cells, however, a shift of the optimal dose of LPS due to IFN-γ treatment did not occur, although the levels of O_2^- generation were greatly enhanced by IFN-γ, compared with those in JA-4 cells, in the LPS concentration range of 0.01 to 10 μg/ml. These results suggest that IFN-γ was able to correct the defect in the induction of O_2^- generating activity by LPS in the mutant cells but that there are still some differences in the effects of IFN-γ on JA-4 and LPS1916 cells in terms of sensitization of these cells to smaller amounts of LPS.

DISCUSSION

In this report, we describe the isolation of an LPS-resistant mutant, LPS1916, from a subline of a macrophage-like cell line, J774.1. The mutant is highly resistant to *E. coli* LPS (Fig. 1 and 2), showing cross-resistance to other LPS and lipid A (Fig. 3 and Table 1) and LPS responses which could reflect such criteria of activated macrophages as production of IL-1 (Fig. 5) and TNF (Fig. 6) and release of arachidonic acid (Fig. 7). However, some of the typical changes observed in macrophage activation (19, 23) were not evident in the case of this mutant; morphological changes were not as great as those of the parental cells (Fig. 4), and O_2^- generation and H_2O_2 generation were very low (Fig. 8 and 9). These characteristics of these mutant cells seem to be useful for elucidating (i) the mechanisms underlying LPS resistance and (ii) the mechanisms underlying macrophage activation due to LPS.

As for the mechanisms underlying LPS resistance, the lack of LPS binding could be one of the clearest explanations for this mechanism. In fact, we have recently established another LPS-resistant mutant that exhibits defective binding of LPS and thus has no responses to LPS (19). However, such a mutant is not in our present scope for studying the mechanisms of LPS-macrophage interactions, and preliminary experiments showed similar binding of fluorescein isothiocyanate-labeled LPS by parental and LPS1916 cells (data not shown). Another explanation for LPS resistance is that some products of activated macrophages become toxic to the cells and thus prevent growth (12). Prostaglandins D_2 and E_2 and H_2O_2 could be such toxic substances, because J774.1 cells produce prostaglandins E_2 (19) and D_2 (5) and H_2O_2 (Fig. 9) on treatment with LPS. However, none of these substances showed selective toxicity toward either LPS1916 or JA-4 cells (Table 1). Additional experiments should be performed to determine whether or not LPS1916 cells exhibit a deficiency in the production of these substances when LPS is added to the culture. In this regard, the much reduced O_2^- generating activity of LPS-treated mutant cells (Fig. 8) may be a clue to this question, because O_2^- rapidly becomes H_2O_2 in water and is easily trapped in the unsaturated lipids of the membrane. Differences between the parent and mutant cells in the susceptibility of growth to

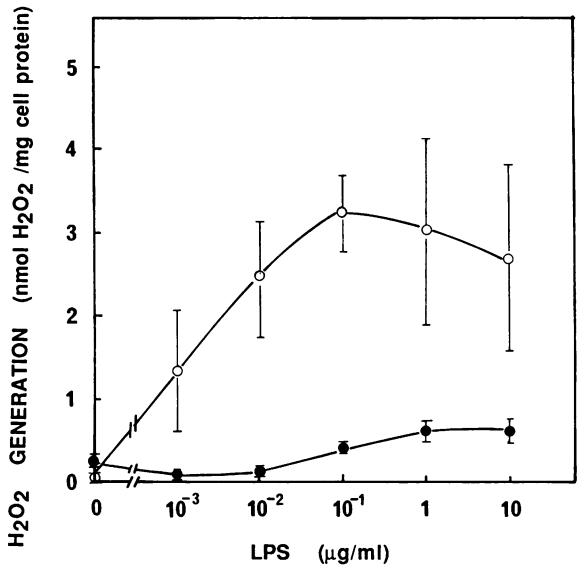

FIG. 9. Induction of H_2O_2-generating activity by LPS. JA-4 (○) and LPS1916 (●) cells were treated with LPS as described in the legend to Fig. 8. H_2O_2 release was detected by the scopoletin fluorescence assay with horseradish peroxidase, and the actual amounts of H_2O_2 were quantitated by a standard curve for H_2O_2 in this system. The results are expressed as specific activities of the cells, and the values are means ± standard errors for three independent experiments involving duplicate assays.

IL-1 (Fig. 5) and TNF (Fig. 6) may be other clues to the mechanisms of LPS resistance.

