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Induction of Macrophage-Mediated Production of Tumor Necrosis Factor Alpha by an L-Form Derived from *Staphylococcus aureus*

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We investigated the capability of an L-form derived from *Staphylococcus aureus* to induce tumor necrosis factor alpha (TNF-α) production in murine peritoneal macrophages. The activity for TNF-α induction was found in the membrane fraction of the L-form but not in the cytoplasmal fraction purified by the sucrose step gradient centrifugation. TNF-α mRNA was also detected in macrophages stimulated with L-form membranes. L-form induced TNF-α production in macrophages from both lipopolysaccharide-responsive and -unresponsive mouse strains. Regardless of the presence of polymyxin B, the activity of TNF-α induction of L-form was mostly found in the phenol layer, but not in the aqueous layer, both of which were prepared by phenol extraction method. Fractions of L-form membranes representing molecular masses of approximately between 29 and 36 kDa were primarily responsible for inducing the production of TNF-α consistently. Moreover, this stimulatory effect was abolished by digestion with *Streptomyces griseus* protease. In Western blot (immunoblot) analysis with anti-lipoteichoic acid antibody, two bands (65 and 45 kDa) were observed in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the phenol layer, whereas one band (14 kDa) was observed in either the aqueous layer or lipoteichoic acid of *S. aureus*. These results suggest that the component in the membrane of the L-form, distinct from cell wall components such as teichoic acid or lipopolysaccharide, possesses the capability to stimulate TNF-α production by macrophages.

Tumor necrosis factor alpha (TNF-α) was originally found in the sera of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)-primed mice that had been challenged with lipopolysaccharide (LPS) or the other cell wall components (6). TNF-α is known not only to cause hemorrhagic necrosis of experimental tumors when injected into tumor-bearing animals (14) but also to be associated with inflammatory diseases (10, 29) and septic shock with a fatal outcome in various bacterial etiologies such as meningococcus and other microorganisms (4, 35).

It is known that L-forms derived from bacteria possess no rigid cell wall and morphological similarities to mycoplasmas under certain conditions. Recently, we and others have reported that several mycoplasmas could induce TNF-α production by murine macrophages (1, 11, 30) and by a human monocytic cell line (31), suggesting that pathological findings caused by these microorganisms might be in part attributed to TNF-α released from macrophages. Although L-forms were isolated from chronic and relapsing infectious diseases, these microorganisms have not been proven to be the etiological agents in any specific diseases (3, 5, 7, 12, 15, 16, 19, 21, 40).

This study was undertaken to determine whether L-form derived from *Staphylococcus aureus* in the presence of cloxacillin could stimulate macrophages to produce TNF-α. The results showed that the L-form stimulated macrophages to produce TNF-α and its activity resided in the membranes and differed from components of the cell wall such as teichoic acid in parental bacteria.

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MATERIALS AND METHODS

Induction of the L-form of *S. aureus*. For induction of the L-form, gradient plate techniques were employed as described previously (26). Briefly, *S. aureus*, derived from a clinical specimen, was cultured for 18 h in brain heart infusion broth. Agar plates (90 by 15 mm) with 5% NaCl were inoculated with 0.2 ml of broth culture. After distributing the inoculum over the surface of the agar, a paper disk containing 1,000 μg of cloxacillin (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was placed on an agar surface, and the plate was incubated at 37°C in the moisture chamber. Typical colonies of L-form appearing on the plate between the antibiotics trough and bacterial growth were transferred to the new agar plate containing 5% NaCl and 500 μg of cloxacillin per ml by the agar block technique. After more than 10 transfers from agar medium, the agar blocks containing L-form colonies were transferred to brain heart infusion broth with 10% NaCl, 5% horse serum (GIBCO Laboratories, Life Technologies, Inc., Grand Island, N.Y.), and 500 μg of cloxacillin per ml. The established L-form could not be grown in the brain heart infusion agar or liquid medium without 10% NaCl and antibiotics.

