INFECTION AND IMMUNITY, Aug. 1996, p. 3188–3195 Vol. 64, No. 8
0019-9567/96/$04.00+0

Induction of Cytokine Gene Expression by Listeriolysin O and Roles of Macrophages and NK Cells

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Received 22 December 1995/Returned for modification 29 February 1996/Accepted 24 May 1996

To determine the role of listeriolysin O (LLO) of *Listeria monocytogenes* in the host response at the initial stage of infection, cytokine gene expression in mouse peritoneal exudate macrophages and spleen cells was examined by reverse transcription-PCR. Expression of various cytokine mRNAs, especially those of interleukin-1 (IL-1), tumor necrosis factor alpha, gamma interferon (IFN-γ), and IL-12, was observed to occur in spleen cells after direct stimulation with an LLO preparation purified to a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Induction of mRNA expression by LLO was not blocked by cholesterol, which abrogated the hemolytic activity of LLO. After the depletion of NK cells in spleen cells by treatment with anti-asialo GM1 antibody plus complement, LLO-induced expression of IFN-γ mRNA was decreased, indicating that NK cells were the main source of IFN-γ. After depletion of macrophages by passing spleen cells over a Sephadex G-10 column, expression of macrophage-derived cytokines, including IL-1α, tumor necrosis factor alpha, and IL-12, was diminished. In addition, IFN-γ mRNA expression was impaired, indicating that IFN-γ mRNA expression from NK cells required signaling from macrophages. It is suggested that LLO is capable of inducing endogenous cytokines of mice, and both NK cells and macrophages are involved in the host cytokine response to LLO.

Listeria monocytogenes is a facultative intracellular bacterium which causes listeriosis in animals and humans, chiefly in immunocompromised patients (23, 39). By transposon insertion mutagenesis, listeriolysin O (LLO) has been shown to be a major virulence factor of this bacterium (6, 10, 36). LLO, a 58-kDa protein, has been purified from culture supernatants of *L. monocytogenes* (13, 21, 33). LLO shows pH-dependent hemolytic activity (13), and its activity is blocked by oxidation (21, 33) or small amounts of cholesterol (24).

The ability to produce LLO is unique to virulent *L. monocytogenes* (8), and only the LLO-producing strain is capable of inducing T-cell-mediated immunity, which plays a critical role in the acquired resistance of the host (3, 15). Virulent strains with ability to produce LLO are known to induce expression of various cytokines in the infected host (20, 38) and in macrophage-like cell line P388D1 (26). Inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin-1α (IL-1α), IL-6, and gamma interferon (IFN-γ), produced at the early stage of infection with *L. monocytogenes* are believed on the basis of findings from experiments using cytokine-specific neutralizing antibodies and recombinant cytokines to be necessary for protection of mice (9, 14, 17, 30). Using various *Listeria* strains with different LLO production abilities, we have shown that the generation of cell-mediated acquired immunity in mice is achieved only after infection with LLO-producing, virulent strains and is dependent on the expression of inflammatory cytokines at an early stage of infection (32, 47).

Our previous finding that only an LLO-producing strain was capable of inducing macrophage production of IL-1 (19, 29) prompted us to examine the role of LLO in this aspect. Using purified LLO, we have shown that IL-1 production is actually induced by LLO itself (44, 48). Among the various cytokines produced in the infected host, IFN-γ appeared to play a distinct role in the induction of cell-mediated acquired immunity (47). In the present study, therefore, using purified LLO, we have examined the expression of various cytokines in vitro, with special interest in IFN-γ expression.

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MATERIALS AND METHODS

Experimental animals. Male mice of the C3H/He strain (Charles River Japan, Atsugi), raised and maintained in a specific-pathogen-free environment, were used for experiments at 7 to 9 weeks of age.

Bacterial strain and LLO purification. A virulent and hemolytic strain of *L. monocytogenes*, EGD (serovar 1/2a), was used. LLO was prepared by a procedure described previously (44, 48). In short, an overnight bacterial culture in 10 ml of brain heart infusion broth (Eiken Chemical Co., Ltd., Tokyo, Japan) was grown in 3 liters of fresh broth for 18 h at 37°C with shaking. A cell-free supernatant was obtained by centrifugation at 11,000 × *g* for 30 min at 4°C followed by filtration through a 0.45-μm-pore-size Millipore filter unit (Millipore Corp., Bedford, Mass.). The sterilized supernatant was concentrated by centrifugation at 11,000 × *g* after addition of ammonium sulfate to give a final concentration of 60%. The concentrated crude supernatant was then applied to a DEAE-Sephacel column (Pharmacia, Uppsala, Sweden) and eluted with a 0 to 0.3 M NaCl gradient. Several fractions showing high levels of hemolytic activity were pooled and subjected to gel filtration on a Sephadex G-100 column (Pharmacia). Active fractions were pooled and stored at −20°C until used at a protein concentration of 500 μg/ml in phosphate-buffered saline (PBS). The purity of the LLO preparation was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% [wt/wt] polyacrylamide separating gel) with voltage run at a constant current. As shown in Fig. 1, a single band of approximately 58 kDa was stained with Coomassie blue. The level of endotoxin, as a possible contaminant, was determined by using a Toxinometer (Wako Pure Chemical, Osaka, Japan) and revealed to be less than 0.1 ng/ml when suspended in complete medium at a protein concentration of 10 μg/ml. A concentrated crude supernatant was also prepared from the broth culture of *L. monocytogenes* ATCC 15313, which is shown to be an *hlyA*-defective and non-LLO-producing strain (5, 32), and used for comparison.

