INFECTION AND IMMUNITY, Apr. 1993, p. 1334–1339
0019-9567/93/041334-06$02.00/0

Vol. 61, No. 4

Membrane Damage and Interleukin-1 Production in Murine Macrophages Exposed to Listeriolysin O

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Received 20 October 1992/Accepted 15 January 1993

To obtain some insight into the interaction between listeriolysin O (LLO) and the macrophage membrane, we examined the effect of purified *Listeria monocytogenes* hemolysin on the viability and functions of mouse peritoneal exudate macrophages. The study showed that purified LLO impaired a variety of functions of the macrophages. First, it suppressed the luminol-dependent chemiluminescence response of macrophages. Second, it suppressed the phagocytic ingestion of opsonized sheep erythrocytes and latex beads. Third, exposure of macrophages to LLO resulted in an increase in dead cells, as determined by the trypan blue dye exclusion method. An interesting observation of this study is that the LLO-induced production of interleukin-1 from macrophages could not be blocked by preincubation with cholesterol, while the membrane-damaging ability could be blocked by cholesterol. The dissociation of the blocking effects of cholesterol suggests that the interleukin-1-inducing ability of LLO may be distinct from its membrane-damaging ability.

Listeria monocytogenes, a gram-positive, facultative intracellular bacterium, can cause severe infections, mainly in newborns and immunocompromised patients (7, 19). The major symptomatic manifestations of *Listeria* infections are septicemia and meningitis. The virulence mechanism enabling *L. monocytogenes* to cause these invasive diseases is thought to be its ability to survive and replicate in cells of the infected host, especially cells of the macrophage lineage (13).

The best characterized virulence factor of *L. monocytogenes* necessary for intracellular survival is listeriolysin O (LLO), a hemolytic cytolysin. LLO has been successfully isolated from culture supernatants of certain strains of *L. monocytogenes* (6, 10, 16). LLO is regarded as one of the sulfhydryl-activated toxins, since the hemolytic activity is reversibly enhanced by reducing agents and is suppressed by oxidation (10, 16) and by exposure to cholesterol (11) or anti-streptolysin O antibodies (10).

A recent approach involving transposon mutagenesis revealed that the inactivation of hemolysin gene expression resulted in the loss of the ability to multiply in infected macrophages and the loss of virulence in vivo (5, 18). In addition, *Bacillus subtilis* into which the LLO gene had been integrated became capable of growing in a macrophage-like cell line, J774 (2). In an electron microscopic study of macrophages infected with *L. monocytogenes*, it was found that a hemolysin-producing strain of *L. monocytogenes* could escape from the phagosome to become free in the cytoplasm. On the other hand, mutants lacking hemolysin-producing ability could not disrupt the phagosomal membrane and accordingly could not escape from the phagosomal compartment into the cytoplasm (20).

Thus, there is no doubt that LLO is mainly responsible for the virulence and intracellular parasitism of *L. monocytogenes*. However, the biological activity of LLO has been examined by use of erythrocytes as target cells (6), and there is no concrete evidence showing that LLO actually causes damage to the macrophage membrane. In an attempt to obtain some insight into the interaction between LLO and the macrophage membrane, we have examined the effect of purified *L. monocytogenes* hemolysin on the viability and functions of mouse peritoneal exudate macrophages.

MATERIALS AND METHODS

Bacterial strain and hemolysin purification. A hemolytic *L. monocytogenes* strain, EGD, serovar 1/2a, was used. *L. monocytogenes* hemolysin was prepared by a procedure described previously (21). In brief, an overnight bacterial culture in 10 ml of brain heart infusion broth (Eiken Chemical Co., Ltd., Tokyo, Japan) was grown in 3 liters of brain heart infusion broth for 18 h at 37°C with shaking. The bacterial cells were removed by centrifugation at 11,000 × *g* for 30 min at 4°C and filtered through a 0.45-μm-pore-size filter unit (Millipore Corp., Bedford, Mass.). The supernatant fluid was concentrated by the addition of ammonium sulfate to yield a final concentration of 60%. The concentrated crude supernatant was then applied to a DEAE-Sephacel column (Pharmacia, Uppsala, Sweden) and eluted with a linear gradient of 0 to 0.5 M NaCl. Several fractions exhibiting high levels of hemolytic activity were pooled and subjected to gel filtration on a Sephadex G-100 column (Pharmacia). Active fractions were pooled, concentrated, and stored at −20°C until use at a protein concentration of 500 μg/ml in phosphate-buffered saline.