TNF-α production by macrophages. Peritoneal exudate macrophages were harvested from BALB/c, C3H/HeN, or C3H/HeJ mice (Inoue Animal Center, Kumamoto, Japan) 3 days following intraperitoneal injection of 1 U of OK432 (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) penicillin- and heat-treated *Streptococcus pyogenes* A3 (23), as described previously (1). The culture for the macrophages was carried out in flat-bottom microtest plates (Falcon, Becton Dickinson, Co., Oxnard, Calif.). Each well contained 40×10^5 peritoneal cells in 200 μl of RPMI 1640 supplemented with 5% fetal calf serum FCS (hereafter termed RPMI/FCS).

After 2 h of incubation at 37°C in a 5% CO_2 incubator, these wells were vigorously washed three times with warmed Hanks' balanced salt solution (Nissui Seiyaku Co., Tokyo, Japan) containing 0.1% gelatin (Difco Laboratories, Detroit, Mich.) and then fed with 100 μl of RPMI/FCS; next, an equal volume of various stimulators were added at the indicated doses. More than 70% of the macrophages in the original cell preparations were recovered at 70 to 80% purity, as revealed by myeloperoxidase staining. Endotoxin in medium was less than 1 ng/ml by the *Limulus* test (Endotoxin test-D; Seikagaku Kogyo Co. Ltd., Tokyo, Japan). L-form and the parental *S. aureus* were inactivated at 80°C for 10 min.

Assay for cytotoxicity. After 24 h of cultivation of L cells at a concentration of 3×10^4 cells per 100 μl per well in a microtest plate (Falcon), serial dilutions of test supernatants were added to the wells in the presence of 1 μg of actinomycin D (Banyu Pharmaceutical Co., Ltd., Osaka, Japan) per ml. After 24 h of incubation, the cells were fixed and stained in 0.2% crystal violet–2% ethanol in distilled water for 5 min. The stained plates were washed extensively with running water and dried. Dye uptake was determined by a Titertek Multiskan (Flow Laboratories, McLean, Va.) at 540 nm. Percent cytotoxicity was calculated from the following equation: (1 − optical density of test/optical density of control in five wells) × 100. Cytotoxic titers were expressed as reciprocal values of the dilution which showed 50% cytotoxicity of target cells, as described previously (1).

Isolation of L-form membranes. Membrane of L-form was isolated by osmotic lysis as described previously (25). Briefly, L-form cultured in 10 liters of liquid medium at 37°C for 5 days was collected by centrifugation at 25,000 × *g* for 30 min and washed three times with 10% NaCl solution to avoid lysis. The pellet was resuspended in a small volume of distilled water and sonicated at 20 kC for 15 min, dialyzed against distilled water for 24 h at 4°C, and lyophilized. Recoveries of soluble and insoluble fraction of hypotonically treated L-form were, respectively, 34 and 59% (each fraction/starting amounts [in dry weight]). The powder of L-form was resuspended at a dose of 250 mg/ml and centrifuged at 38,000 × *g* for 45 min. The precipitates were resuspended at a dose of 2 mg/ml. The suspension (500 μl) was layered on a step gradient composed of an upper layer of 2.5 ml of 35% sucrose and a lower layer of 2.5 ml of 50% sucrose and centrifuged at 25,000 rpm for 3 h in the SW25 rotor as described previously (8). The bands at the interface of each fraction were then removed with Auto Densi-Flow model H-C (Haake Buchler Instruments Co., Ltd.) and suspended in beta buffer (0.156 M NaCl, 0.05 M Tris, 0.01 M 2-mercaptoethanol, in distilled water, adjusted to pH 7.4 with HCl). The each sample was centrifuged at 37,000 × *g* for 30 min, and the amount was measured by monitoring the A_{600}. A standard curve was constructed from the data produced by twofold serial dilutions of L-form membranes. The components were interpolated from the standard curve. These materials were lyophilized and stocked at −20°C until use.

