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Molecular and Structural Requirements of a Lipoteichoic Acid from *Enterococcus hirae* ATCC 9790 for Cytokine-Inducing, Antitumor, and Antigenic Activities

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Comparison was made between the immunobiological and antigenic properties of two lipoteichoic acid (LTA) fractions (LTA-1 and -2) from *Enterococcus hirae* ATCC 9790, their glycolipid portions, and synthetic compounds partially mimicking the above bacterial products. The more lipophilic LTA-2 fraction was capable of inducing serum tumor necrosis factor alpha and interleukin-6 in muramyldipeptide-primed mice and serum gamma interferon in those primed with *Propionibacterium acnes*. The LTA-2 fraction also induced tumor necrosis factor alpha, interleukin-6, and thymocyte-activating factor (essentially interleukin-1) in murine peritoneal macrophage cultures. Consecutive intravenous injections of muramyldipeptide and the LTA-2 fraction in Meth A fibrosarcoma-bearing BALB/c mice caused hemorrhagic necrosis and marked regression leading to complete regression of the tumor with no accompanying weakening or lethal effects. The LTA-2 fraction was at least 10,000-fold less pyrogenic in rabbits than a reference endotoxic lipopolysaccharide. The more hydrophilic LTA-1 fraction, on the other hand, showed at most marginal activity in the in vivo and in vitro assays. Natural glycolipids (NGL-1 and -2) which were prepared from a chloroform-methanol extract of *Streptococcus pyogenes* and *E. hirae* cells, and comparable in structure to the lipid moieties of the LTA-1 and -2 fractions, respectively, were practically inactive in all of the assays. None of the test synthetic compounds was immunobiologically active, although synthetic partial counterparts of the structure of LTA proposed by W. Fischer (Handb. Lipid Res. 6:123–234, 1990) reacted with murine monoclonal antibody TS-2, which was raised against OK-432, a penicillin-killed *S. pyogenes* preparation, and capable of neutralizing the cytokine-inducing activities of the LTA-2 fraction.

Lipoteichoic acids (LTAs) are biologically active cell surface layer constituents which are widely, though not ubiquitously, distributed in gram-positive bacteria (7, 19). These amphipathic compounds generally consist of a poly(*sn*-glycero-1-phosphate) backbone (which in some cases is substituted with D-alanyl esters or glycosidic groups at the 2-*O* position in the glycerol residues) covalently linked to C_6 hydroxy groups at the nonreducing hexose residue of glycolipids or phosphatidylglycolipids associated with the cytoplasmic membrane (7). A series of studies on the tumor necrosis factor (TNF)-inducing and antitumor activities of *Streptococcus pyogenes* LTA was carried out by Yamamoto et al. (37), Usami et al. (33, 34), and Kotani (21). Subsequently Tsutsui et al. (32) prepared two LTA fractions, LTA-1 and -2, from hot aqueous-phenol extracts of *Enterococcus hirae* ATCC 9790 according to the method of Fischer et al. (8) and found that the more hydrophobic LTA-2 was a potent inducer of TNF-α, alpha/beta interferon (IFN-α/β), and IFN-γ in *Propionibacterium acnes*-primed ICR mice, while LTA-1 was only weakly active. They also showed that both LTA-1 and -2 in combination with muramyldipeptide (MDP) priming exhibited strong antitumor activity against Meth A fibrosarcoma established in BALB/c mice and that both fractions were capable of inducing thymocyte-activating factor (TAF; essentially interleukin-1 [IL-1]) in murine peritoneal macrophage cultures. Cytokine induction by LTA was subsequently confirmed by Bhakdi et al. (2) and Keller et al. (18). These investigators reported that LTA specimens derived from various gram-positive bacterial strains, including several enterococcal species, induced IL-1β, IL-6, and TNF in human monocyte cultures and TNF in rat bone marrow cell cultures.

Tsutsui et al. (32) reported that a phosphatidylglycolipid fraction derived from the acid-hydrolyzed LTA-2 was active in all of the aforementioned bioassays, although alkaline treatment of LTA-2 for deacylation abolished its bioactivities. This finding suggests that a lipid anchor portion of LTA is the biologically active center of the parent molecule and may lead to the conclusion that the entire molecular structure of *E. hirae* LTA is not required for exhibition of its immunobiological activities. However, the bioactive phosphatidylglycolipid preparation tested in the study of Tsutsui et al. was not isolated in a satisfactorily pure form. Therefore, the possibility cannot be excluded that the reported bioactivities of the glycolipid fraction are in reality due to the intact parent molecules which

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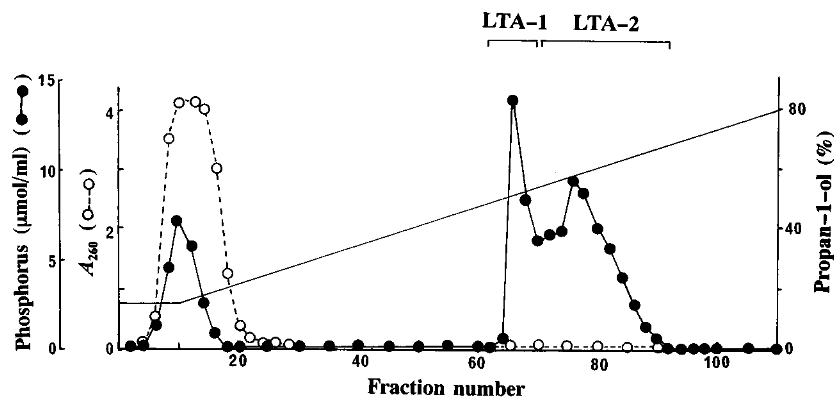

FIG. 1. Hydrophobic interaction chromatography of a crude LTA from *E. hirae* ATCC 9790 on an Octyl-Sepharose column. The crude LTA preparation was fractionated on an Octyl-Sepharose CL-4B column (2.6 by 60 cm) equilibrated with 0.1 M sodium acetate buffer (pH 4.5) containing 15% propan-1-ol. The column was successively eluted with the buffer described above and a linear gradient of 15 to 80% propan-1-ol in the same buffer. Fractions (6 ml each) were collected and monitored by the phosphorus content and A_{260}, which probably represents contaminating nucleic acids. The fractions indicated by the bars were separately pooled as LTA-1 and LTA-2 fractions, respectively.

escaped acid hydrolysis and contaminated the test glycolipid specimen, as pointed out by Kotani (21). Thus, we reexamined the biological activity of intact glycolipid specimens that were isolated without acid hydrolysis from chloroform-methanol extracts of *S. pyogenes* and *E. hirae* cells and correspond to the glycolipid moiety of the diacyl- and tetraacyl-type LTAs proposed by Fischer (7), respectively. We further synthesized four specimens that partially mimicked these LTAs and their glycolipid moieties to test their immunobiological activities. In this report, we also describe the antigenic reactivity of these bacterial and synthetic products with monoclonal antibodies (MAbs) (27, 28) raised against a penicillin-killed *S. pyogenes* ATCC 21060 (strain Su) preparation (OK-432) which has been clinically used in Japan as an antitumor agent.

MATERIALS AND METHODS

LTAs and their glycolipid moieties. (i) Bacterial products. LTA-1 and -2 fractions were prepared from *E. hirae* ATCC 9790 grown aerobically at 37°C for 6 h in Trypticase-tryptone-yeast extract medium (13) as described previously (32). Briefly, delipidated cells (40 g [dry weight]) were extracted with hot aqueous-phenol solution. The extracts were subjected to DNase and RNase digestion, and the digests were fractionated by Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column chromatography with monitoring of the A_{260} and phosphorus content (micromoles per milliliter). The first peak fractions in terms of the phosphorus content were pooled as a crude LTA fraction. This fraction was then subjected to hydrophobic interaction chromatography on an Octyl-Sepharose CL-4B (Pharmacia) column to separate the LTA-1 and -2 fractions, which have different hydrophobicities. The elution profile is shown in Fig. 1, and the yields of LTA-1 and -2 fractions were 100.5 and 133.1 mg (0.25 and 0.33% of the starting delipidated cells), respectively.