SDS-PAGE. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The sample was boiled for 5 min in an equal volume of sample buffer, consisting of 3% SDS, 5% 2-mercaptoethanol, 0.1 g of glycerol, and 0.5 mg of bromophenol blue per ml in 0.06 M Tris-HCl buffer (pH 6.8). Electrophoresis was performed with a slab gel of 12% (wt/vol) acrylamide at 15 mA for 6 h. The protein was visualized by staining with Coomassie brilliant blue.

Preparation of PEC. Peritoneal exudate cells (PEC) were recovered from ICR mice (Charles River Japan, Atsugi, Japan) 3 days after an intraperitoneal injection of 1.5 ml of

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10% proteose peptone (Difco Laboratories, Detroit, Mich.). The PEC were washed and suspended in RPMI 1640 complete medium (RPMI 1640 CM), which is 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–50 mM 2-mercaptoethanol.

Treatment of PEC with hemolysin. PEC were suspended in an appropriate medium at a concentration of 10^7/ml. The cells were incubated for 5 to 30 min at 37°C in the presence of various concentrations of hemolysin before being analyzed for functional changes.

Treatment of LLO with cholesterol. Cholesterol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in ethanol to yield a concentration of 1 mg/ml. LLO (25 μg/100 μl) was incubated with 10 μg of cholesterol for 30 min at 37°C.

Luminol-dependent chemiluminescence. Luminol-dependent chemiluminescence was determined by use of a lumiphotometer (TD-4000; Labo Science, Tokyo, Japan) as reported previously (4). PEC were suspended to yield 10^8/ml in 50 μl of buffer II, consisting of 10 mM HEPES, 5 mM KCl, 145 mM NaCl, and 5.5 mM glucose (pH 7.4). After incubation for 5 to 20 min at 37°C with or without hemolysin, to the cells were added 100 μl of luminol solution, 100 μl of buffer I, consisting of buffer II supplemented with 1 mM $CaCl_2$, and 50 μl of phorbol myristate acetate (PMA; 20 μg/ml). Chemiluminescence was monitored by use of the lumiphotometer for 10 min and expressed in relative light units.

Viability of PEC after treatment with LLO. PEC (10^6/50 μl) suspended in Hanks' balanced salt solution (HBSS) were incubated with LLO at a final concentration of 6.25 to 50 μg/ml for 10 to 30 min at 37°C. After incubation, the PEC were stained with 0.004% trypan blue. The stained cells were counted as dead cells under a microscope in a hemocytometer.

Phagocytosis assay. A PEC suspension (2×10^6/500 μl) in HBSS or RPMI 1640 CM in microcentrifuge tubes was incubated with or without hemolysin (final concentration, 25 μg/ml) for 30 min at 37°C. The tubes were centrifuged at 150 × *g* for 5 min to remove the hemolysin. The pellet of PEC was resuspended in RPMI 1640 CM and incubated for 30 min at 37°C with opsonized sheep erythrocytes (SRBC) (1%/ml) or a 0.1% suspension of latex beads (1 ml). The tubes were centrifuged at 150 × *g* for 5 min to remove nonphagocytosed SRBC or latex beads. The pellet was resuspended in RPMI 1640 CM, and the suspension was smeared on a glass slide. The numbers of phagocytosed SRBC per 100 macrophages and macrophages phagocytosing latex beads in a high-power microscopic field were counted.