Phenol extraction of L-form membrane. Phenol extraction was performed as described previously (39). Briefly, the crude cell membranes were shaken with an equal volume of 90% aqueous phenol at 4°C for 1 h. After centrifugation at 1,500 × *g* for 10 min, the aqueous layer was removed and the phenol layer was washed with 1 volume of water. Both the aqueous and phenol layers were dialyzed against distilled water, and then both fractions were lyophilized. Total phosphorus, glycerol, and protein by phenol treatment of the L-form were determined by the methods of Bartlett (2), Lambert and Neish (20), and Lowry et al. (22), respectively.

Isolation of RNA. Extraction of RNA was performed as described previously (13). Briefly, 10^7 macrophages were incubated with the L-form membrane or the L-form cytoplasm at a dose of 20 μg/ml for 2 h at 37°C. Cells were centrifuged and resuspended in 250 μl of cold 100 mM Tris-HCl (pH 7.5)–0.15 M NaCl–1.5 mM $MgCl_2$–0.65% Nonidet P-40. After vigorous vortexing for 20 s, the suspended pellet was centrifuged at 4,000 rpm for 5 min at 4°C. Supernatant (200 μl) was transferred to a fresh microtube containing 200 μl of 7 M urea, 1% sodium dodecyl sulfate (SDS), 0.35 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.5), and 400 μl of phenol-chloroform (50:50). After mixing for 1 min, the microtube was centrifuged at 15,000 rpm for 2 min at 4°C. The RNA was recovered by precipitation of supernatant with 95% ethanol at −20°C and applied to formaldehyde-agarose gels.

Northern (RNA) blot analysis. RNA (10 μg) was denatured with formaldehyde, electrophoresed on a 1% agarose gel, and transferred to the membrane (Hybond N+; Amersham, Buckinghamshire, United Kingdom) as described previously (28). The membrane was preincubated in the buffer (Rapid hybridization buffer; Amersham) for 30 min at 65°C and subsequently hybridized at 65°C with 10^6 cpm of ^{32}P-labeled murine TNF-α cDNA (Asahi Chemical Inc., Tokyo, Japan) per ml for 2 h. After hybridization, the blot was washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS for 10 min at room temperature, once with 1× SSC plus 0.1% SDS for 15 min at 65°C, and finally twice with 0.7× SSC plus 0.1% SDS for 15 min at 65°C. The membrane was then exposed to film with intensifying screens at −70°C for 48 h. The *Bgl*II-*Eco*RI fragment (614 bp) of the murine TNF-α cDNA was labeled up to a specific activity of 10^8 cpm/μg with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mM; Amersham).

Macrophage Western blot (immunoblot). Phenol extract was mixed with an equal volume of reducing sample buffer (4% SDS, 20% [vol/vol] glycerol, 10% 2-mercaptoethanol, 1.5% Tris-HCl [pH 6.75]), heated to 100°C for 2 min, and applied to a 9% acrylamide gel as described previously (36). After electrophoresis, the gel was stained with Coomassie brilliant blue and rhodamine G6. The gel was also transferred to nitrocellulose paper, rinsed in phosphate-buffered saline, and stained with Aurodye (Amersham). For the detection of the TNF-α-producing activity on the nitrocellulose paper, the paper was cut into 3-mm horizontal strips, transferred to a 96-well microtiter plate, and incubated with peritoneal exudate cells (40×10^4 cells per well) at 37°C in 5% CO_2 for 24 h. The cytotoxic activity of the supernatant was determined as described previously (1). Densitometric analysis of Aurodye-stained bands on the nitrocellulose paper was performed by densitometer (personal densitometer model PD110; Molecular Dynamics, Co. Ltd., Tokyo, Japan). The ratio of protein in each fraction was calculated from absorbance (optical density at 481 nm) per total applied protein.