The glycolipid moieties of the LTA-1 and -2 fractions were prepared by two methods. One pair of preparations was obtained by hydrolysis of the LTAs with 1 N HCl at 95°C for 20 min (32). The hydrolysate was evaporated to remove HCl and was then shaken with chloroform-methanol-water (1:1:1, by volume) to obtain the glycolipid portion dissolved in the chloroform layer (HGL-1 and -2, respectively, where H indicates acid hydrolysis product). The second pair of glycolipid preparations was extracted from bacterial cells without acid hydrolysis to prepare intact and more homogeneous glycolipids. One of these was obtained from *S. pyogenes* ATCC 21059 (strain Sv) by the method of Ishizuka and Yamakawa (17). Briefly, the lyophilized bacterial cells were extracted with chloroform-methanol (2:1, by volume) at room temperature for 2 h. The extract was then concentrated and shaken with a mixture of chloroform-methanol-water (15:15:13.5, by volume). The separated chloroform layer was withdrawn and reserved, and the remaining water and methanol phases were combined and extracted with an appropriate volume of chloroform. The first and second chloroform-soluble portions were pooled and dried. The dried residue was dissolved in a small amount of chloroform and applied to chromatography on a silicic acid (100 mesh; Mallinckrodt Inc., St. Louis, Mo.) column to obtain the pure LTA-1-type glycolipid. The other glycolipid preparation was obtained by extraction of *E. hirae* ATCC 9790 wet cells with a chloroform-methanol-aqueous sodium acetate buffer (pH 4.7) mixture (1:2:1, by volume) at 60 to 70°C overnight. The extract was evaporated, and the dried residue was then subjected to silica gel column chromatography (Merck Kieselgel 60, 180 g; solvent, chloroform-methanol [4:1, by volume]). The fraction containing the glycolipid, which comigrated with a synthetic specimen (SGL-2; see below) with an R_f value of 0.4 on silica gel thin-layer chromatography (Merck Kieselgel 60 F_{254}; solvent, chloroform-acetone-methanol-acetic acid-water [50:20:10:10:0.5, by volume]), was concentrated. A portion of this fraction was subjected to column chromatography on Sephadex LH-20 (solvent, chloroform-methanol [1:1, by volume]) four times to obtain the pure LTA-2-type bacterial glycolipid. The specimens thus obtained from *S. pyogenes* and *E. hirae* were designated NGL-1 and -2 (N indicating natural product), respectively. The purity (no contamination with the LTAs) of NGL-1 and -2 was confirmed by comparison of their ^{1}H nuclear magnetic resonance spectra with those of the corresponding synthetic glycolipid specimens, which were prepared as described below.

(ii) Synthetic compounds. Two LTA analogs, SLTA(4)-1 and SLTA(4)-2, and two glycolipid analogs, SGL-1 and SGL-2 (S indicating synthetic compound), were synthesized as described elsewhere (10, 11) by referring to the chemical structure of diacyl- and tetraacyl-type LTAs proposed by Fisher (7) (Fig. 2). In brief, SGL-1 and -2 were prepared by the stepwise formation of α-glycosidic bonds and the subsequent removal of protecting groups. Then, SLTA(4)-1 and -2 were synthesized by coupling the corresponding glycolipid portion (SGL-1 and SGL-2, respectively) with the poly(glycerophosphate) (PGP) portion; the latter was synthesized by a simple repeating procedure for the construction of the 1,3-phosphodiester linkage. The chemical structures of these synthetic compounds are shown in Fig. 3. There were no differences between the nuclear magnetic resonance spectra of SGL-1 and -2 and those of NGL-1 and -2, respectively, except the existence of a double bond in the acyl moieties of the bacterial products (10, 11).

Reference compounds and other reagents. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP), used as a priming agent for cytokine induction in mice, was a gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). We also used formalin-killed *P. acnes* whole cells prepared at the Tokyo Institute for Immunopharmacology as an additional priming agent (37). Ultrapurified lipopolysaccharide (LPS) prepared from *Salmonella abortusequi* (12) was a generous gift from C. Galanos, Max-Planck-Institut für Immunbiologie, Freiburg, Germany, and served as a reference compound in most assays for cytokine induction. The LPS specimens prepared from *Salmonella enteritidis* and *Escherichia coli* O111:B4 by the hot phenol-water extraction method (Difco Laboratories, Detroit, Mich.) were used as reference compounds in the antitumor and *Limulus* tests, respectively. Phytohemagglutinin (HA-16) was purchased from Wellcome Diagnostics (Temple Hill, United Kingdom). Recombinant human TNF-α (36) and recombinant human IL-6 (14, 15) were gifts from the Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan) and T. Hirano, Osaka University, Osaka, Japan, respectively. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) was purchased from Wako Pure Chemicals (Osaka, Japan), and [^{125}I]deoxyuridine was obtained from Dupont, NEN Research Products (Boston, Mass.).

Anti-*S. pyogenes* MAbs. Two murine immunoglobulin M-type MAbs, TS-1 and TS-2, both of which were raised against OK-432, namely, penicillin-killed *S. pyogenes* ATCC 21060 cells, were obtained as described previously (27, 28).

Cell lines. L929 cells (NCTC clone 929; ATCC CCL-1) were purchased from Dainippon Pharmaceutical Co. Ltd. The IL-6-dependent cell line MH60.BSF2 (24) was a gift from T. Kishimoto, Osaka University.

Chemical analysis. The chemical compositions of fractions LTA-1 and -2 were analyzed as follows. Phosphorus was determined according to the method of Bartlett (1), with a slight modification, using sodium phosphate as a standard. Fatty acids were analyzed as described by Ikemoto et al. (16) by gas chromatog-

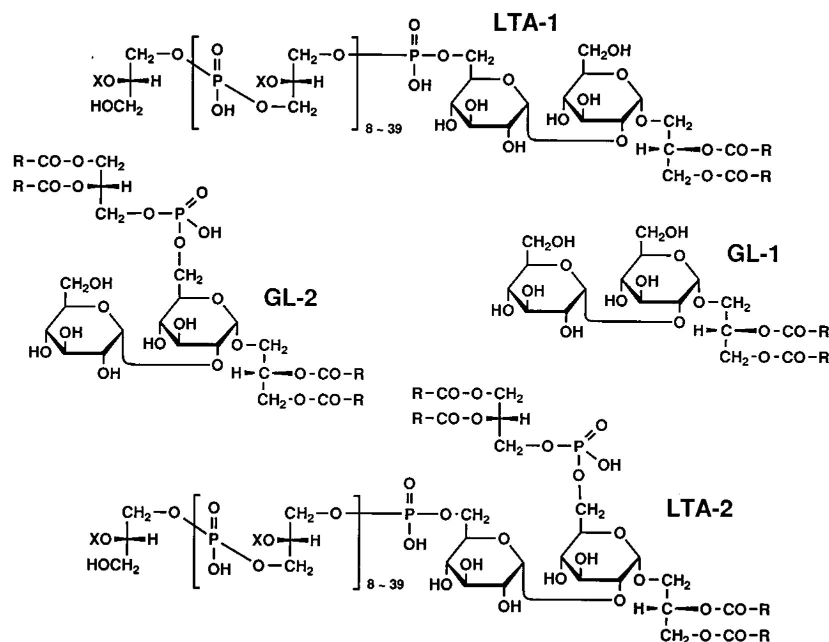

FIG. 2. Chemical structures of *E. hirae* LTA-1 and -2 and their glycolipid moieties proposed by Fischer (7). X, mono-, di-, tri-, or tetra-α-glucosyl residue; R, acyl group.

raphy (GC) using eicosanoic acid as an internal standard. Glucose was analyzed by the alditol acetate method (31), using GC. To estimate glycerol contents, test specimens were hydrolyzed with 2 M HCl at 125°C for 48 h in a sealed tube under nitrogen. Mannitol was added as an internal standard, and the mixture was extracted with hexane to remove fatty acids. After removal of HCl by repeated coevaporation with methanol, the hydrolyzed residue was treated with alkaline phosphatase (from *Escherichia coli*; Wako Pure Chemicals) at 37°C for 24 h in 0.04 M ammonium carbonate buffer at pH 9.0. After repeated coevaporation with methanol, the residue was converted to its trimethylsilyl derivative with 1,1,1,3,3,3-hexamethyldisilazane/pyridine/trimethylsilyl chloride and then analyzed by GC with conditions similar to those used in the analysis of glucose (column temperature, 85 to 200°C). Alanine content was determined as described previously (32), using an amino acid analyzer (Hitachi KLA-5 equipped with a Hitachi 655A apparatus; Hitachi, Tokyo, Japan).