LPS-induced IL-1 production by PEC treated with LLO. PEC (3×10^6) in RPMI 1640 CM were incubated for 1.5 h at 37°C in 5% CO_2 in the wells of a 24-well culture dish (Costar, Cambridge, Mass.). After incubation, the nonadherent cells were removed by washing with warm HBSS, and new RPMI 1640 CM (1 ml) was added to the adherent cells. LLO, cholesterol, or cholesterol-treated LLO was added to the wells to yield a final concentration of 25 μg/ml. After incubation for 30 min, the adherent cells were washed with warm HBSS, new RPMI 1640 CM was added, and lipopolysaccharide (LPS) was added to yield a final concentration of 25 μg/ml. After incubation for 36 h, the culture supernatants were removed. The interleukin-1 (IL-1) activity of the supernatants was measured by a thymocyte costimulator assay (15).

IL-1 production by macrophages after stimulation with LLO or cholesterol-treated LLO. PEC suspended in RPMI 1640 CM (3×10^6) were incubated in 5% CO_2 in a 24-well culture dish for 1.5 h at 37°C. After incubation, the adherent cells were treated as described above and then incubated with LLO or cholesterol-treated LLO (25 μg/ml) in fresh RPMI 1640 CM for 36 h. After incubation, the IL-1 activity of each culture supernatant was measured by a thymocyte costimulator assay.

Determination of IL-1 activity. IL-1 activity was measured by the standard costimulator assay. In the presence of a substimulatory dose of phytohemagglutinin (Difco), 10^6 thymocytes from C3H/HeJ mice were cultured in 200 μl of serially diluted culture supernatant for 3 days. Cells in each well were pulsed with 18.5 kBq (0.5 μCi) of [^{3}H]thymidine (ICN Biomedicals Inc., Costa Mesa, Calif.) 18 h before harvesting. IL-1 activity was expressed as counts per minute of [^{3}H]thymidine incorporated into thymocytes.

RESULTS

Purification of LLO. Starting with the ammonium sulfate precipitate of the supernatant of a culture of *L. monocytogenes*, hemolysin was purified by ion-exchange chromatography and gel filtration. The hemolytic activity was determined on the basis of the endpoint of complete hemolysis of a 1% SRBC suspension after the addition of the same amount of a twofold-diluted sample. The specific activity of the finally obtained hemolysin was approximately 300 times higher than that of the starting material. For the maximal expression of hemolytic activity in an in vitro assay, the presence of at least 2 mM L-cysteine or dithiothreitol was always required. When the finally obtained protein (500 μg/ml) was incubated in the presence of 10 μg of cholesterol per ml, the hemolytic activity was totally abrogated (data not shown). These findings were indicative of the properties of a so-called thiol-activated hemolysin (1).

In SDS-PAGE analysis, the purified hemolysin produced a single band with an approximate molecular mass of 58 kDa (Fig. 1), a size consistent with the previously reported molecular mass of LLO (6) and also with that predicted from the DNA sequence of the gene coding for LLO (14). On the basis of these characteristics, the purified hemolysin protein was used in the present study to determine the effect of LLO on macrophages.

Luminol-dependent chemiluminescence of PEC treated with LLO. For investigation of the effect of LLO on the macrophage membrane, the luminol-dependent chemiluminescence of PEC was determined after treatment of the PEC with LLO. The kinetics of the chemiluminescence response were monitored for 10 min after stimulation with PMA (Fig. 2). The chemiluminescence response of PEC treated with 25 μg of LLO per ml for 5 min was suppressed compared with that of PEC not treated with LLO. When PEC were treated with 25 μg of LLO per ml for 10 min before PMA stimulation, the response was suppressed almost completely. There was a dose-dependent suppression of the peak chemiluminescence response of PEC treated with various concentrations of LLO for 10 min (data not shown).