Preparation of anti-LTA antibody of *S. aureus*. The procedures for preparation of anti-lipoteichoic acid (LTA) antibody were followed by the method of Fiedel and Jackson as described previously (9). Briefly, antiserum against LTA was prepared by precipitating LTA of *S. aureus* (Sigma Chemical Co., St. Louis, Mo.; lot 89F4061) with methylated bovine serum albumin (Sigma; lot 90H9313), emulsifying the precipitate in incomplete Freund adjuvant (Difco) and 1% Tween 80, and injecting rabbits subcutaneously three times at 2-week intervals. One week after the third injection, an ear vein bleed was performed.

Western blotting. The procedures followed the method of

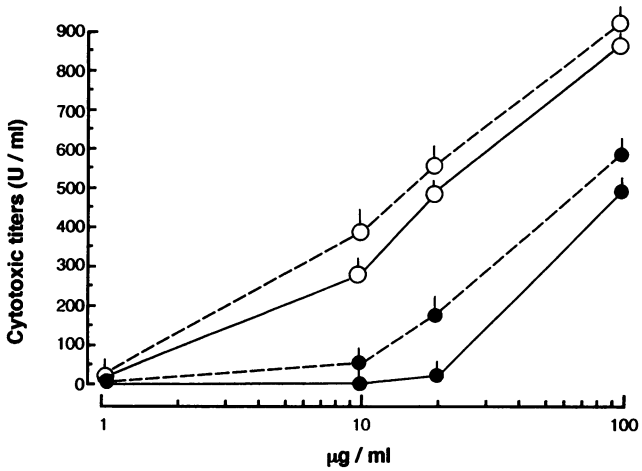

FIG. 1. Effects of *S. aureus* L-form or parental *S. aureus* on the cytotoxic activity of the supernatants of macrophage cultivation. Macrophages (2×10^5) were incubated with heat-inactivated L-form (——) or *S. aureus* (– – –) at the indicated dose for 4 h (●) or 18 h (○). Each value represents the mean of triplicate determinations.

Towbin et al. (34). Nonspecific binding was inhibited by incubation of the nitrocellulose papers in 1% gelatin solution for 1 h at 40°C. The papers were rinsed in saline and incubated with anti-LTA rabbit serum diluted 1:500 in 1% gelatin in saline. After 12 h of incubation at room temperature, the nitrocellulose paper was washed in saline and incubated for 12 h with peroxidase-conjugated rabbit anti-goat immunoglobulin diluted 1:8,000 (Organon Teknika Corp. Cappel Research Products, Durham, N.C.) at room temperature. For the color reaction, the blots were soaked in a solution of 25 μg of *o*-dianisidine per ml, 0.01% H_2O_2, 10 mM Tris-HCl, pH 7.4. The reaction was terminated after 20 to 30 min by washing with water.

Reagents. Polyclonal rabbit anti-mouse TNF-α antibody (Genzyme, Boston, Mass.), monoclonal hamster anti-murine gamma interferon (IFN-γ) antibody (Genzyme), and polyclonal rabbit anti-IFN-α/β antibody (Lee Biochemical Research Inc., San Diego, Calif.) were used for a neutralization test at the indicated dose. LPS of *Escherichia coli* (O26:B6), protease type XIV (Pronase) from *Streptomyces griseus*, and polymyxin B were purchased from Sigma.

RESULTS

Capability of the L-form to stimulate macrophages to produce cytotoxic factors. We examined the cytotoxic activities in culture supernatants of peritoneal macrophages stimulated with heat-inactivated L-form or parental *S. aureus* (Fig. 1). Macrophages (2×10^5) were cultured with L-form or *S. aureus* at various concentrations at 37°C for 4 or 18 h, and then their cytotoxic activity against L cells was tested. *S. aureus* induced the cytotoxic activity at concentrations of ≧ 20 μg/ml for 4 h of incubation, whereas L-form did so only at 100 μg/ml. Both L-form and *S. aureus* induced the cytotoxic activity in their supernatants at concentrations of more than 10 μg/ml for 18 h of incubation. The cytotoxic activity induced by *S. aureus* was slightly higher than that by L-form.