Cytokine induction in mice. To prepare test serum specimens for TNF-α and IL-6 assays, groups of three male C3H/HeN mice (7 to 12 weeks old; Clea Japan, Osaka, Japan) were primed with an intravenous (i.v.) injection of MDP (100 μg in 0.2 ml of physiological saline). Four hours later, the mice received i.v. injections of test specimens in 0.2 ml of saline. After 90 min, animals were bled from the axillary vein. For the induction of IFN-γ, groups of three C3H/HeN mice were primed with an intraperitoneal injection of formalin-killed *P. acnes* whole cells (1.2 mg [dry weight] in 0.2 ml of saline), followed after 7 days by i.v. injection of test specimens. Four hours after the eliciting injection, the mice were bled to obtain test sera.

Cytokine induction in murine peritoneal macrophage cultures. The experimental methods were described previously (30). Briefly, thioglycolate medium-induced peritoneal macrophages (10^5/100 μl per well of a flat-bottom microculture plate) of C3H/HeN mice were cultured in RPMI 1640 medium with or without test materials at 37°C for 24 h. Triplicate culture supernatants for each test point were pooled as cell-free specimens to determine TNF-α, IL-6, and cell-free TAF activity. Residual adherent macrophages on the plate added with fresh medium (100 μl per well) were disrupted by freezing (at −80°C) and thawing (at 25°C) three times. The triplicate disrupted cell suspensions were pooled as cell-associated specimens for the TAF assay.

Cytokine assays. Assay methods were described previously (30). Briefly, TNF activities in test serum and cell-free macrophage culture specimens were determined by measuring cytotoxic activity against L929 cells. On the basis of a ratio of a 50% cytotoxic dose of a test specimen to that of standard recombinant human TNF-α, TNF levels were expressed as nanograms (strictly speaking, nanogram equivalents to human TNF-α) per milliliter. The TNF activity induced

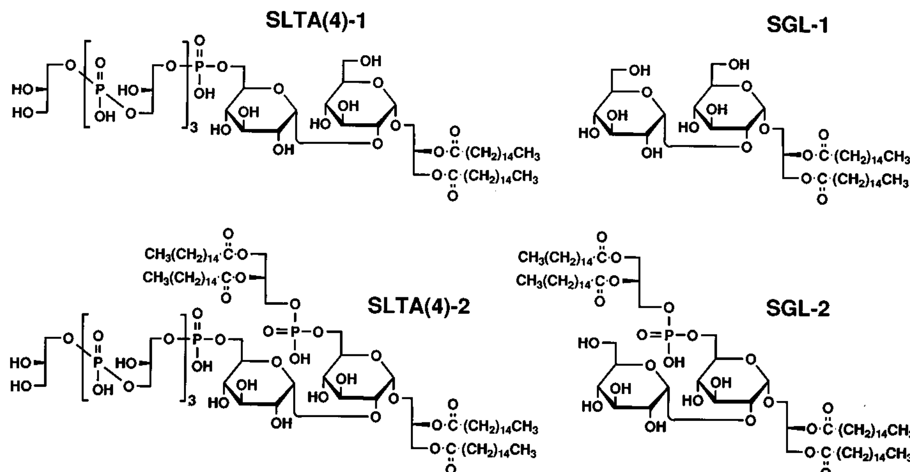

FIG. 3. Chemical structures of synthetic analogs of LTA-1 and -2 [SLTA(4)-1 and -2, respectively] and their glycolipids (SGL-1 and -2, respectively).

by test specimens in this study was confirmed to be due to TNF-α by complete neutralization of the cytotoxic activity of representative test specimens against L929 cells with an anti-murine TNF-α rabbit antibody, which was kindly supplied by Suntory Inc. (Osaka, Japan). The IL-6 activity of test sera and cell-free macrophage culture specimens was determined by measuring the capacity to support the in vitro growth of the IL-6-dependent cell line MH60.BSF2. The IL-6 activities in test specimens, determined by referring to the titration curve of the standard recombinant human IL-6, were expressed as units (equivalent to human IL-6) per milliliter. IFN-γ levels in test murine serum, expressed as nanograms per milliliter, were determined by using murine IFN-γ enzyme-linked immunosorbent assay (ELISA) kits (Genzyme, Boston, Mass.). TAF activity in test cell-associated specimens was determined by a conventional comitogenic assay in the presence of phytohemagglutinin (1.0 μg/ml) for IL-1 measurement, using thymocytes from C3H/HeJ mice as the target cells. The results were expressed as the stimulation index (SI), where SI = [^{125}I]deoxyuridine uptake (counts per minute) in the presence of test stimulant/[^{125}I]deoxyuridine uptake (counts per minute) in medium containing phytohemagglutinin alone.

Antitumor effects against Meth A fibrosarcoma established in BALB/c mice. A Meth A fibrosarcoma (solid type) which had been maintained at the Tokyo Institute for Immunopharmacology was inoculated intradermally into BALB/c mice (6 to 7 weeks old) at 2.5×10^5 cells per mouse. The animals bearing Meth A tumors 7 to 8 mm in average diameter 8 days after tumor inoculation were pretreated by i.v. injection of MDP (100 μg per mouse). Four hours after MDP priming, the mice received i.v. injections of test specimens. The antitumor effects of these consecutive treatments were determined by comparing the following parameters in the test and control groups: size ([length by width]$^{1/2}$ [millimeters]), hemorrhagic necrosis, and complete regression of Meth A tumors; and body weight, general health, and death. These parameters were determined at appropriate intervals for an observation period of 28 to 31 days. The extent of hemorrhagic necrosis was evaluated by measuring the area of necrosis as a percentage of the total tumor area and scored as follows: 3, extensive (≥50%); 2, moderate (50 to 25%); 1, slight (<25%); and 0, no change (5). At the end of the observation period, the tumors were resected and weighed in experiments B, C, and D.

Pyrogenicity. The pyrogenicity test was performed as described previously (20). Briefly, Japanese domestic rabbits, each weighing around 3 kg, were injected i.v. with LTA-1 and -2 (100 μg/ml/kg) in saline. The rectal temperature of each rabbit was measured and recorded continuously for 5 h to determine the maximum increase of body temperature.

***Limulus* test.** *Limulus* activity was quantitatively determined with Endospecy (Seikagaku Co.), an endotoxin-specific chromogenic *Limulus* test reagent (26). LPS from *Escherichia coli* O111:B4 (Difco) was used as a reference standard.

Serological assay. ELISA was carried out with MAbs TS-2 and -1. Microtiter plates (Falcon 3915; Becton Dickinson and Co., Paramus, N.J.) were coated with test materials (100 μl per well), which were serially 10-fold diluted from 1 to 10^{-5} mg/ml, and blocked with Block Ace (Dainippon Pharmaceutical Co.). After an incubation at room temperature for 1 h, 50-μl aliquots of TS-2 or -1 (5 μg of protein per ml) were added to the wells and incubated overnight at 4°C. The wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20, then 200-fold-diluted peroxidase conjugated to goat anti-mouse immunoglobulin (Cappel, West Chester, Pa.) was added, and the plates were incubated at room temperature for 1 h. *o*-Phenylenediamine reagent (25 mg of *o*-phenylenediamine, 5 ml of methanol, 25 μl of 31% peroxide, 45 ml of 0.2 M citrate buffer [pH 5.5]) was added to the wells. Five minutes later, the reaction was stopped with 2 N sulfuric acid, and the A_{492} was measured, with reference to the A_{655}.

Neutralization of cytokine-inducing activities of LTA-2 by MAb TS-2. For the in vivo assay, LTA-2 (4 μg) or a reference *S. abortusequi* LPS (0.8 μg) was incubated with or without TS-2 (160 μg of protein) in 500 μl of physiological saline at 37°C for 2 h. The mixture was injected i.v. into MDP-primed C3H/HeN mice. The levels of TNF and IL-6 were determined with serum specimens collected 90 min after the eliciting injection. For the in vitro assay, aqueous LTA-2 (80 μg/ml) was incubated with an equal volume of TS-2 (0 to 450 μg/ml) at 37°C for 2 h. The mixture was diluted with culture medium to a final concentration of 10 μg of LTA per ml and then added to the monolayer of peritoneal macrophages from C3H/HeN mice. Twenty-four hours thereafter, TNF and IL-6 activities in the culture supernatants were measured.