Viability of PEC after treatment with LLO. To obtain some insight into the interaction between LLO and the macrophage membrane, we examined PEC for viability after treatment with various doses of LLO by the trypan blue dye exclusion method. The decrease in viability depended on the

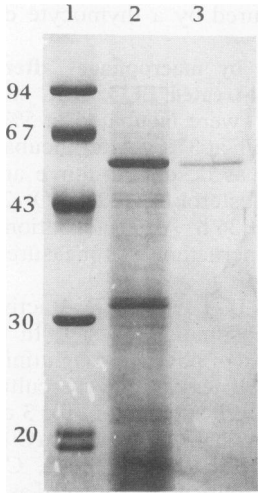

FIG. 1. SDS-PAGE analysis of purified *L. monocytogenes* hemolysin. Lanes: 1, molecular mass standards (in kilodaltons); 2, ammonium sulfate precipitate of the culture supernatant; 3, finally obtained hemolysin preparation.

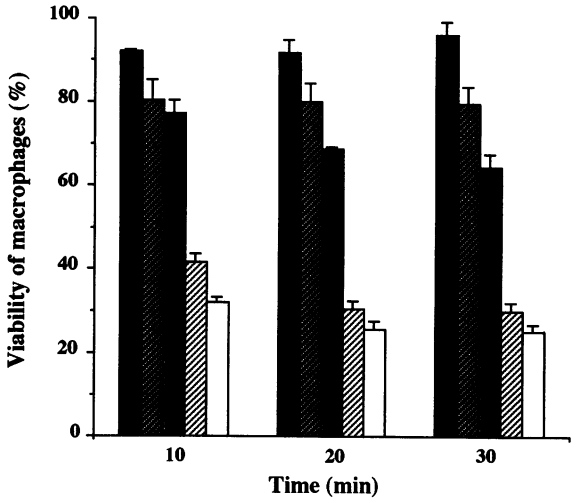

FIG. 3. Kinetic changes in the viability of macrophages after treatment with various doses of LLO. PEC (10^6) suspended in HBSS were incubated with LLO at final concentrations of 0, 6.25, 12.5, 25, and 50 μg/ml (bars from left to right). PEC were incubated with each dose of LLO for 10, 20, and 30 min.

concentration of LLO and the length of incubation with LLO (Fig. 3).

Macrophage phagocytosis after incubation with or without LLO. For determination of the effect of LLO on macrophage phagocytosis, PEC were incubated with or without LLO in HBSS or RPMI 1640 CM and examined under a light microscope for the ability to phagocytose opsonized SRBC (Fig. 4). Phagocytosis of opsonized SRBC by PEC treated with 25 μg of LLO per ml for 30 min in HBSS decreased to 3% of that by untreated PEC. Morphological examination by light microscopy after Giemsa staining revealed that 78% of the total macrophages became ghost cells, abnormally enlarged cells surrounded by a discrete cell membrane with a few nuclear remnants. However, phagocytosis of opsonized SRBC by PEC treated with the same concentration of LLO in RPMI 1640 CM decreased to 20% of the control level, and the percentage of ghost cells was 66.8%. It was speculated that the cholesterol in RPMI 1640 CM might have neutralized the cytotoxic activity of LLO to some extent. Similar results were obtained with latex beads (data not shown).

Restoration of LLO-induced suppression of macrophage function by preincubation of LLO with cholesterol. For determination of the effect of cholesterol on LLO-induced suppression of macrophage viability and function, LLO was incubated with cholesterol and examined for toxic activity for macrophages (Fig. 5). The toxic effect of LLO, as determined on basis of the viability of macrophages, could be neutralized completely by incubation of LLO with cholesterol. The suppressive effects on phagocytosis and chemiluminescence were also significantly reduced.