In order to characterize the cytotoxic activity of the supernatants, we performed the neutralization test with anti-TNF-α and anti-IFN antibodies. Anti-mouse TNF-α antibody neutralized the cytotoxic activity of the supernatant by approximately 90%. In contrast, anti-IFN-α/β and anti-IFN-γ antibodies failed to inhibit the cytotoxic activity in the supernatants. These results indicate that most of the cytotoxic activity would be due to TNF-α.

TABLE 1. Induction of TNF-α production by macrophages stimulated with L-form membranes[a]

Fraction	Recovery (%)	TNF-α titers[b] (U/ml) stimulated with following amt (μg/ml) of L-form membrane:			
		Expt 1, 10	Expt 2		
			10	1	0.1
Top on 35% sucrose	18.7	<4	29	<4	<4
Interface between 35% and 50% sucrose	19.8	100	145	100	15
Bottom on 50% sucrose	58.8	<4	<4	<4	<4

[a] Peritoneal macrophages (2×10^5 cells) of BALB/c mice were incubated with each fraction for 18 h at 37°C. Each value represents the mean of triplicate determinations.
[b] See Materials and Methods.

Induction of TNF-α production by L-form membranes. Soluble and insoluble fractions of hypotonically treated L-form were tested for the capability to stimulate macrophage-mediated TNF-α production, to determine whether the production of TNF-α from peritoneal macrophages is due to the membrane of the L-form. The precipitate considered as the membrane component of L-form obtained by the centrifugation at 38,000 × *g* for 45 min induced TNF-α (94 U/ml), but the soluble fraction failed to induce TNF-α (4 U/ml). The precipitate, obtained as described above, was further purified by a step gradient centrifugation. The cell membrane fraction at interface between 35 and 50% sucrose induced TNF-α production by macrophages (Table 1). However, the top and bottom fractions failed to induce TNF-α production. The TNF-α titers were also obtained when lower concentrations of membrane fraction (experiment 2) were added to macrophage culture. We performed Northern blot analysis to detect TNF-α mRNA in macrophages stimulated with L-form membranes (Fig. 2). TNF-α mRNA was highly expressed in macrophages stimulated with L-form membranes. Macrophages stimulated with L-form cytoplasm showed apparently lesser amounts of TNF-α mRNA than that of L-form membrane-stimulated macrophages. To rule

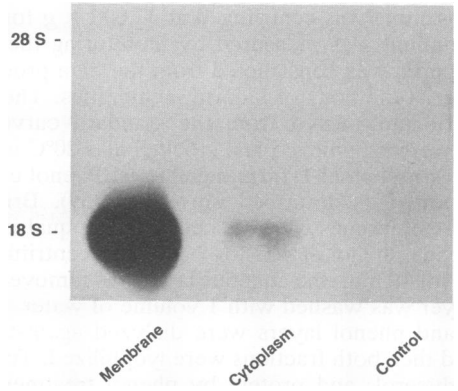

FIG. 2. Northern blot analysis of TNF-α mRNA in macrophages stimulated with L-form membrane or L-form cytoplasm.

TABLE 2. TNF-α activity in the culture fluids of the peritoneal macrophages of LPS-responsive and -unresponsive mice

Stimulator and concn (μg/ml)	TNF-α titers[a] (U/ml)		
	BALB/c	C3H/HeN	C3H/HeJ
S. aureus			
100	108	63	91
20	64	23	38
L-form			
100	100	98	91
20	45	43	75
LPS			
100	46	46	<4
20	13	18	<4
None	<4	<4	<4

[a] Peritoneal macrophages (2×10^5 cells) of different strains were incubated with *S. aureus*, L-form, or LPS for 18 h at 37°C. Each value represents the mean of triplicate determinations.

out the possible involvement of LPS in the medium, TNF-α production by peritoneal macrophages from LPS-responsive mice (BALB/c and C3H/Hen) was compared with those from LPS-unresponsive mice (C3H/HeJ). Both *S. aureus* and its L-form induced the production of TNF-α from macrophages of both LPS-responsive and -unresponsive mice, whereas LPS induced TNF-α production only by macrophages of LPS-responsive mice (Table 2). These results suggest that the membrane components of L-form possess the capacity of TNF-α induction in macrophages.