Miscellaneous. All test specimens for cytokine assays were stored at −80°C until use. The statistical significance of the differences between each test and its respective control was determined by Student's *t* test. However, with some assays in which the calculation of the mean and standard error of the mean did not seem to be realistic, standard error of the mean values are not shown. Including these cases, the range of induced cytokine levels in triplicate assays was within 20% of the respective mean value. We took care throughout this study to prevent possible contamination of test specimens and instruments with extraneous bacterial endotoxins.

RESULTS

Chemical composition of LTA-1 and -2 fractions. Tables 1 and 2 summarize the data from chemical analyses performed with LTA-1 and -2 fractions, the major test bacterial products used in this study. The marked difference in fatty acid contents between LTA-1 and -2 (0.16 mmol/mg for LTA-1 and 0.26 mmol/mg for LTA-2) and the structures of LTA proposed by Fischer (7) led us to conclude that our LTA-1 and -2 contained two and four fatty acid residues, respectively. On the basis of this assumption and the proposed structures of LTA (Fig. 2), the PGP portion of LTA-1 appears to contain 12 glycerol residues substituted with a total of 34 glucose and 5 alanine residues. LTA-2 contains, in contrast, 19 glycerols with 44 glucose and 7 alanine residues. It must be admitted, however, that an additional 7 and 2 mol of phosphorus were present in LTA-1 and -2, respectively; these might be present in the form of phosphomonoesters and/or phosphodiesters linked to the molecules. The main fatty acids present were $C_{18:1}$ and $C_{16:0}$ in both LTA-1 and -2 fractions (Table 2).

Induction of serum TNF-α and IL-6 by bacterial LTA and glycolipids in MDP-primed mice. As summarized in Table 3, the LTA-2 fraction and the reference *E. hirae* whole cells induced high levels of serum TNF-α and IL-6 in MDP-primed mice. A dose as small as 0.8 μg (unless otherwise stated, dose per mouse) of the LTA-2 was active for TNF-α induction, and the potency of a dose of 20 μg was comparable to that of the reference LPS (0.8 μg). In contrast, the LTA-1 fraction exhibited no activity at the highest test dose used (100 μg). None of the glycolipid specimens, i.e., HGL-1 and -2 and NGL-1 and -2, irrespective of the type of parent products or the preparation method, exhibited any significant capacity to induce TNF-α at a dose of 100 μg. In view of the possible overdose suppression

TABLE 1. Chemical composition of LTA-1 and -2 fractions obtained from *E. hirae* ATCC 9790[a]

Test fraction	Concn (mmol/mg [molar ratio[b]])				
	Phosphorus	Fatty acids[c]	Glucose	Glycerol	Alanine
LTA-1					
Found	1.55 (19.3)	0.16 (2)	2.86 (35.5)	1.05 (13.2)	0.40 (4.9)
Reference[d]	1.82 (15.1)	0.24 (2)	2.46 (20.4)	1.81 (14.9)	0.44 (3.6)
LTA-2					
Found	1.39 (21.8)	0.26 (4)	2.93 (46.0)	1.34 (21.0)	0.43 (6.8)
Reference	1.95 (18.0)	0.48 (4)	2.24 (20.6)	1.60 (14.7)	0.29 (2.6)

[a] Contents of constituents of LTA-1 and -2 amounted to about 80% of the test specimens on the basis of weight. No sugars or amino acids other than those listed were detected in an appreciable amount.
[b] Relative molecular ratio based on the fatty acid contents in the respective LTA fraction (see text).
[c] Estimated on the basis of the fatty acids and their relative ratios (shown in Table 2) detected by GC analysis.
[d] Calculated on the basis of analytical data presented in reference 32 (Table 1).

TABLE 2. Fatty acid composition of LTA fractions obtained from *E. hirae* ATCC 9790

Fatty acid	LTA-1		LTA-2	
	Amt (wt %)[a]	Molar ratio[b]	Amt (wt %)	Molar ratio
$C_{14:0}$	0.1	0.07	0.17	0.07
$C_{16:1}$	0.25	0.16	0.47	0.17
$C_{16:0}$	1.51	0.97	2.14	0.76
$C_{18:1}$	1.72	1	3.11	1
$C_{18:0}$	0.25	0.14	0.36	0.11
Unknown	0.49		0.63	
Total	4.32		6.88	

[a] In the LTA fraction.
[b] Molar ratio based on $C_{18:1}$ in the respective LTA fraction.

TABLE 3. Induction of serum TNF-α and IL-6 by i.v. injection of whole cells, LTAs, and glycolipids prepared from *E. hirae* into MDP-primed C3H/HeN mice[a]

Test material	Dose (μg/mouse)	Mean level ± SEM	
		TNF-α (ng/ml)	IL-6 (U/ml)
Whole cells	1,000	233 ± 14	886 ± 244
	500	158 ± 40	406 ± 72
LTA-1	100	<0.1	27.6 ± 13.4
LTA-2	100	297 ± 33	969 ± 48
	20	300 ± 27	665 ± 33
	4	113 ± 46	311 ± 15
	0.8	30 ± 1	239 ± 12
HGL-1	100	13 ± 3	9.8 ± 2.6
HGL-2	100	<0.1	17 ± 9
NGL-1	100	<0.1	0.8 ± 0.6
NGL-2	100	<0.1	0.5 ± 0.1
LPS[b]	0.8	225 ± 37	2,230 ± 325
Saline		<0.1	0.7 ± 0.2

[a] Groups of three male C3H/HeN mice (7 to 12 weeks old) were primed with an i.v. injection of MDP (100 μg). Four hours later, the mice received i.v. injections of test specimens (indicated doses) for the induction of TNF-α and IL-6. The cytokine levels were determined in serum specimens collected 90 min after elicitation as described in the text.

[b] Ultrapurified LPS specimen prepared from *S. abortusequi.*

of cytokine induction, with the HGL-2 specimen, a wide range of doses (from 100 to 0.8 μg per mouse) was tested for TNF-α induction but with totally negative results (data not shown). Induction of IL-6, on the other hand, is a more sensitive indicator of the cytokine-inducing activity of test specimens than TNF-α induction. Thus, in addition to the LTA-2 and whole cells, some of the preparations incapable of inducing TNF-α in MDP-primed mice, namely, LTA-1, HGL-2, and HGL-1, caused weak but detectable IL-6 induction. However, both NGL-2 and NGL-1 were totally inactive, suggesting that the detected activity of HGL-2 or -1 is due to the active LTA fractions escaping acid hydrolysis.

Induction of serum IFN-γ by LTA fractions and glycolipids in *P. acnes*-primed mice. Of four test specimens (LTA-1, LTA-2, HGL-1, and HGL-2), only LTA-2 elicited the induction of a significant level of IFN-γ in the serum of *P. acnes*-primed mice. However, its potency was much lower than that of the reference LPS in terms of both the extent of induction and the effective dose. The other three bacterial products induced only marginal levels of IFN-γ production (Table 4).

Induction of cytokines by bacterial LTA fractions and glycolipids in murine macrophage cultures. Table 5 shows that

TABLE 4. Induction of serum IFN-γ by i.v. injection of *E. hirae* LTAs and glycolipids into *P. acnes*-primed C3H/HeN mice[a]

Test material	Dose (μg/mouse)	Mean serum IFN-γ level (ng/ml) ± SEM
LTA-1	100	2.0 ± 1.1
LTA-2	100	20.5 ± 6.4
HGL-1	100	4.0 ± 1.1
HGL-2	100	5.2 ± 0.6
LPS	0.8	120.7 ± 9.7
Saline		ND[b]

[a] Groups of three male C3H/HeN mice (7 to 12 weeks old) were primed by i.p. injection of formalin-killed *P. acnes* cells (1.2 mg [dry weight]) administered 7 days before the i.v. injection of test specimens (indicated doses). IFN-γ levels in serum specimens collected 4 h after elicitation were measured with an ELISA kit.

[b] ND, not detected.