IFN-γ and IL-1α assay for ELISA. The IFN-γ titer and the IL-1α titer were determined by a two-site sandwich enzyme linked immunosorbent assay (ELISA). For the IFN-γ assay, wells of enzyme immunoassay (EIA) plates (Costar, Cambridge, Mass.) were precoated with 1.5 μg of rat anti-mouse IFN-γ monoclonal antibody (R4-6A2) per ml and 0.5% bovine serum albumin (BSA) in carbonate-bicarbonate buffer (pH 9.6). Next, the test supernatant or standard murine IFN-γ was added to each well. After incubation for 60 min, the plates

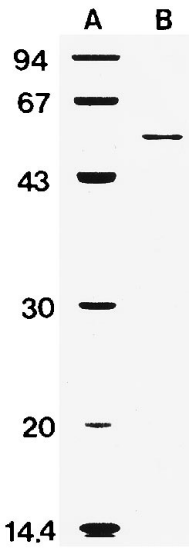

FIG. 1. SDS-PAGE analysis of the purity of the LLO preparation used in this study. Lane A, molecular mass marker (numbers indicate kilodaltons); lane B, LLO preparation (3 μg of protein per lane).

were washed with PBS containing 0.05% Tween 20 (PBS-Tween) and incubated with rabbit anti-mouse IFN-γ polyclonal antibody (a gift from Dai-ichi Pharmaceutical Co., Ltd., Tokyo, Japan) for 60 min. For the IL-1α assay, the test supernatant or standard IL-1α (recombinant murine IL-1α; Genzyme, Cambridge, Mass.) was added to wells precoated with hamster anti-mouse IL-1α monoclonal antibody (Genzyme) and 0.5% BSA in carbonate-bicarbonate buffer. After incubation for 60 min, the plates were washed. Next, rabbit anti-mouse IL-1α polyclonal antibody (Genzyme) was added and the mixture was incubated for 60 min. After that, for both assays, the plates were washed and peroxidase-conjugated anti-rabbit immunoglobulin G (Zymed Laboratories, Inc., South San Francisco, Calif.) was added. After incubation for 60 min, the plates were washed and a substrate solution, 100 μl of 0.4-mg/ml orthophenylenediamine in phosphate-citrate buffer (pH 5.0) containing 0.003% H_2O_2, was added. The A_{490} was measured after termination of the reaction with 50 μl of 2.5 N H_2SO_4.

Treatment of LLO with cholesterol. Cholesterol (Wako Pure Chemical Industries, Ltd.) was dissolved in ethanol to give a final concentration of 2 mg/ml. LLO (50 μg/100 μl) was incubated with 10 μg of cholesterol for 30 min at 37°C. This treatment resulted in the complete abrogation of hemolytic activity of LLO against sheep erythrocytes, as reported elsewhere (48).

Preparation of cells. Peritoneal exudate cells (PEC) were recovered from ICR mice 3 days after intraperitoneal injection of 1.5 ml of 3% thioglycolate medium (Difco). The PEC were washed with Hanks' balanced salt solution (HBSS) and suspended in RPMI 1640 complete medium (RPMI 1640 CM) consisting of RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 10% heat-inactivated fetal calf serum (Flow), 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 0.075% sodium bicarbonate, 2 mM L-glutamine, 100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 50 mM 2-mercaptoethanol. PEC were cultured in culture plates for 1.5 h at 37°C, and then nonadherent cells were removed by washing with warmed HBSS and adherent cells were used as macrophages. The enrichment for macrophages was monitored by an assay to determine phagocytic activity. Macrophages ingested a 0.02% suspension (vol/vol) of nonopsonized latex particles (average diameter, 0.81 mm; Difco). Spleens were aseptically removed from normal C3H/He mice and teased between two sterile glass slides. After treatment with 0.83% ammonium chloride in 0.17 mM Tris-HCl (pH 7.6) to lyse erythrocytes, spleen cells were washed with HBSS and suspended in RPMI 1640 CM.

Depletion of spleen cell subset. To deplete NK cells, spleen cells were treated with anti-asialo GM1 polyclonal antibody (Wako) plus complement (Cedarlane Laboratory Ltd., Ontario, Canada). In order to assess NK cell depletion after the treatment, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-IL-2 receptor β-chain (IL-2Rβ) monoclonal antibody and phycoerythrin-conjugated anti-CD3 monoclonal antibody (Becton Dickinson, Mountain View, Calif.). Stained cells were subjected to a two-color flow cytometric analysis by using a FACScan cell sorter (Becton Dickinson). In order to assess NK cell depletion, a standard ^{51}Cr release assay was also carried out (46). Target cells, murine T-cell lymphoma YAC-1 cells, were labeled with ^{51}Cr and suspended to a concentration of 10^5/ml. Spleen cells were mixed with ^{51}Cr-labeled target cells to give an effector-to-target cell ratio of 50:1 in U-bottom microtiter plates. The centrifuged plates were incubated at 37°C for 4 h. After centrifugation (400 × *g* for 5 min), one-half of the supernatant of each well was assayed for radioactivity by counting in a γ-counter. To deplete macrophages, spleen cells were passed twice through a Sephadex G-10 (Pharmacia) column (16). In order to confirm macrophage depletion, cells were stained with FITC-conjugated F4/80 monoclonal antibody and were subjected to single-color flow cytometric analysis by using a FACScan cell sorter.

Preparation of culture supernatant of spleen cells stimulated with LLO. Cells were plated at 5×10^6 per well in a 24-well flat-bottom tissue culture plate (Costar). The purified LLO was added to yield a final concentration of 2, 5, 10, 20, or 50 μg/ml. The culture supernatant was collected after incubation of plates for 48 h, centrifuged at 1,500 × *g* for 15 min, and passed through a 0.22-μm-pore-size membrane filter. The culture supernatant of spleen cells stimulated with a crude supernatant of EGD or ATCC 15313 was also prepared.