LPS-induced IL-1 production by PEC treated with LLO. For determination of the functional aspects of LLO other than cytotoxic activity, the effect of LLO on LPS-induced IL-1 production by PEC was investigated (Fig. 6). There was no difference in IL-1 production among PEC treated with cholesterol or LLO or left untreated. PEC treated with LLO incubated with cholesterol showed higher IL-1-producing activity than the other PEC upon stimulation with LPS, although there was no statistically significant difference ($P > 0.05$) between treatment with LLO and treatment with cholesterol-treated LLO.

IL-1 production by macrophages after stimulation with LLO or cholesterol-treated LLO. For examination of the ability of LLO or cholesterol-treated LLO to directly stimulate macrophage IL-1 production, IL-1 activity in the culture supernatants was determined after stimulation with these preparations (Fig. 7). Macrophages stimulated with LLO or cholesterol-treated LLO showed a very high level of IL-1 production compared with that of unstimulated macrophages. Incubation of LLO with cholesterol had no effect on LLO-induced IL-1 production by macrophages.

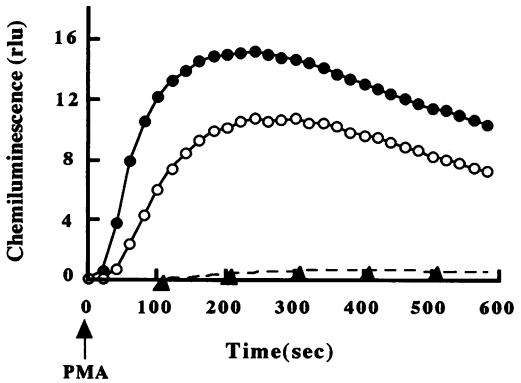

FIG. 2. Chemiluminescence response of PEC without treatment (●), PEC incubated with 25 μg of LLO per ml for 5 min (○), and PEC incubated with 25 μg of LLO per ml for 10 min (▲) after stimulation with PMA. rlu, relative light units.

DISCUSSION

The cell biology of *L. monocytogenes* infection can be divided into four broad stages: internalization, escape from phagosomes, nucleation of actin filaments, and cell-to-cell spread (20). Genes involved in each of these steps have now been identified: *inlA*, *hlyA*, *actA*, and *plcB*; they code for internalin, LLO, a surface protein necessary for *L. monocytogenes* actin assembly, and lecithinase, respectively (17).

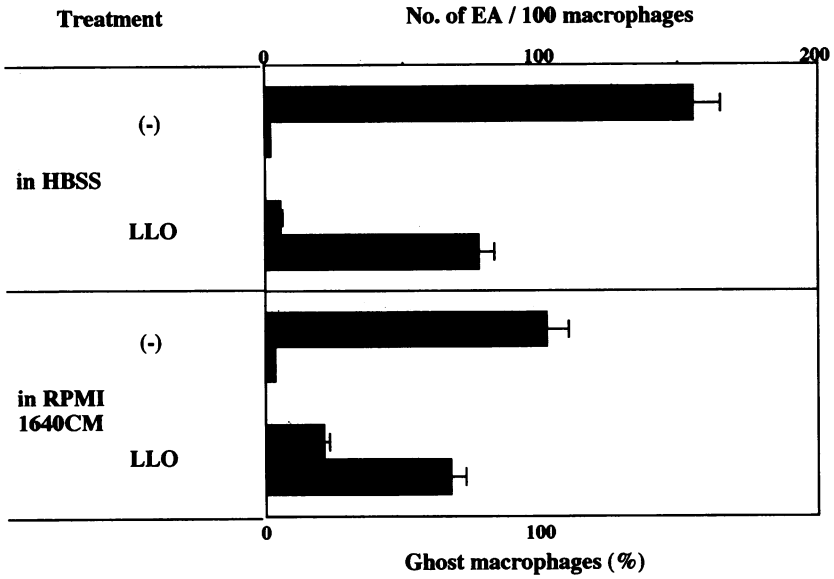

FIG. 4. Macrophage phagocytosis of opsonized SRBC (EA) after treatment of PEC with 25 μg of LLO per ml for 30 min in HBSS or RPMI 1640 CM or after no treatment of PEC. The numbers of opsonized SRBC phagocytosed by 100 macrophages (black bars) and the percentages of ghost macrophages (gray bars) are shown.