TABLE 5. Induction of cytokines by *E. hirae* LTAs and glycolipids in murine peritoneal macrophage cultures[a]

Test material	Dose (μg/ml)	TNF-α (ng/ml)	IL-6 (U/ml)	CA-TAF (SI; mean ± SEM)[b]
LTA-1	100	0.2	12.5	6.0 ± 0.9**
	10	<0.05	15.0	9.6 ± 1.6**
	1	<0.05	11.5	5.7 ± 0.3**
	0.1	<0.05	8.0	4.2 ± 0.9*
LTA-2	100	1.9	15.5	4.5 ± 0.2**
	10	0.2	188.0	15.1 ± 0.7**
	1	<0.05	95.0	12.1 ± 2.4*
	0.1	<0.05	22.5	4.7 ± 0.5**
HGL-1	100	0.7	12.5	11.6 ± 0.5**
	10	<0.05	11.5	7.9 ± 2.1*
	1	<0.05	9.5	4.0 ± 0.5*
	0.1	<0.05	7.0	3.1 ± 0.5
HGL-2	100	<0.05	15.0	6.7 ± 1.2*
	10	<0.05	17.0	3.5 ± 0.6*
	1	<0.05	27.5	3.2 ± 0.5**
	0.1	<0.05	6.5	2.9 ± 0.3*
LPS	0.1	1.3	45.0	13.6 ± 1.4**
None	0	<0.05	<2.0	2.6 ± 0.4

[a] Monolayer cultures of thioglycolate-induced C3H/HeN peritoneal macrophages were incubated in triplicate with test or reference specimens for 24 h. The pooled culture supernatants were assayed for TNF-α and IL-6, and the macrophage specimens disrupted by alternate freezing and thawing were tested for cell-associated TAF (CA-TAF) as described in the text.

[b] CA-TAF activity in test culture significantly higher than in the control culture (none of the test specimens) (Student's *t* test; *, $P \leq 0.05$; **, $P \leq 0.01$).

the LTA-2 fraction stimulated murine peritoneal macrophage cultures to produce cell-free TNF-α and IL-6 and cell-associated TAF activity attributable to IL-1. Overdose suppressive effects were noted with IL-6 and cell-associated TAF induction by the LTA-2 fraction. Test compounds other than LTA-2, including the glycolipid fractions, caused weak but detectable induction of cytokines. This finding may indicate that the dependency of cytokine induction on the structure of test compounds was less strict with the stimulation of macrophage cultures compared with the structural dependency for the elicitation of MDP-primed mice.

Regression of Meth A fibrosarcoma in MDP-primed BALB/c mice by LTA-2 administration. The i.v. injection of the LTA-2 fraction (200 μg per mouse) caused, within a few days, marked hemorrhagic necrosis (average score of 2.5 in a scoring system in which the maximum value is 3.0) in all six of the MDP-primed BALB/c mice bearing Meth A fibrosarcomas (experiment A; Fig. 4). Four of six mice which received injections of the LTA-2 fraction showed complete regression of the established tumors, while one mouse died on day 28 as a result of tumor growth. Under the same assay conditions, three of six tumor-bearing mice died within a few days after receiving i.v. injections of *S. enteritidis* LPS (1 μg). These early deaths may be due to an enhanced susceptibility to the lethal effects of LPS by MDP priming and tumor bearing. The residual three mice showed complete disappearance of the tumors. In contrast, LTA-1 (200 μg) showed no significant antitumor effects in terms of causing significant hemorrhagic necrosis or tumor regression. The bacterial glycolipids NGL-1 and -2 (100 μg) were virtually ineffective in both induction of hemorrhagic necrosis and tumor regression (experiment B; data not shown).

Figure 5 shows the weight of Meth A tumors resected at the end of the observation period on day 28 or 29; calculations were based on the data for three different experiments (B, C, and D). A group of 14 MDP-treated Meth A tumor-carrying mice which received the LTA-2 fraction (200 μg; experiment

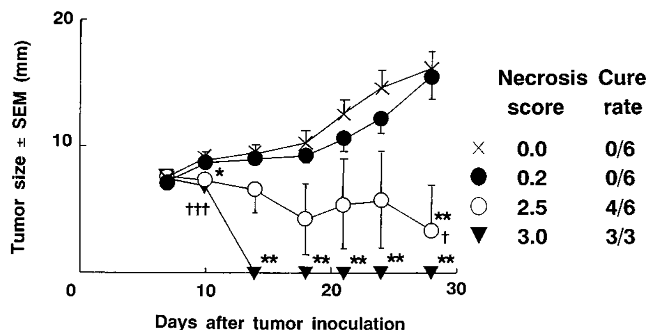

FIG. 4. Regressive and curative effects of LTA-1 and -2 prepared from *E. hirae* and the reference LPS on Meth A fibrosarcoma established in BALB/c mice (experiment A). Groups of six BALB/c mice bearing Meth A tumors (7 to 8 mm in average diameter) were given i.v. injections of MDP (100 μg per mouse). Four hours later, the animals received i.v. injections of test material (200 μg per mouse except for the reference LPS [1.0 μg per mouse]): LTA-2 (○), LTA-1 (●), and the reference LPS of *S. enteritidis* (Difco) (▼). A group that received MDP alone served as the control (x). Three of six mice that received LPS died from the enhanced lethal effects of LPS within 3 days after the i.v. injection of the test LPS (†). The necrosis score represents the mean score for hemorrhagic necrosis 3 days after the i.v. injection of test and reference specimens (the maximum score being 3.0). The cure rate is expressed as the number of mice in which Meth A tumors showed complete regression over the number of mice surviving at the end of the experimental period of 32 days. Statistically significant differences from the control (animals that received MDP alone) as determined by Student's t test were $P < 0.01$ (**).

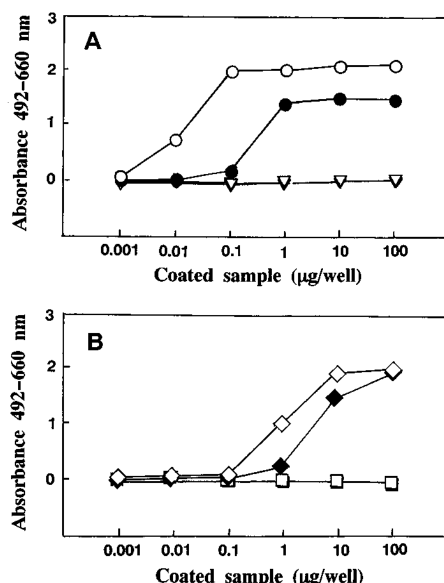

FIG. 6. Serological reactivities (in ELISA) of bacterial LTAs, glycolipids, and related synthetic compounds with anti-penicillin-killed *S. pyogenes* (OK-432) MAb TS-2. See the text for assay conditions. (A) ○, LTA-2; ●, LTA-1; ▽, NGL-2; ▼, NGL-1. (B) ◇, SLTA(4)-2; ◆, SLTA(4)-1; □, SGL-2; ■, SGL-1.

D) showed marked reductions in the weight of established tumors similar to the reductions found for the groups of mice given *S. enteritidis* LPS (1 μg; experiments B and C). Ten of fourteen mice in the LTA-2 fraction (200 μg) group showed complete curing (disappearance) of the tumors. Four of seven mice treated with *S. enteritidis* LPS (1 μg) in experiment B that survived the lethal endotoxic effects showed complete regression of the tumors, and a further group of seven mice similarly treated with the LPS (1 μg; experiment C) also showed a marked reduction of tumor weight. Smaller doses (100, 20, and 4 μg) of the LTA-2 fraction (experiment B) also exhibited low but definite antitumor activity accompanied by significant hemorrhagic necrosis. Under essentially the same assay conditions,

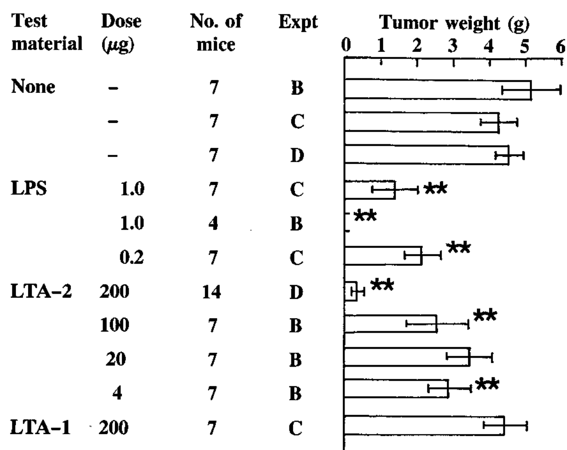

FIG. 5. Resected tumor weight at the end of the observation period on day 28 or 29 after tumor inoculation. The number of mice shown is that at the end of the observation period. In experiment B, three of seven mice in an LPS group died within a few days after the LPS injection. The experimental conditions were essentially as described in the legend to Fig. 4. Statistically significant differences from the respective controls, determined by Student's t test, were $P < 0.05$ (*) and $P < 0.01$ (**).

the LTA-1 fraction (200 μg; experiment C) was totally ineffective.