RNA extraction. Cells were plated at 5×10^6 per well in a 6-well flat-bottom tissue culture plate. The cells were stimulated with LLO for 6 h, and then total RNA was extracted by the acid guanidinium-phenol-chloroform method. In brief, cells were collected and solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) was added. The cell pellets were disrupted by being passed through a 21-gauge needle, and 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml of water-saturated phenol, and 0.2 ml of chloroform-isoamyl alcohol were added. The final suspension was vigorously spun in a vortex mixer for 20 s and then cooled on ice for 10 min. Samples were centrifuged at 10,000 × *g* for 20 min at 4°C. After centrifugation, the aqueous phase containing RNA was transferred to a new tube, mixed with the same volume of isopropanol, and placed at −20°C to precipitate RNA. After centrifugation at 10,000 × *g* at 4°C, the RNA pellet was dissolved in 0.5 ml of solution D and precipitated with the same volume of isopropanol at −20°C for 2 h. RNA was collected by centrifugation for 15 min at 4°C, washed once with 75% ethanol, dried, and dissolved in 20 μl of distilled water. The RNA concentration was measured by determining A_{260} by using a spectrophotometer (GeneQuant; Pharmacia LKB Biochem Ltd., Cambridge, United Kingdom).

RT-PCR. Production of cDNA by reverse transcription (RT) was done by the following method as described previously (47). Total RNA extracted (5 μg) was mixed with 4 μl of RT buffer, 2 μl of 0.1 M dithiothreitol, 0.5 μl of RNasin (Promega, Madison, Wis.), 1 μl of 10 mM deoxynucleoside triphosphates (dNTPs) (Pharmacia), 2 μl of random primer (Pharmacia), 0.5 μl of reverse transcriptase (Gibco-BRL, Life Technologies, Inc., Gaithersburg, Md.), and distilled water to give a final volume 20 μl. The mixture was incubated at 42°C for 60 min and boiled at 95°C for 3 min, and then samples were kept at −20°C until used.

The PCR mixture consisted of 1 μl of sample cDNA, 5 μl of PCR amplification buffer, 2 μl of 25 mM $MgCl_2$, 4 μl of 2.5 mM dNTPs, 0.3 μl of *Taq* DNA polymerase (5 U/μl; Promega), 2 μl of 20 mM primer, and double-distilled water to give a final volume of 50 μl. The sequences of oligonucleotide primers used were as follows: 5′-CTCTAGAGCACCATGCTACAGAC-3′ and 5′-TGGAATCCAGGGGAAACACTG-3′ for IL-1α, 5′-AGCGGCTGACTGAACTCAGATTGTAG-3′ and 5′-GTCACAGTTTTCAGCTGTATAGGG-3′ for IFN-γ, 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′ and 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′ for TNFα, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ for β-actin, 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′ and 5′-TCTGACCACAGTGAGGAATGTCCAC-3′ for IL-6, 5′-TCCTTAATGCAGGACTTTAAGGGTTACTTG-3′ and 5′-GACACCTTGGTCTTGGAGCTTATTAAAATC-3′ for IL-10, and 5′-AACTGGCGTTGGAAGCACGG-3′ and 5′-GAACACATGCCCACTTGCTG-3′ for IL-12 (p40). The predicted sizes of the amplified products for IL-1α, IFN-γ, TNFα, β-actin, IL-6, IL-10, and IL-12 (p40) were 288, 213, 309, 348, 130, 240, and 368 bp, respectively. The primers were made by Kurabo Biomedicals (Osaka, Japan) according to our sequence design. The PCR was performed by a thermal cycler (TP cycler-100; Toyobo, Osaka, Japan). The PCR program was one cycle of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Samples were amplified at 22 cycles for β-actin, 35 cycles for IL-12 (p40), and 25 cycles for other cytokines. The most appropriate number of amplification cycles was chosen from a preliminary experiment. The reaction was terminated by incubation at 72°C for 7 min, and the products were kept at 4°C in the cycler. Analysis of the PCR product was performed by agarose gel electrophoresis using a 1% low-melting-point agarose gel (Wako) in 1× TAE (Tris-acetate–EDTA) buffer supplemented with 0.005% ethidium bromide. A 10-μl volume of PCR product and 2 μl of marker dye were applied to each well. The bands were visualized by a UV transilluminator and photographed.

Statistical analysis. The statistical difference of the data was analyzed by Student's *t* test, and $P < 0.05$ was taken as the level of significance.

RESULTS

Cytokine gene expression by spleen cells stimulated with LLO. Normal spleen cells were stimulated with purified LLO at 10 μg/ml for 6 h, and then total RNA was extracted and cytokine-specific mRNA expression was examined by the RT-PCR method. PCR products for IL-1α, IFN-γ, TNFα, IL-6, IL-10, and IL-12 were detected (Fig. 2). To determine whether the

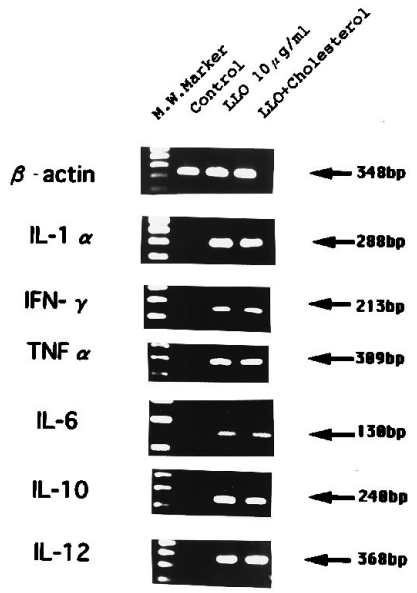

FIG. 2. PCR detection of cytokine-specific mRNA produced by spleen cells. Normal spleen cells (5×10^6 cells per ml) were stimulated with either untreated LLO at a concentration of 10 μg/ml or cholesterol-treated LLO. After 6 h, total RNA was extracted and RT-PCR was performed by using cytokine-specific primer pairs for β-actin, IL-1α, IFN-γ, TNFα, IL-6, IL-10, and IL-12. Next, PCR products were electrophoresed. The predicted band sizes of PCR products are indicated. M.W., molecular weight.

hemolytic activity of LLO, which can be blocked by cholesterol, is required for the induction of these cytokines, spleen cells were stimulated with cholesterol-pretreated, nonhemolytic LLO. The cytokine-inducing activity of LLO was not affected at all by cholesterol treatment, suggesting that this activity is not related to membrane damage by LLO. As we have reported previously (48), this dose of the LLO preparation did not lyse the cells as long as complete medium supplemented with sera was used.