TNF-α-inducing activity in phenol extraction. The membranes were extracted with a phenol-water mixture, in order to exclude the possible involvement of teichoic acid in the membrane of L-form for the induction of TNF-α. The activity of TNF-α induction was mainly found in the phenol layer, but was also found to a lesser extent in the aqueous layer, where lipoglycan of membranes was generally thought to be accumulated (Table 3). Total phosphorus was accumulated in the aqueous layer, whereas the relative large amount of glycerol and protein were detected in the phenol layer. Furthermore, the TNF-α-inducing activities of L-form in the phenol layer were not inhibited by the addition of polymyxin B to the macrophage culture (Table 4).

Each fraction was run on SDS-polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue and rhodamine 6G (Fig. 3). Many protein bands were revealed in the phenol layer, but not in the aqueous layer. By lipid staining, two major bands of approximately 10 kDa and three sparse bands at approximately 70, 62, and 55 kDa were revealed in the phenol layer and three sparse bands at 97, 35, 23, and 10 kDa were observed in the aqueous layer.

TABLE 3. Chemical analysis and TNF-α production of phenol extraction of L-form membranes

Material	Phosphorus (μmol/g)	Glycerol (μmol/g)	Protein (%)	TNF-α titers[a] (U/ml) stimulated with following amt (μg/ml) of L-form membrane:		
				20	5	1
Membrane	1,147	121	77	38	<4	<4
Phenol layer	152	198	52	133	113	20
Aqueous layer	4,757	56	1	20	5	<4

[a] Each material at the indicated doses was incubated with peritoneal macrophages (2×10^5 cells) for 18 h at 37°C. Each value of cytotoxic titers represents the mean of triplicate determinations.

TABLE 4. Effects of polymyxin B on TNF-α induction by L-form

Stimulator	Polymyxin B[a]	TNF-α titer[b]
L-form	−	64
	+	64
Phenol layer	−	64
	+	64
LPS	−	120
	+	9
None	−	<4
	+	<4

[a] Peritoneal macrophages (2×10^5 cells) were incubated with 20 μg of L-form or phenol layer per ml and 10 μg of LPS per ml for 8 h at 37°C in the presence (+) or absence (−) of 20 μg of polymyxin B per ml.
[b] Each value of cytotoxic titers represents the mean of triplicate determinations.

Analysis of L-form components to induce TNF-α by the macrophage Western blot. Identification of the components responsible for TNF-α induction was performed by macrophage Western blot as described by Wallis et al. (36). The phenol layer of L-form membranes was subjected to SDS-PAGE and was transferred to a nitrocellulose membrane (Fig. 4), cut into 3-mm horizontal strips, and incubated with peritoneal macrophages. Figure 5 shows a representative result of five experiments in which cytotoxic activities of the supernatant of macrophages stimulated with each strip of nitrocellulose were assayed. The activity of TNF-α production was found mainly in fractions 9 to 12, corresponding to molecular weights of 29,000 to 36,000. The other fractions failed to induce significant TNF production. Also, the activities of TNF-α induction in each fraction did not coincide with the amounts of protein analyzed by densitometry. The phenol layer was incubated with protease before SDS-PAGE to confirm that proteins were actually responsible for the TNF-α production. Protein degradation was confirmed by

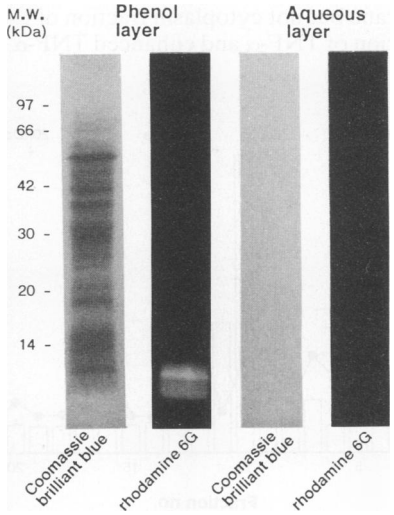

FIG. 3. Protein and lipid staining. Fifteen micrograms of phenol layer or aqueous layer was run on SDS-PAGE and were stained with Coomassie brilliant blue and rhodamine 6G.