As for the mechanisms underlying macrophage activation, some interesting phenomena can be pointed out from our results. JA-4, the parental cell line, was activated by LPS and expressed phenotypes of activated macrophages, such as secretion of IL-1 and TNF, morphological changes (spreading), arachidonic acid release, and induction of O_2^- generation and H_2O_2 generation. All of these changes became evident within 24 h after LPS treatment of JA-4 cells, but there were some differences in the time delays before the phenomena were seen; morphological changes, secretion of IL-1, and release of arachidonic acid appeared within about 6 h after LPS addition (data not shown), as did increased secretion of TNF, which is continuously released into the culture medium at low levels, even in the absence of LPS. However, O_2^- generating activity became detectable at about 16 h after LPS treatment (data not shown). These results, together with the characteristics of LPS-treated LPS1916 cells, suggest that there are some pathways for induction of these phenotypes in macrophages by LPS. We are currently focusing our attention on the regulatory mechanisms for induction of the O_2^- generating system, because the greatest difference in the induction of this system by LPS was observed between JA-4 and LPS1916 cells (Fig. 8), whose regulatory mechanisms are largely unknown. In this regard, however, we have recently found that murine recombinant IFN-γ alone is able to induce O_2^- generating activity in both JA-4 and LPS1916 cells; IFN-γ at 100 U/ml induced O_2^- generation as much as 47 ± 1 or 36 ± 4 nmol of O_2^- per mg of cell protein per 90 min in JA-4 and LPS1916 cells, respectively, when 0.5 mg of zymosan per ml was added to the reaction mixture as a stimulant. Besides, we showed in this report that IFN-γ at 10 U/ml could greatly elevate O_2^--generating activity in LPS1916 cells induced by LPS (Fig. 10), suggesting that LPS1916 cells do not completely lack an O_2^- generating system but have some defects in the regulatory mechanisms for induction of O_2^-- generating activity through LPS-mediated pathways.

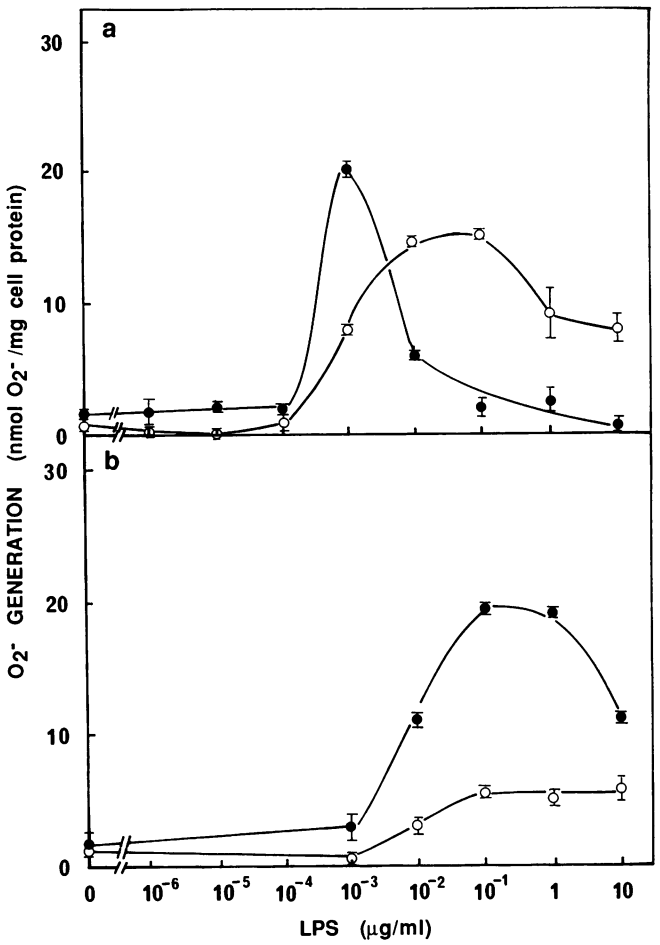

FIG. 10. Effect of IFN-γ on induction of O_2^- generating activity by LPS. JA-4 (a) and LPS1916 (b) cells were treated with various amounts of LPS simultaneously with (●) or without (○) murine recombinant IFN-γ at 37°C for 24 h. O_2^--generating activity was assayed with 5 μg of PMA per ml as the stimulant as described in the text. The results are expressed as specific activities of the cells and are means ± standard deviations for duplicate samples from a typical experiment.

During the preparation of this report, an interesting variant of J774 macrophagelike cells which is defective in both O_2^--generating activity and responses to IFN-γ was reported (10). However, a series of studies about J774 cells (8, 15, 25) did not concern the mechanisms underlying LPS-induced O_2^- generating activity. Both biochemical characterization of and approaches involving LPS1916 somatic cell genetics will increase our understanding of the mechanisms underlying the interaction between macrophages and LPS.

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