The corrected body weight was calculated by subtracting the resected tumor weight from the total body weight as determined immediately before tumor resection. There were no significant differences in the corrected body weights between the LTA-2 fraction (200-μg dose)-treated group and the control groups that had received MDP alone. No toxic effects on general health were noted with any of the test mice which received the highest and highly antitumorigenic dose of the LTA fraction (200 μg; experiment D; data not shown).

Pyrogenicity. The LTA-1 and -2 fractions were tested for pyrogenicity in rabbits. Eight of twelve rabbits which received i.v. injections of the LTA-2 fraction (100 μg/kg) exhibited a positive febrile response, i.e., an increases in rectal temperature of more than 0.6°C over a 5-h observation period. The maximum rectal temperature increases in these 12 animals were 1.20, 1.10, 1.00, 0.95, 0.95, 0.95, 0.85, 0.65, 0.55, 0.45, 0.40, and 0.30°C. Thus, the pyrogenicity of the LTA-2 fraction proved to be at least 10,000-fold less than that of a reference *S. abortusequi* LPS, which exhibited definite pyrogenicity at a dose of 0.01 μg/kg. In contrast, none of the four rabbits which received i.v. injections of the LTA-1 fraction (100 μg/kg) showed febrile responses. Endospecy test indicated that possible contamination of extraneous LPS in both LTA-2 and -1 fractions was at most less than 0.002%. This finding suggests that the observed pyrogenicity of the LTA-2 fraction was due to an inherent activity within the fraction itself.

Reactivity of bacterial LTA-1 and -2 fractions and their synthetic analogs with anti-*S. pyogenes* MAbs by the ELISA method. Figure 6A shows that both LTA-1 and -2 fractions exhibited strong serological reactivity with MAb TS-2, but neither NGL-1 nor -2, bacterial products corresponding to the glycolipid moieties of LTA-1 and -2, respectively, reacted with TS-2. Considering both the effective dose and the maximum extent of the reaction, the LTA-2 fraction was more reactive than the LTA-1 fraction. Among synthetic compounds partially mimicking bacterial LTAs and their glycolipids, both

TABLE 6. Inhibitory effect of MAb TS-2 on the cytokine-inducing activities of LTA-2 in MDP-primed C3H/HeN mice[a]

Test material	Serum TNF-α		Serum IL-6	
	Concn (ng/ml)	% of control	Concn (U/ml)	% of control
LTA-2 (4 μg)	52.1 ± 17.4	100	859 ± 109	100
LTA-2 (4 μg) + TS-2 (160 μg)	3.5 ± 1.6[b]	6.7	244 ± 25[c]	28.4
LPS (0.8 μg)	119 ± 18	100	955 ± 276	100
LPS (0.8 μg) + TS-2 (160 μg)	95 ± 23	80	1,007 ± 74	105.4

[a] LTA-2 (4 μg) and LPS of *S. abortusequi* (0.8 μg) were preincubated with or without TS-2 (160 μg of protein) in 500 μl of physiological saline at 37°C for 2 h. The mixture was injected i.v. into C3H/HeN mice primed by i.v. injection of MDP (100 μg) 4 h before. Cytokine levels were determined in serum specimens collected 90 min after elicitation with test materials.

[b] Statistically significant difference from the positive control without preincubation with MAb TS-2 as determined by Student's *t* test ($P < 0.05$).

[c] Statistically significant difference from the positive control without preincubation with MAb TS-2 as determined by Student's *t* test ($P < 0.01$).

TABLE 7. Inhibitory effect of MAb TS-2 on the cytokine-inducing activities of LTA-2 in murine macrophage cultures[a]

Concn (μg/ml)			TNF-α		IL-6	
In preincubation		LTA-2 in culture	Concn (ng/ml)	% of control	Concn (U/ml)	% of control
LTA-2	TS-2					
80	450	10	ND[b]	0	5.0	7
80	150	10	0.25	20	8.4	12
80	15	10	1.30	103	16.9	24
80	0	10	1.26	100	71.9	100
0	0	0	ND		0.2	

[a] Aqueous LTA-2 (80 μg/ml) was incubated with an equal volume of TS-2 (0 to 450 μg/ml) at 37°C for 2 h. The mixture was diluted with culture medium to a final LTA concentration of 10 μg/ml and then added to the monolayer of peritoneal macrophages from C3H/HeN mice. After 24 h, TNF and IL-6 activities in the culture supernatant were measured as described in the text.

[b] ND, not detected.

SLTA(4)-1 and -2 were moderately reactive with TS-2, although little difference was noted between the two compounds (Fig. 6B). The extent of reactivity of these synthetic compounds with TS-2 was about 100-fold less than that of the LTA-2 fraction and comparable to that of the LTA-1 fraction. Neither SGL-1 nor -2 was reactive. Another anti-*S. pyogenes* MAb, TS-1, did not react with any test materials except SGL-1, which showed moderate reactivity for an unidentified reason (data not shown).

Neutralization of cytokine-inducing activities of the LTA-2 fraction by MAb TS-2. In view of the strong reactivity of MAb TS-2 with the LTA-2 fraction, we studied the possibility that this MAb may be capable of neutralizing the cytokine-inducing activities of the LTA-2 fraction. Table 6 shows that the TNF-α- and IL-6-inducing activities of the LTA-2 fraction (4 μg per mouse) in MDP-primed C3H/HeN mice were markedly reduced by preincubation of LTA-2 with TS-2 before i.v. injection. The inhibition rates were 93.3 and 71.6% for TNF-α and IL-6, respectively. In contrast, the induction of serum TNF or serum IL-6 by a highly purified LPS preparation from *S. abortusequi* was scarcely inhibited by the MAb TS-2. Similar neutralizing effects of TS-2 on cytokine induction by the LTA-2 fraction were also noted in the in vitro assay system. MAb TS-2 almost completely neutralized the TNF-α- and IL-6-inducing activities of LTA-2 in murine peritoneal macrophage cultures, although the neutralization of TNF-α induction required more antibody than that of IL-6 (Table 7).

Lack of biological activities of the synthetic compounds. Since synthetic partial counterparts of LTAs, SLTA(4)-1 and -2, exhibited definite reactivity with MAb TS-2, which is capable of neutralizing TNF-α- and IL-6-inducing activities of the LTA-2 fraction, we examined the cytokine-inducing and antitumor activities of these synthetic compounds. However, neither SLTA(4)-2 nor -1 nor their glycolipid analogs, SGL-2 and -1, caused the induction of any detectable serum TNF-α, and at most induced marginal serum IL-6 in MDP-primed C3H/HeN mice. Similarly, none of the synthetic compounds were significantly active in enhancing the induction of TNF-α, IL-6, and TAF by murine peritoneal macrophage cultures (data not shown).

Synthetic compounds (200 μg per mouse) were further tested for antitumor effects when injected i.v. into MDP-primed Meth A tumor-bearing BALB/c mice. None of the synthetic test compounds, including SLTA(4)-2, a synthetic partial analog of the proposed structure of LTA-2, caused any statistically significant regressive effects on the tumors except in some mice which showed weak hemorrhagic tumor necrosis (data not shown).

DISCUSSION

We have demonstrated that among LTA-related bacterial compounds isolated from *E. hirae* ATCC 9790, only the LTA-2 fraction exhibited powerful cytokine-inducing activity in both appropriately primed mice and murine peritoneal macrophage cultures and that this fraction, in combination with MDP priming, showed strong antitumor effects ranging from simple hemorrhagic necrosis through marked to complete regression of Meth A fibrosarcoma established in BALB/c mice. In contrast, the LTA-1 fraction, which was distinct from the LTA-2 fraction in chemical composition, was nearly inactive in in vivo assays, although weak IL-6- and TAF-inducing activities were noted in murine peritoneal macrophage cultures. This finding is not consistent with those of the previous study by Tsutsui et al. (32) in some respects. They reported a powerful induction of IL-1 (TAF) in murine peritoneal macrophage cultures by their LTA-1 preparation and its strong regressive and curative effects in combination with MDP priming on Meth A tumors established in BALB/c mice, although marked differences were noted in the induction of TNF, IFN-α/β, and IFN-γ in *P. acnes*-primed mice between the LTA-1 and -2 fractions.