Electron microscopic analysis of *plcB* mutants has suggested that lecithinase also may be involved in the lysis of the double-membrane vacuole that is found during cell-to-cell spread (22). Thus, membrane damage may not be solely due to LLO; it is likely that lecithinase is also involved. There are no experimental data regarding the direct effect of LLO on the macrophage membrane. About two decades ago, Kingdon and Sword examined the effect of a hemolytic factor of *L. monocytogenes* on lysosomes and phagocytic cells (12). They observed that the hemolytic factor caused the release of lysosome-associated enzymes from the isolated lysozyme-containing large granule fraction and caused the degranulation of both rabbit and mouse peritoneal cells. Although it was clearly shown in their study that the culture supernatant possessed the ability to lyse the macrophage membrane, the activity cannot be ascribed to 58-kDa LLO, since the hemolytic factor used was a crude concentrate of the culture supernatant possibly containing lecithinase as well as LLO. To investigate the interaction between LLO and the macrophage membrane, we purified an extracellular hemolysin produced by *L. monocytogenes* through the use of ion-exchange chromatography and gel filtration. The purified hemolysin finally produced a single band with a molecular mass of 58 kDa in SDS-PAGE.

The present results showed that purified LLO impaired a variety of functions of peritoneal exudate macrophages. First, a suppressive effect on the luminol-dependent chemiluminescence response of macrophages was exhibited. Since macrophage chemiluminescence triggered by PMA is highly dependent on the generation of a superoxide anion resulting from the activation of the respiratory burst oxidase system located in the intact plasma membrane (3), the functional impairment of the chemiluminescence response after treatment with LLO suggested direct damage to the macrophage membrane. Second, purified LLO suppressed the phagocytic ingestion of opsonized SRBC and latex beads. Third, the exposure of macrophages to LLO resulted in an increase in the number of dead cells, as determined by the trypan blue dye exclusion method. The surface membrane of living cells is able to exclude dyes selectively, whereas dead cells permit

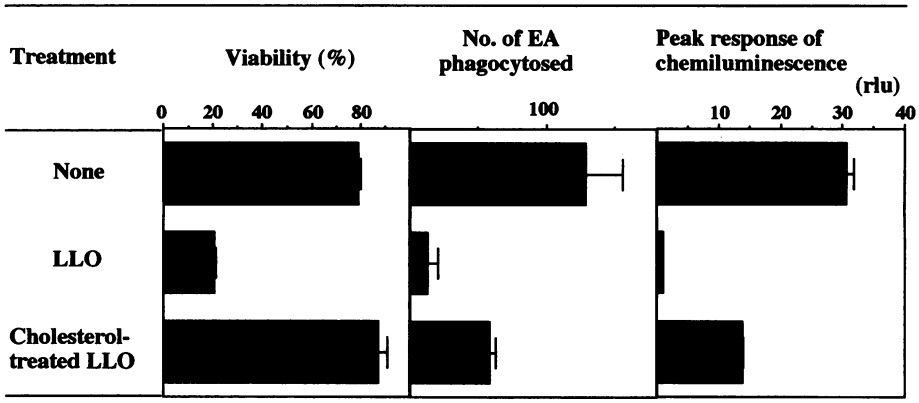

FIG. 5. Restoration of LLO-induced suppression of macrophage viability, phagocytosis, and chemiluminescence by incubation of LLO with cholesterol. EA is as defined in the legend to Fig. 4. rlu, relative light units.