Production of IL-1α and IFN-γ by spleen cells stimulated with untreated LLO or cholesterol-treated LLO. Among the various cytokines induced by LLO, IL-1α and IFN-γ play important roles, especially for inducing protective immunity after listerial infection. To examine the actual production of these two cytokines induced by LLO, the culture supernatants of spleen cells stimulated with various doses of untreated LLO or cholesterol-treated LLO for 48 h were examined for IL-1α and IFN-γ titers by ELISA. IL-1α and IFN-γ were produced at a significant level, in comparison with the levels for unstimulated spleen cells, after stimulation of cells with LLO at a concentration of 5 μg/ml or more (Fig. 3). The result shown in Fig. 2 shows that production of cytokine-specific mRNA by spleen cells could be induced after stimulation with LLO at 10 μg/ml, and so we stimulated cells with LLO at this concentration in subsequent experiments. There was no significant difference between the abilities of naive LLO and that treated with cholesterol to induce cytokine production at the dose range examined. Production of IL-1α and IFN-γ was not detected in the culture supernatant without LLO stimulation. As indicated in Materials and Methods, a very low level of endotoxin was detected in the culture containing purified LLO (0.1 ng/ml); however, addition of this amount of *Escherichia coli* lipopolysaccharide to spleen cells never induced cytokines to a detectable level (data not shown), indicating that the possible involvement of endotoxin can be ruled out.

Though LLO was purified as a protein exhibiting a single band on SDS-PAGE, the possibility that cytokine induction depends on some contaminants other than LLO could not be ruled out. To address this issue, we compared the cytokine-inducing abilities of the crude supernatant of strain EGD and that of *hlyA*-negative non-LLO-producing strain ATCC 15313 (5, 32). As shown in Fig. 4, the concentrated supernatant from ATCC 15313 failed to induce the production of IL-1α and IFN-γ. This result confirmed that LLO is responsible for inducing cytokine expression by spleen cells in vitro.

Cytokine gene expression by peritoneal macrophages stimulated with LLO. Spleen cells are a heterogeneous population of cells; however, most of the cytokines expressed on LLO

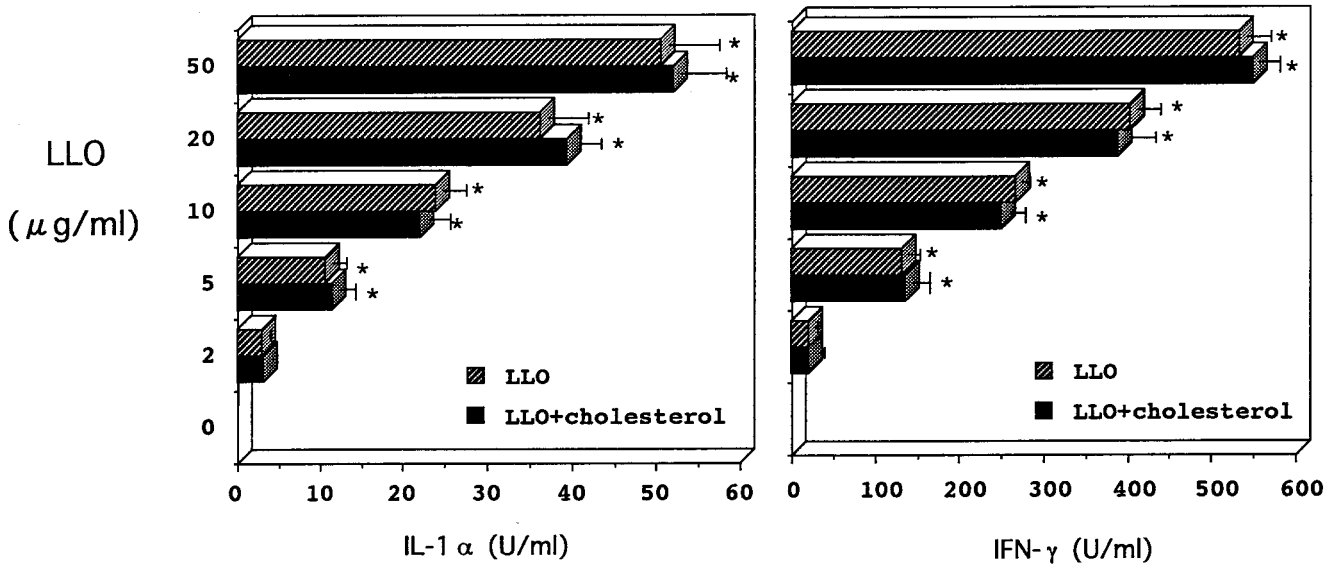

FIG. 3. IL-1α and IFN-γ production by spleen cells stimulated with untreated LLO or cholesterol-treated LLO. Normal spleen cells (5×10^6 cells per ml) were stimulated with LLO or cholesterol-treated LLO at various concentrations for 48 h. IL-1α and IFN-γ titers in the culture supernatants were assayed by ELISA. The asterisks in the figure indicate statistical significance ($P < 0.05$) relative to control values. Error bars indicate standard deviations.