The chemical compositions of the two LTA fractions (LTA-1 and -2) used here were not exactly the same as those used previously by Tsutsui et al. (32) (Table 1). In brief, the present and previous LTA-1 and -2 preparations showed similar chemical compositions, but the content of each component was different. Compared with the previous LTA fractions, those used in the present study (both LTA-1 and -2) contained higher levels of glucose, consistent with previous reports of usually high glucose substitution in *E. hirae* LTA (4, 22) but less phosphorus, fatty acids, and glycerol. Both present and previous LTA-1 fractions had essentially the same alanine contents, whereas the LTA-2 used in this study showed a higher content of alanine than that used by Tsutsui et al. These observations indicate that LTA fractions obtained from *E. hirae* ATCC 9790 according to the method of Fischer et al. (8) were not homogeneous; their composition may deviate depending on minute differences in the growth conditions and/or preparation procedures, although the details of the parameters responsible for these differences have not yet been clarified. Part

of the structural deviation might be also due to the polydispersity of the glycerophosphate moiety in the LTA fraction, which was noted previously by Leopold and Fischer (23) for the LTA fraction from *Enterococcus faecalis*. Fischer's group (8, 22) also obtained two LTA fractions which had diacyl and tetraacyl glycolipids, respectively, from *E. hirae* ATCC 9790 and reported their chemical compositions. Extensive analysis was performed for the diacyl glycolipid-type LTA, which was the major fraction in their preparation and is assumed to correspond to our LTA-1 fraction because of its lower fatty acid contents in comparison with LTA-2. The most significant difference between the data of Fisher's group and our results was the presence of alanine residues in our LTA-1 fraction, in contrast to the absence in their corresponding fraction. From the molar ratios of the components in LTA-1 and -2 (Table 1), the main molecular species existing in these fractions in this study may be the diacyl-type and the tetraacyl-type glycolipids containing PGP, respectively, as reported by Fischer et al. (9), although alanine substitution may exist in addition to the oligoglucoside substitution on the 2-*O* position in the glycerol moiety of our LTA fractions. Since the present study confirmed that the LTA-1 fraction exhibits much less immunological activity than the LTA-2 fraction, the previously reported higher activity found in the LTA-1 fraction (32) might have been due to contamination by the LTA-2 fraction, the biological activities of which were established in our present assay system.

Another noteworthy discrepancy between the findings of Tsutsui et al. (32) and those of the present study concerns the bioactivity of the glycolipid moieties of LTAs. Tsutsui et al. (32) suggested that the glycolipid portion of the tetraacyl-type LTA structure proposed by Fischer (7), i.e., the phosphatidylglycolipid moiety, may play an important role in the manifestation of the biological activities of the LTA-2 fraction, on the basis of their experimental results obtained with a chloroform-soluble fraction derived from an acid-hydrolyzed LTA-2 comparable to the HGL-2 used in the present study. To confirm the activity of the glycolipid part of the LTA-2 fraction, we directly isolated the glycolipid (NGL-2) from the cells of *E. hirae* ATCC 9790 and tested its activity. Surprisingly, NGL-2 showed almost no activity in any assay. We then hydrolyzed LTA fractions to obtain acid-treated products (HGL-1 and -2) comparable to those of Tsutsui et al., and again, both HGL-1 and -2 fractions showed no or marginal biological activity in all assays performed. These observations strongly suggest that even if the glycolipid moiety was important for the immunobiological activities of the LTA-2 fraction, the lipid portion alone was not sufficient for these bioactivities. The acid-treated fractions reported by Tsutsui et al. might have been contaminated with the intact parent molecules or residual bioactive partial structures which escaped acid hydrolysis.

Okamoto et al. (27) prepared murine immunoglobulin M-type MAb TS-2 by immunization of mice with penicillin-killed *S. pyogenes* ATCC 21060 (OK-432) and found that the MAb was able to neutralize the IFN-γ-inducing activity of OK-432 in *P. acnes*-primed mice. We previously found by immunoblotting that TS-2 reacted with various bacterial LTA preparations, including the LTA-1 and -2 fractions of *E. hirae* (29). Okamoto et al. (27) also mentioned the reactivity of TS-2 with a bacterial LTA fraction without presenting any experimental data. The immunodeterminants of LTAs of various gram-positive bacteria are reported to be mainly located in their PGP moieties, i.e., the PGP molecule itself and its glycosyl (35) and alanyl (25) substituents. The LTA-2 and -1 fractions of *E. hirae*, which consist of PGP and glycolipid portions according to Fischer (7), exhibited high reactivity with TS-2, while the glycolipid moiety itself, either NGL-1 (LTA-1 type) or NGL-2 (LTA-2 type), was totally unreactive. These findings, taken together, suggest that the PGP moiety is immunodeterminant in the reaction of *E. hirae* LTA with TS-2. Because MAb TS-2 powerfully neutralized the induction of cytokines by LTA-2 (Table 6), TS-2 combined with the PGP portion may hinder the manifestation of the cytokine-inducing activity of the LTA-2 fraction by interfering with the interaction between the bioactive center of the active molecule and the receptor of target cells. Recently, Dziarski and Gupta (6) reported that LTAs from various bacterial species bound to the 70-kDa LPS binding protein on murine lymphocytes. Brade et al. (3) also reported that LTA preparations from various gram-positive bacteria bound to a 28-kDa protein in normal mouse serum which was shown to bind to the inner core region of LPS. However, MAb TS-2 did not react with LPS in either the bioassay (Table 6) or the ELISA (data not shown).

Synthetic LTA analogs SLTA(4)-1 and -2, like bacterial LTA-1 and -2 fractions, reacted with TS-2, but all of the test preparations other than the LTA-2 fraction, either bacterial or synthetic, were biologically inactive. This finding suggests that a structure shared by these biologically inactive preparations is insufficient in exhibition of the bioactivities of the LTA-2 fraction. The synthetic LTA analog SLTA(4)-2, which was prepared by partially mimicking *E. hirae* LTA, differed from the bacterial tetraacyl-type LTA proposed by Fischer (7) with respect to the following points: (i) parts of the four fatty acid residues on the lipid anchor portion of bacterial LTA were unsaturated, whereas all of the four acyl groups in the synthetic products were saturated; (ii) the degree of polymerization of *sn*-glycero-1-phosphate in the *E. hirae* PGP backbone is assumed to be between 9 and 40 repeating units in the bacterial product (22), whereas that of the synthetic analog was only 4; and (iii) there were no substituents in the tetra(glycerophosphate) moiety of the synthetic compound, in contrast to the reported unusually high glucosyl substitution consisting of mono-, di-, tri-, and tetraglucosyl residues in *E. hirae* LTA (4, 22) and an appreciable content of alanine residues in the present bacterial LTA fractions. The possibility that the observed bioactivities of the LTA-2 preparation is derived from extraneous LPS contamination can be entirely excluded by the negligible *Limulus* activity (less than 0.002% of the reference LPS) of the bacterial LTA-2 fraction.

In view of the fact that the present LTA-2 fraction was not homogeneous, we are pursuing experiments with the aim of isolating a key compound or structure present in the LTA-2 fraction in a satisfactorily homogeneous state. Investigations along this line may allow complete characterization of the chemical entity or a structure responsible for the reported immunobiological activity of *E. hirae* LTA and furthermore may facilitate the synthesis of a compound(s) responsible for the bioactivity of the bacterial product. This may in turn culminate in the creation of novel compounds whose biological activities shift in favor of the host and which have a highly favorable balance between beneficial and harmful effects.

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REFERENCES

1. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234:**466–468.