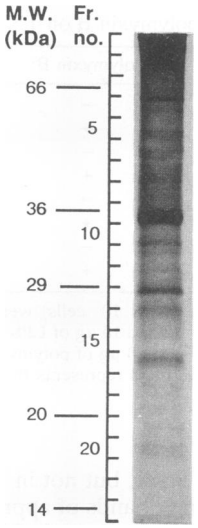

FIG. 4. Aurodye stain of nitrocellulose paper transferred from SDS-polyacrylamide gels of phenol layer. Fr., fraction.

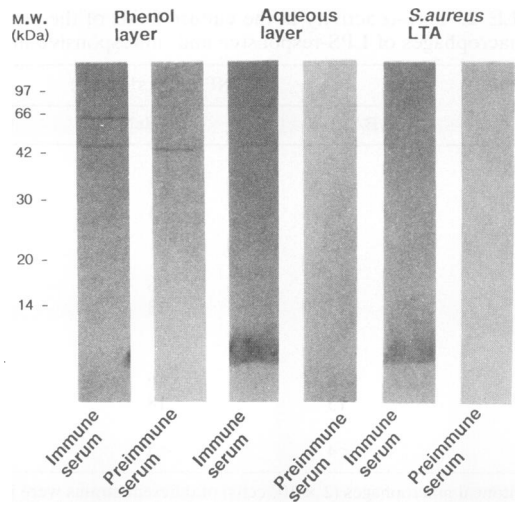

FIG. 6. Western blot analysis by anti-LTA antibody. Phenol or aqueous layer (15 μg) or *S. aureus* LTA (1 μg) was run on SDS-PAGE and transferred to the nitrocellulose paper. Each blot was reacted with rabbit anti-LTA serum and preimmune serum.

staining of the nitrocellulose transfer, and this nitrocellulose paper failed to induce TNF-α production (data not shown).

Western blotting analysis. Western blot analysis was carried out for detection of LTA by using anti-LTA antibody (Fig. 6). Two bands of 45 and 65 kDa were revealed in the phenol layer by using hyperimmune serum, and one band (45 kDa) was also detected by the preimmune serum. One major band of under 14 kDa was detected clearly in the aqueous layer and the purified LTA of *S. aureus* by using hyperimmune serum, but not by preimmune serum. These results suggest that the component in the membrane of L-form which possesses the activity to induce the production of TNF-α might be distinguished from LTA in the L-form membranes.

DISCUSSION

We have demonstrated that L-form derived from *S. aureus* induced TNF-α production by peritoneal macrophages. The membrane but not cytoplasm fraction of L-form induced the production of TNF-α and enhanced TNF-α mRNA in the peritoneal macrophages as well as the inductions of TNF-α mRNA in macrophages by LPS stimulation (17, 32).

It is well known that teichoic acid, a component of gram-positive bacterial cell wall as well as LPS of gram-negative bacilli and lipoarabinomannan of *Mycobacterium tuberculosis*, possesses potent activity to produce TNF-α by macrophages. Toon et al. (33) and Wicken (39) have reported the presence of a little amount of teichoic acid-lipid in the cytoplasmic membranes of *Enterobacter faecalis* and *Lactobacillus fermenti*. Pratt (24), however, described that none of the L-form strains derived from *S. aureus* were found to contain the cell wall ribitol teichoic acid. Weibull et al. (37) also reported that material extracted with the aqueous phase of phenol extraction from the membrane fraction of the L-forms was about as toxic and pyrogenic as the typical enterobacterial endotoxins isolated from cell walls of normal bacteria, but the phenol layer of L-form membrane did not exhibit any endotoxic activities. As the activity for TNF-α induction was found in the phenol layer of L-form membranes but not in the aqueous layer in the present experiments, the results suggest that the components of L-form membranes for TNF-α induction might differ from those of LPS or teichoic acid of the bacterial cell wall.