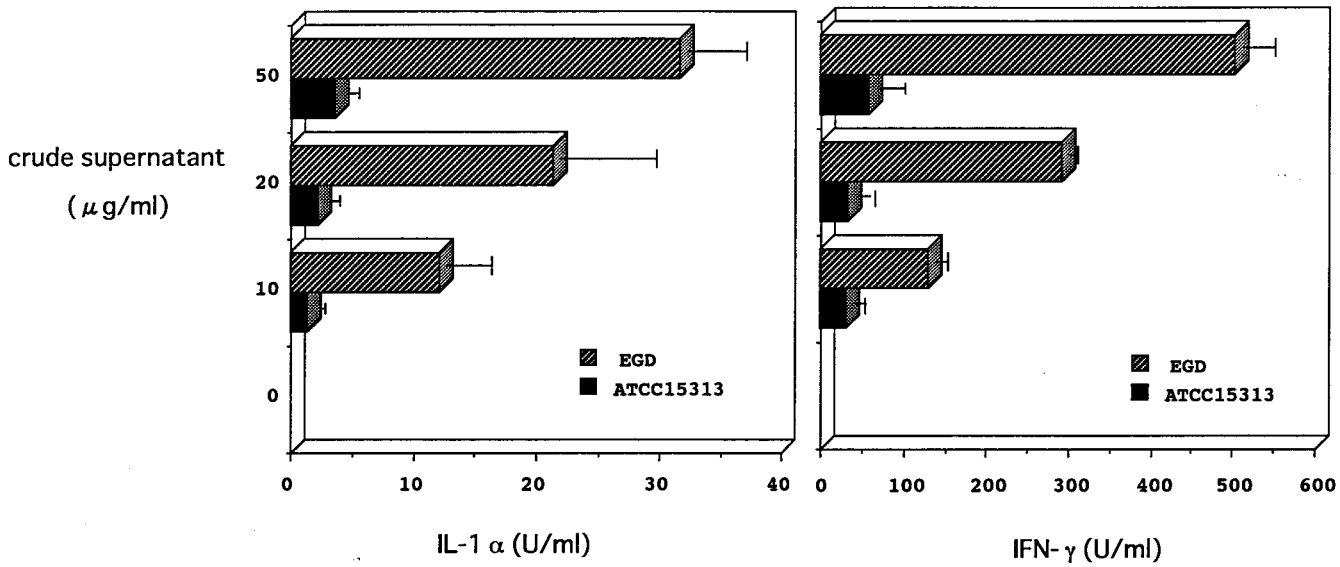

FIG. 4. IL-1α and IFN-γ production by spleen cells stimulated with concentrated crude supernatant of *L. monocytogenes* EGD or ATCC 15313. Normal spleen cells (5×10^6 cells per ml) were stimulated with the crude supernatant at various concentrations for 48 h. IL-1α and IFN-γ titers in the culture supernatants were assayed by ELISA. Error bars indicate standard deviations.

stimulation were those which are produced mainly from macrophages. To determine the response of macrophages to stimulation with LLO, adherent macrophages were prepared from PEC. More than 96% of adherent cells employed in this experiment were active in phagocytosis of latex beads. After 6 h of stimulation with LLO, total RNA was extracted and cytokine gene expression was determined by RT-PCR. PCR products for IL-1α, TNFα, and IL-12 were detected, but those for IFN-γ, IL-6, and IL-10 were not (Fig. 5). Stimulation with cholesterol-treated LLO resulted in the same profile of cytokine expression.

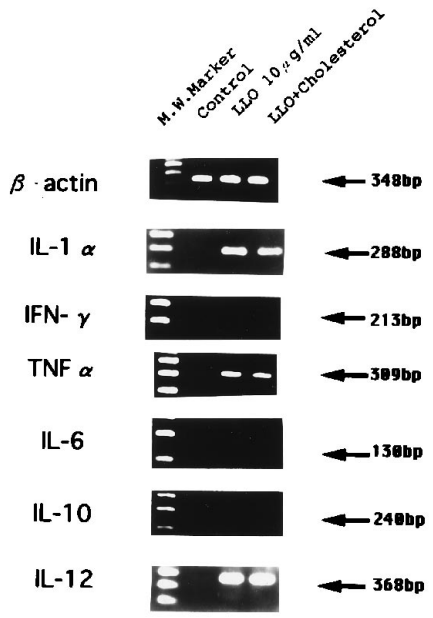

FIG. 5. PCR detection of cytokine-specific mRNA produced by macrophages. Macrophages (5×10^6 cells per ml) were stimulated with untreated LLO or cholesterol-treated LLO. After 6 h, total RNA was extracted and RT-PCR was done by using cytokine-specific primer pairs for β-actin, IL-1α, IFN-γ, TNFα, IL-6, IL-10, and IL-12. PCR products were electrophoresed, and the predicted sizes of PCR products are indicated. M.W., molecular weight.

LLO-induced cytokine gene expression and IFN-γ production by NK cell-depleted spleen cells. To determine the role of NK cells in cytokine expression, particularly in IFN-γ expression induced by LLO, spleen cells were treated with anti-asialo GM1 antibody plus complement to deplete NK cells and cytokine gene expression was examined after stimulation with LLO. NK cell depletion was assessed by flow cytometric analysis. It has been reported that IL-2Rβ$^+$ CD3$^-$ cell populations are NK cells (1). After the treatment, the NK cell population was decreased from 6.1 to 0.4%, showing NK cell depletion of more than 90% (Fig. 6). NK cell activity as determined by the YAC-1 killing assay also confirmed the depletion of NK cells by the treatment (4.8% ± 1.0% before treatment versus less than 0.3% after treatment). When these NK cell-depleted spleen cells were stimulated with LLO, expression of IL-1α, TNFα, and IL-12 mRNAs was observed at almost the same level as that in whole spleen cells; however, the level of IFN-γ mRNA expression was decreased after treatment (Fig. 7). The titer of IFN-γ produced in the culture was examined by EIA 48 h after stimulation with LLO. The level of IFN-γ production in NK cell-depleted spleen cells was apparently lower than that in nontreated cells and cells treated with complement alone (Fig. 8).