2. **Bhakdi, S., T. Klonisch, P. Nuber, and W. Fischer.** 1991. Stimulation of monokine production by lipoteichoic acids. Infect. Immun. **59:**4614–4620.
3. **Brade, L., H. Brade, and W. Fischer.** 1990. A 28 kDa protein of normal mouse serum binds lipopolysaccharides of Gram-negative and lipoteichoic acids of Gram-positive bacteria. Microb. Pathog. **9:**355–362.
4. **Cabacungan, E., and R. A. Pieringer.** 1985. Evidence for a tetraglucoside substituent on the lipoteichoic acid of *Streptococcus faecium* ATCC 9790. FEMS Microbiol. Lett. **26:**49–52.
5. **Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA **72:**3666–3670.
6. **Dziarski, R., and D. Gupta.** 1994. Heparin, sulfated heparinoids, and lipoteichoic acids bind to the 70-kDa peptidoglycan/lipopolysaccharide receptor protein on lymphocytes. J. Biol. Chem. **269:**2100–2110.
7. **Fischer, W.** 1990. Bacterial phosphoglycolipids and lipoteichoic acids. Handb. Lipid Res. **6:**123–234.
8. **Fischer, W., H. U. Koch, and R. Haas.** 1983. Improved preparation of lipoteichoic acids. Eur. J. Biochem. **133:**523–530.
9. **Fischer, W., M. Nakano, R. A. Laine, and W. Bohrer.** 1978. On the relationship between glycerophosphoglycolipids and lipoteichoic acids in Gram-positive bacteria. I. The occurrence of phosphoglycolipids. Biochim. Biophys. Acta **528:**288–297.
10. **Fukase, K., T. Matsumoto, N. Ito, T. Yoshimura, S. Kotani, and S. Kusumoto.** 1992. Synthetic study on lipoteichoic acid of Gram positive bacteria. I. Synthesis of proposed fundamental structure of *Streptococcus pyogenes* lipoteichoic acid. Bull. Chem. Soc. Jpn. **65:**2643–2654.
11. **Fukase, K., T. Yoshimura, S. Kotani, and S. Kusumoto.** 1994. Synthetic study of lipoteichoic acid of Gram positive bacteria. II. Synthesis of the proposed fundamental structure of *Enterococcus hirae* lipoteichoic acid. Bull. Chem. Soc. Jpn. **67:**473–482.
12. **Galanos, C., O. Lüderitz, and O. Westphal.** 1979. Preparation and properties of a standardized lipopolysaccharide from *Salmonella abortus equi* (Novo-Pyrexal). Zentralbl. Bakteriol. Abt. 1 Orig. **243:**226–244.
13. **Hamada, S., and M. Torii.** 1978. Effect of sucrose in culture media on the location of glucosyltransferase of *Streptococcus mutans* and cell adherence to glass surfaces. Infect. Immun. **20:**592–599.
14. **Hirano, T., T. Taga, N. Nakano, K. Yasukawa, S. Kashiwamura, K. Shimizu, K. Nakajima, K. H. Pyun, and T. Kishimoto.** 1985. Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). Proc. Natl. Acad. Sci. USA **82:**5490–5494.
15. **Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, S. Tsunasawa, F. Sakiyama, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kishimoto.** 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature (London) **324:**73–76.
16. **Ikemoto, S., K. Katoh, and K. Komagata.** 1978. Cellular fatty acid composition in methanol-utilizing bacteria. J. Gen. Appl. Microbiol. **24:**41–49.
17. **Ishizuka, I., and T. Yamakawa.** 1968. Glycosyl glycerides from *Streptococcus hemolyticus* strain D-58. J. Biochem. **64:**13–23.
18. **Keller, R., W. Fischer, R. Keist, and S. Bassetti.** 1992. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. Infect. Immun. **60:**3664–3672.
19. **Knox, K. W., and A. J. Wicken.** 1973. Immunological properties of teichoic acids. Bacteriol. Rev. **37:**215–257.
20. **Komuro, T., C. Yomota, T. Kimura, and C. Galanos.** 1989. Comparison of R- and S-form lipopolysaccharides fractionated from *Escherichia coli* UKT-B lipopolysaccharide in pyrogen and *Limulus* tests. FEMS Microbiol. Lett. **60:** 79–84.
21. **Kotani, S.** 1992. Bacterial cell surface biological response modifiers and their synthetic counterparts. Adv. Exp. Med. Biol. **319:**145–164.
22. **Leopold, K., and W. Fischer.** 1991. Separation of the poly(glycerophosphate) lipoteichoic acids of *Enterococcus faecalis* Kiel 27738, *Enterococcus hirae* ATCC 9790 and *Leuconostoc mesenteroides* DSM 20343 into molecular species by affinity chromatography on concanavalin A. Eur. J. Biochem. **196:** 475–482.
23. **Leopold, K., and W. Fischer.** 1992. Heterogeneity of lipoteichoic acid detected by anion exchange chromatography. Arch. Microbiol. **157:**446–450.
24. **Matsuda, T., T. Hirano, and T. Kishimoto.** 1988. Establishment of an interleukin 6 (IL 6)/B cell stimulatory factor 2-dependent cell line and preparation of anti-IL 6 monoclonal antibodies. Eur. J. Immunol. **18:**951–956.
25. **McCarty, M.** 1964. The role of D-alanine in the serological specificity of group A streptococcal glycerol teichoic acid. Proc. Natl. Acad. Sci. USA **52:** 259–265.
26. **Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai.** 1985. A new chromogenic endotoxin-specific assay using recombined limulus coagulating enzymes and its clinical applications. Clin. Chim. Acta **149:**55–65.
27. **Okamoto, M., R. Kaji, H. Kasetani, H. Yoshida, Y. Moriya, M. Saito, and M. Sato.** 1993. Purification and characterization of interferon-γ-inducing molecule of OK-432, a penicillin-killed streptococcal preparation, by monoclonal antibody neutralizing interferon-γ-inducing activity of OK-432. J. Immunother. **13:**232–242.
28. **Sato, M., R. Kaji, M. Urata, H. Yoshida, T. Yanagawa, K. Miyamoto, M. Azuma, N. Furumoto, and M. Saito.** 1988. Monoclonal antibody to the streptococcal preparation OK-432: tissue OK-432 localization and analysis of interaction between OK-432 and macrophages or NK cells in human salivary adenocarcinoma-bearing nude mice given OK-432. J. Biol. Response Modif. **7:**212–228.
29. **Takada, H., R. Arakaki, M. Saito, M. Sato, and S. Kotani.** Unpublished data.
30. **Takada, H., Y. Kawabata, M. Tamura, K. Matsushita, H. Igarashi, H. Ohkuni, Y. Todome, T. Uchiyama, and S. Kotani.** 1993. Cytokine induction by extracellular products of oral viridans group streptococci. Infect. Immun. **61:**5252–5260.
31. **Torello, L. A., A. J. Yates, and D. K. Thompson.** 1980. Critical study of the alditol acetate method for quantitating small quantities of hexoses and hexosamines in gangliosides. J. Chromatogr. **202:**195–209.
32. **Tsutsui, O., S. Kokeguchi, T. Matsumura, and K. Kato.** 1991. Relationship of the chemical structure and immunobiological activities of lipoteichoic acid from *Streptococcus faecalis* (*Enterococcus hirae*) ATCC 9790. FEMS Microbiol. Immunol. **76:**211–218.
33. **Usami, H., A. Yamamoto, Y. Sugawara, S. Hamada, T. Yamamoto, K. Kato, S. Kokeguchi, H. Takada, and S. Kotani.** 1987. A nontoxic tumour necrosis factor induced by streptococcal lipoteichoic acids. Br. J. Cancer **56:**797–799.
34. **Usami, H., A. Yamamoto, W. Yamashita, Y. Sugawara, S. Hamada, T. Yamamoto, K. Kato, S. Kokeguchi, H. Ohokuni, and S. Kotani.** 1988. Antitumour effects of streptococcal lipoteichoic acids on Meth A fibrosarcoma. Br. J. Cancer **57:**70–73.
35. **Wicken, A. J., and K. W. Knox.** 1975. Lipoteichoic acids: a new class of bacterial antigen. Science **187:**1161–1167.
36. **Yamada, M., Y. Furutani, M. Notake, J. Yamagishi, M. Yamayoshi, T. Fukui, H. Nomura, M. Komiya, J. Kuwashima, K. Nakano, Y. Sohmura, and S. Nakamura.** 1985. Efficient production of human tumour necrosis factor in *Escherichia coli*. J. Biotechnol. **3:**141–153.
37. **Yamamoto, A., H. Usami, M. Nagamuta, Y. Sugawara, S. Hamada, T. Yamamoto, K. Kato, S. Kokeguchi, and S. Kotani.** 1985. The use of lipoteichoic acid (LTA) from *Streptococcus pyogenes* to induce a serum factor causing tumour necrosis. Br. J. Cancer **51:**739–742.