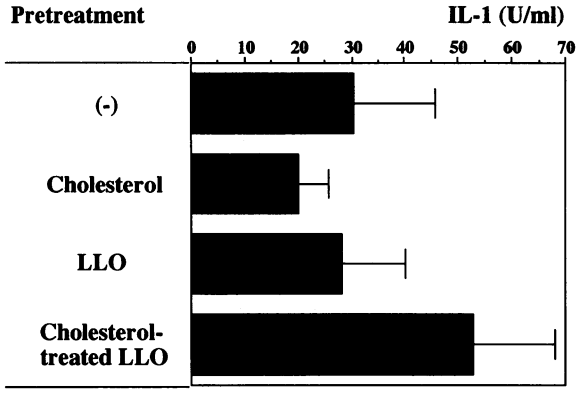

FIG. 6. LPS-induced IL-1 production by untreated macrophages and macrophages incubated with cholesterol, LLO, or cholesterol-treated LLO. After treatment for 30 min, macrophages were stimulated for IL-1 production with LPS. IL-1 activity in the culture supernatant was determined by a thymocyte costimulator assay and expressed in units per milliliter.

dyes to enter easily, and a defect in dye exclusion is a reflection of membrane damage. Furthermore, like the effects of other sulfhydryl-activated toxins, these cytotoxic effects could be mostly abolished by the incubation of LLO with cholesterol.

In an actual infection of a host with *L. monocytogenes*, the membrane-damaging activity of LLO, as observed in the present experiments, may play its role mainly inside macrophages, since the activity is blocked by free cholesterol in the sera even when LLO is produced extracellularly. The bacteria are readily phagocytosed by host macrophages, and a phagosome is formed by invagination of the plasma membrane. Inside the phagosomal compartment, the membrane surrounding the ingested bacteria is the outer leaflet of the plasma membrane (8). Therefore, it is possible that LLO produced inside the phagosome exhibits membrane-damaging activity against the phagosomal membrane as well as the plasma membrane.

In a recent study, we showed that purified LLO is capable of stimulating macrophages, thereby resulting in the induction of IL-1 mRNA transcription and the production of a cell-associated and soluble form of IL-1 (21). An interesting observation of the present study is that LLO induction of IL-1 production by macrophages could not be blocked by the incubation of LLO with cholesterol, while the membrane-damaging ability of LLO could be blocked by cholesterol. This dissociation of the blocking effects of cholesterol suggests that the IL-1-inducing ability of LLO may be distinct from its membrane-damaging ability. In other words, there is a possibility that some site of LLO other than the cholesterol-binding site is involved in the stimulation of IL-1 production or that LLO initiates IL-1 production by macrophages after internalization into the cytoplasm. A very high level of IL-1-inducing activity was also observed in whole cells of *L. monocytogenes* but was limited to viable and hemolysin-producing strains (15). These observations suggest that LLO produced after ingestion of bacteria inside the phagosomal compartment of macrophages not only supports the escape of bacteria from intracellular killing but also contributes to the production of IL-1, which is critical for the development of T cells specific for *L. monocytogenes* (9).

Thus, the present study has revealed two functional aspects of LLO, one being the ability to damage the macrophage membrane and the other being the ability to induce IL-1 production by macrophages. The precise mechanism for these two activities exerted by LLO should be examined further.

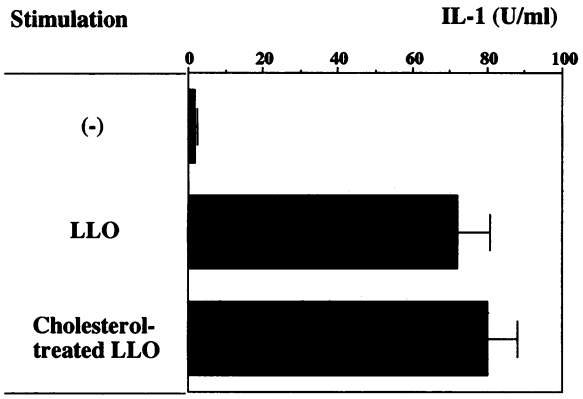

FIG. 7. Effect of treatment of LLO with cholesterol on the ability to directly induce IL-1 production by macrophages. Adherent macrophages were left unstimulated or were stimulated with LLO or cholesterol-treated LLO. IL-1 activity in the culture supernatant after incubation for 36 h is shown.