LLO-induced cytokine gene expression in macrophage-depleted spleen cells. To determine the role of macrophages in the expression of various cytokine genes induced by LLO, spleen cells were passed through Sephadex G-10 columns, which are known to deplete macrophages by adherence (16), and then cytokine gene expression was examined after stimulation with LLO. After the treatment, the percentage of cells recovered was decreased by 33%, which was consistent with what was indicated in a previous publication (16). In order to assess the depletion of macrophages, we employed monoclonal antibody F4/80, which is reported to bind to mouse macrophages specifically (2). After passage through the column, the F4/80-binding cell population was decreased from 7.5 to 1.8%. Though the depletion as determined by the number of F4/80-

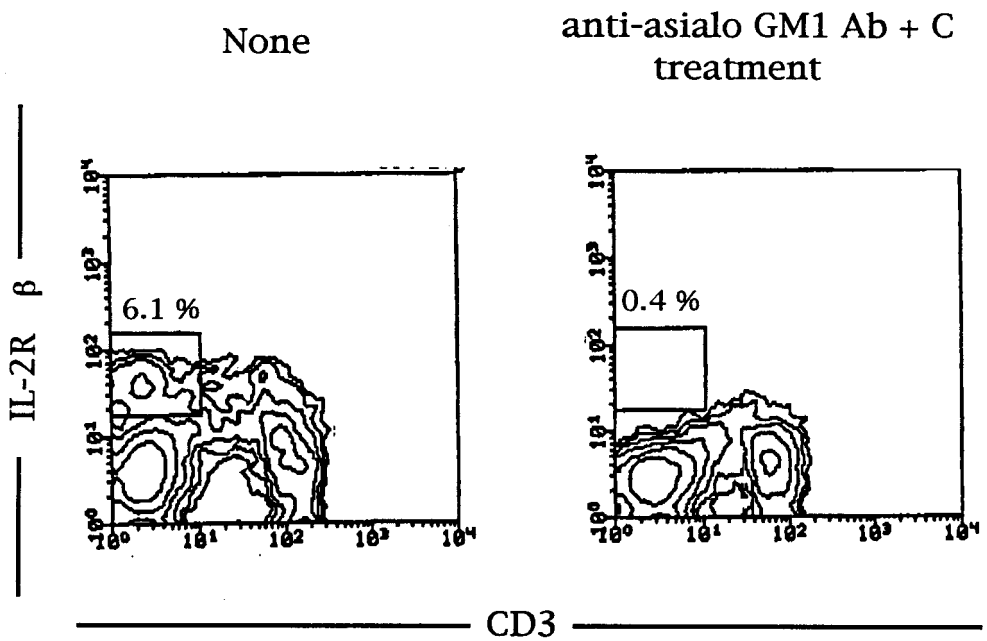

FIG. 6. Flow cytometric analysis of spleen cells after treatment with anti-asialo GM1 antibody (Ab) plus complement (C). Cells were stained with FITC-conjugated anti-IL-2Rβ and phycoerythrin-conjugated anti-CD3 monoclonal Ab and analyzed by flow cytometry. The IL-2Rβ$^+$ CD3$^-$ cells were regarded as NK cells.

binding cells was not complete, the expressions of IL-1α, TNFα, and IL-12 were diminished when these cells were stimulated with LLO. IFN-γ-specific mRNA expression was also decreased (Fig. 9). The NK cell populations in the spleen cells with and without macrophage depletion were examined by flow cytometry. The data showed that the percentage of NK cells was not changed (5.3% before and 6.3% after), indicating that NK cells were not affected by G-10 treatment (Fig. 10).

DISCUSSION

LLO, a 58-kDa secretory protein of *L. monocytogenes*, is the essential virulence factor which enables this bacterium to escape from the phagosomal compartment of macrophages by virtue of its membrane-damaging activity (22, 37). Non-LLO-producing mutants are incapable of multiplying inside macrophages and hence exhibiting in vivo virulence (27). In contrast to our knowledge about this critical role of LLO as a major virulence factor, little is known about the role of LLO in inducing the host response, except that LLO peptide (LLO positions 91 to 99) serves as an antigen recognized by cytotoxic T cells in the context of class I major histocompatibility complex molecules (34).

In the present study, we have fractionated LLO from culture supernatant to single-band purity on SDS-PAGE and examined the cytokine-inducing ability in vitro. It was shown that purified LLO induced the expression of a variety of cytokines in spleen cells and macrophages in vitro. A possible contribution of a contaminant other than LLO can be ruled out by the fact that a concentrated crude supernatant from *L. monocytogenes* ATCC 15313, which is not able to produce LLO (32), did not induce IL-1α and IFN-γ as determined by EIA. LLO is regarded as one of the membrane-damaging toxins which lyse the cell membrane by pore formation with oligomeric toxin molecules like streptolysin O (40), and such an activity is blocked by pretreatment with cholesterol (24, 48). The result obtained by using the cholesterol-treated LLO suggested that the cytokine induction was triggered not by direct binding of LLO to membrane cholesterol but probably by binding to cell membrane at sites other than that of membrane-bound cholesterol or signaling action after internalization of LLO inside the cells. The precise mechanism of this