Although there is the possibility that the protein band is associated with the lipid component of L-form membranes, there was no detection of lipid by rhodamine staining. LTA and protein A are also believed to be associated virtually with gram-positive bacteria which stimulated macrophages and lymphocytes. The TNF-α-inducible component in the phenol layer of L-form might be different from LTA, because the component in SDS-PAGE of the phenol layer which induces TNF-α did not react to anti-LTA antibody in Western blot analysis, which revealed two bands at 60 and 45 kDa. The latter band was also detected with preimmune rabbit serum, as low titers of antibodies reacting with staphylococcus are regularly found in normal rabbit serum (18).

We and the others have reported that mycoplasmas, cell wall-lacking microorganisms closely similar to the L-form in many biological characteristics, exhibited the ability to stimulate TNF-α production by macrophages (1, 11, 30, 31). The

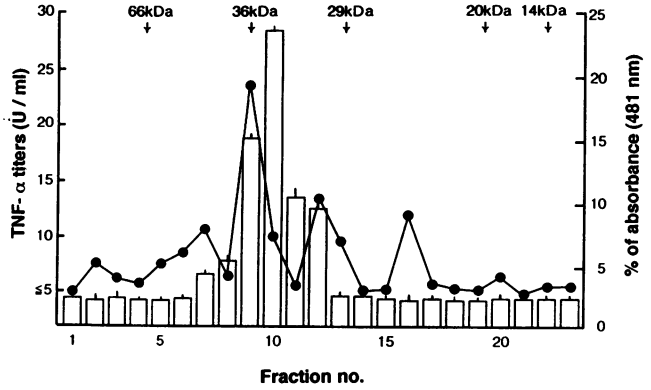

FIG. 5. Induction of TNF-α by nitrocellulose-bound fractions of phenol layer. The cytotoxic titers (□) and ratio of protein (●) in each fraction represent the means of triplicate determinations.

mechanisms of macrophage activation by the membranes of mycoplasmas would differ from those by LPS, because macrophages from C3H/HeJ mice, a low responder to LPS, were shown to increase TNF-α production by mycoplasma membranes (30). Although cell membranes of *Acholeplasma laidlawii* possess lipoglycan, levels of mycoplasmal LPS were extremely less than those of bacterial LPS (38). Furthermore, we have reported that *A. laidlawii* induced the production of TNF-α in a human monocytic cell line, THP-1 cells, by a protein kinase C-independent pathway, while LPS was shown to activate macrophages in a protein kinase C-dependent pathway (31). These evidences suggest that the membranes of L-forms, as well as mycoplasmal membranes, might induce the TNF-α production in a different pathway from that of components of bacterial cell wall such as LPS. Ruuth and Praz (27) described that the mitogenic activity of *Mycoplasma arginini* membranes on B cells was found in the phenol layer and was abolished by the treatment of membranes with proteinase. Wallis et al. have also determined that mycobacterial proteins induce the production of TNF-α by macrophage Western blotting analysis (36). These reports suggested that the protein(s) of microorganisms, unlike LPS, might be responsible for induction of TNF-α production. Although there are several reports describing isolation and detection of L-forms from clinical specimens of inflammatory diseases such as Crohn's disease (3), arthritis (5), and meningitis (19), their pathogenicities remain to be elucidated.

The present results suggest that L-form, induced by exposure of bacteria to antibiotics in infectious disease, possesses the ability to induce TNF-α production and that the released TNF-α might deteriorate inflammatory responses in infectious diseases.

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