ACKNOWLEDGMENTS

This work was supported by a grant (02454176) for scientific research from the Ministry of Education, Science and Culture and by a grant from the Naito Foundation, Tokyo, Japan.

We thank Mary Louise Robbins for critical review of the manuscript.

REFERENCES

1. **Alouf, J. E., and C. Geoffroy.** 1988. Production, purification and assay of streptolysin O. Methods Enzymol. **165**:52–59.
2. **Bielecki, J., P. Youngman, P. Connelly, and D. A. Portnoy.** 1990. *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells. Nature (London) **345**:175–176.
3. **Clark, R. A.** 1990. The human neutrophil respiratory burst oxidase. J. Infect. Dis. **161**:1140–1147.
4. **Fujimura, T., T. Suzuki, M. Mitsuyama, H. Saito, and K. Nomoto.** 1989. Antibody-independent protection against *Pseudomonas aeruginosa* infection in mice after treatment with a homologous strain vaccine. J. Med. Microbiol. **28**:101–108.
5. **Gaillard, J. L., P. Berche, and P. Sansonetti.** 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. **52**:50–55.
6. **Geoffroy, C., J. L. Gaillard, J. E. Alouf, and P. Berche.** 1987. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. Infect. Immun. **55**:1641–1646.
7. **Gray, M. L., and A. H. Killinger.** 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. **30**:309–382.
8. **Horwitz, M. A.** 1982. Phagocytosis of microorganisms. Rev. Infect. Dis. **4**:104–123.
9. **Igarashi, K., M. Mitsuyama, K. Muramori, H. Tsukada, and K. Nomoto.** 1990. Interleukin-1-induced promotion of T-cell differentiation in mice immunized with killed *Listeria monocytogenes*. Infect. Immun. **58**:3973–3979.
10. **Jenkins, E. M., A. N. Njoku-Obi, and E. A. Adams.** 1964. Purification of the soluble hemolysins of *Listeria monocytogenes*. J. Bacteriol. **88**:418–424.
11. **Kingdon, G. C., and C. P. Sword.** 1970. Biochemical and immunological effects of *Listeria monocytogenes* hemolysin. Infect. Immun. **1**:363–372.
12. **Kingdon, G. C., and C. P. Sword.** 1970. Effects of *Listeria*

monocytogenes hemolysin on phagocytic cells and lysosomes. Infect. Immun. **1**:356–362.
13. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116**:381–406.
14. **Mengaud, J., M. F. Vicente, J. Chenevert, J. M. Pereira, C. Geoffroy, B. G. Sanzey, F. Baquero, J. C. Perez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56**:766–772.
15. **Mitsuyama, M., K. Igarashi, I. Kawamura, T. Ohmori, and K. Nomoto.** 1990. Difference in the induction of macrophage interleukin-1 production between viable and killed cells of *Listeria monocytogenes*. Infect. Immun. **58**:1254–1260.
16. **Njoku-Obi, A. N., E. M. Jenkins, J. C. Njoku-Obi, J. Adams, and V. Covington.** 1963. Production and nature of *Listeria monocytogenes* hemolysins. J. Bacteriol. **86**:1–8.
17. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60**:1263–1267.
18. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167**:1459–1471.
19. **Schuchat, A., B. Swaminathan, and C. V. Broome.** 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. **4**:169–183.
20. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J. Cell Biol. **109**:1597–1608.
21. **Tsukada, H., I. Kawamura, T. Fujimura, K. Igarashi, M. Arakawa, and M. Mitsuyama.** 1992. Induction of macrophage interleukin-1 production by *Listeria monocytogenes* hemolysin. Cell. Immunol. **140**:21–30.
22. **Vazquez-Boland, J., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart.** 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. **60**:219–230.