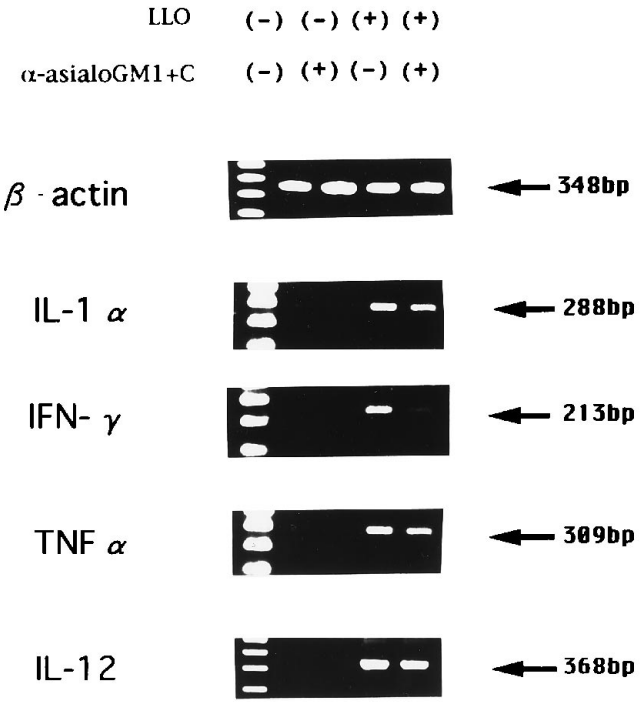

FIG. 7. Expression of cytokine-specific mRNAs after NK cell depletion. Spleen cells treated with anti-asialo GM1 antibody plus complement (C) (5×10^6 cells per ml) and normal spleen cells (5×10^6 cells per ml) were stimulated with LLO for 6 h. Total RNA was extracted and subjected to RT-PCR. PCR products obtained by using specific primers for β-actin, IL-1α, IFN-γ, TNFα, and IL-12 were electrophoresed. The predicted band sizes of PCR products are indicated.

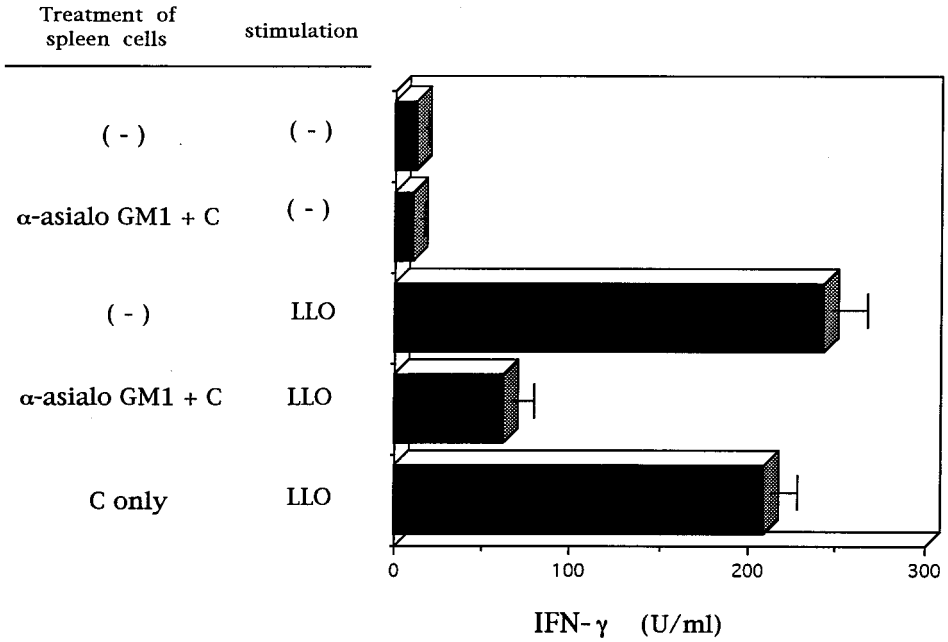

FIG. 8. IFN-γ production after NK cell depletion. Spleen cells treated with anti-asialo GM1 antibody plus complement (α-asialo GM1 + C) or complement (C) alone and normal spleen cells were adjusted to 5×10^6 cells per ml. These cells were stimulated with LLO (10 μg/ml) for 48 h. IFN-γ levels in the culture supernatants were determined by ELISA. Error bars indicate standard deviations.

aspect of cytokine induction by LLO is to be determined in a further study.

Among the inflammatory cytokines induced by LLO, IL-1α, TNFα, and IL-12 appeared to be produced mainly by macrophages, since macrophage depletion resulted in the loss of expression and adherent macrophages showed a response similar to that in whole spleen cells. This is consistent with a report showing that mRNAs for IL-1α and TNFα were detected in P388D1 macrophage-like cells infected with a virulent strain of *L. monocytogenes* (26). IFN-γ is a cytokine which could not be expressed in macrophages. The result after depletion of NK cells from spleen cells suggested that NK cells are the major source of IFN-γ. This is consistent with an in vivo observation of NK cell-dependent production of this cytokine in infected mice (9). It is interesting that IFN-γ expression was abolished even in the presence of NK cells when macrophages were depleted. These results suggested that NK cells express and produce IFN-γ in collaboration with macrophages.

The profile of cytokine expression observed in the present in vitro study was similar to that observed to occur in mice infected with virulent strains of *L. monocytogenes* (47, 49). Macrophage-derived cytokines are known to contribute to the nonspecific protection of the host. The importance of IL-1 and TNF has been established in experiments using recombinant cytokine and neutralizing antibody (17, 30, 31). The protective role of IL-12 has been emphasized recently, IL-12 having been found to induce resistance against not only *L. monocytogenes* (42, 45) but also other intracellular parasites, such as *Leishmania major* (41) and *Toxoplasma gondii* (12). NK cell-dependent IFN-γ is known to be critically important for nonspecific protection of the host (31). The present study indicated that LLO released from virulent *L. monocytogenes* is at least one of the major factors which induce these cytokine responses in the host.

In addition to endogenous cytokines, which play a role in induction of nonspecific resistance, it seems that some cytokines are involved also in the generation of antigen-specific acquired resistance of the host. As we have previously reported, IFN-γ mRNA was induced only by virulent LLO-producing strains and not by non-LLO producers while TNFα was expressed equally by all strains of *L. monocytogenes*, irrespec-

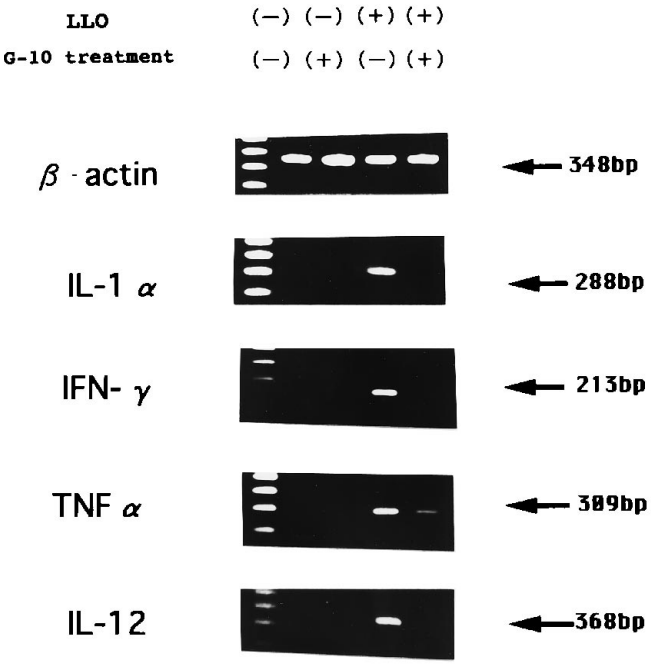

FIG. 9. Expression of cytokine-specific mRNAs after macrophage depletion. Spleen cells treated by being passed through a G-10 column (5×10^6 cells per ml) and normal spleen cells (5×10^6 cells per ml) were stimulated with LLO for 6 h. Total RNA was extracted, and RT-PCR was performed. PCR products obtained by using specific primers for β-actin, IL-1α, IFN-γ, TNFα, and IL-12 were electrophoresed. The predicted band sizes of PCR products are indicated.

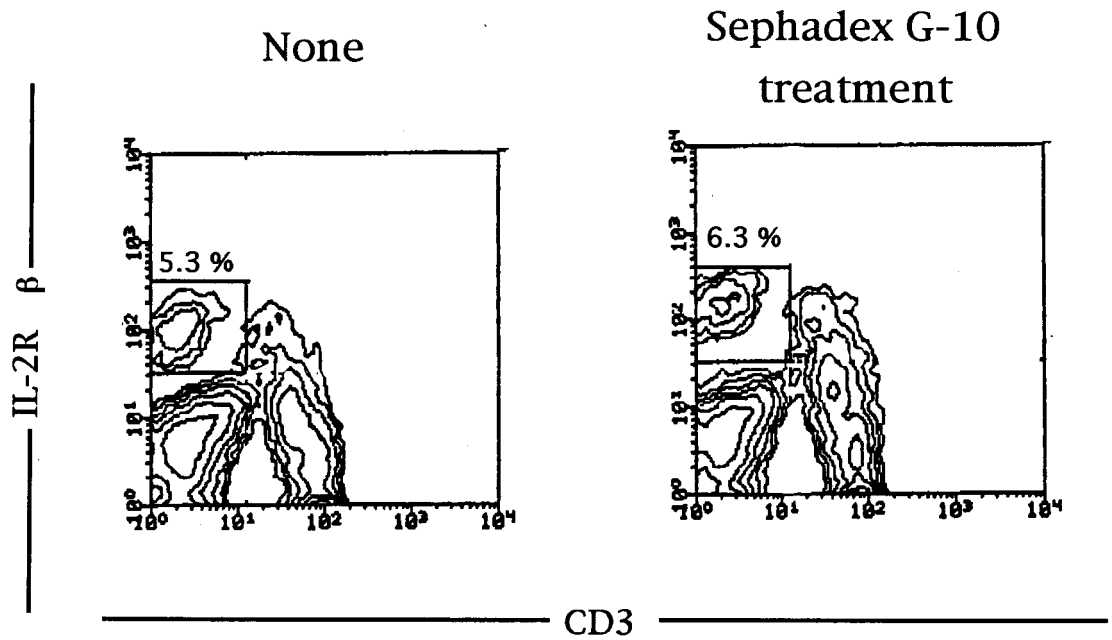

FIG. 10. NK cell populations after treatment by passage through a G-10 column. Spleen cells were passed through a G-10 column to deplete macrophages. Cells were stained with FITC-conjugated anti-IL-2Rβ and phycoerythrin-conjugated anti-CD3 monoclonal antibody and analyzed by flow cytometry. The IL-2Rβ$^+$ CD3$^-$ cells were regarded as NK cells.

tive of LLO-producing ability, and the acquired immunity was generated in mice only when IFN-γ was expressed at the early stage of infection (32, 47). The present study suggested that a macrophage-derived factor is required for the LLO-induced expression of IFN-γ which is known to be crucial for the development of the protective Th1 type of cells (11, 35). IL-12 is reported to induce NK cells to produce IFN-γ alone (4, 7) or in combination with TNFα (43). Taking these findings into consideration, it is possible that macrophage-derived IL-12 plays a role in IFN-γ induction in NK cells; however, some other macrophage-derived factor may be involved in our experimental model, since IL-12 is shown to be produced even after stimulation by non-LLO-producing strains (18, 43) which are incapable of inducing specific protective immunity (25, 28).

In conclusion, the present study indicated that LLO is capable of inducing various cytokines which are believed to be critically important for the expression of nonspecific resistance and the generation of acquired resistance in an infected host. This in vitro model may provide a tool for a further analysis of the relationship between virulence factors of bacteria and the host response.

ACKNOWLEDGMENTS

This study was supported by grants-in-aid of scientific research from the Ministry of Education, Science and Culture of the Japanese government.

We thank Hisami Watanabe for the operation of the FACScan instrument.

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Editor: S. H. E. Kaufmann