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Evidence of Immunostimulating Lipoprotein Existing in the Natural Lipoteichoic Acid Fraction▽

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Lipoteichoic acid (LTA) is a cell surface glycoconjugate of gram-positive bacteria and is reported to activate the innate immune system. We previously reported that purified LTA obtained from *Enterococcus hirae* has no immunostimulating activity, but a subfraction (Eh-AF) in an LTA fraction possesses activity. In this study, we established a mouse monoclonal antibody neutralizing the activity of Eh-AF and investigated its inhibitory effects. Monoclonal antibody (MAbEh1) was established by the immunization of BALB/c mice with Eh-AF, followed by hybridoma screening based on its inhibitory effect for the production of interleukin-6 (IL-6) induced by Eh-AF. MAbEh1 neutralized the production of IL-6 by LTA fraction from not only *E. hirae* but also *Staphylococcus aureus*, while it failed to block that of lipopolysaccharide, suggesting that the antibody recognized a common active structure(s) in LTA fractions. Synthetic glycolipids in these LTAs did not induce cytokine production, at least in our system. Interestingly, the antibody was found to inhibit the activity of immunostimulating synthetic lipopeptides, Pam_3CSK_4 and FSL-1. These results suggest that MAbEh1 neutralizes the activity of lipoprotein-like compounds which is responsible for the activity of the LTA fraction of *E. hirae* and *S. aureus*.

Lipoteichoic acid (LTA) is a macroamphiphile distributing on the cell surfaces of gram-positive bacteria and is reported to exhibit immunostimulatory and inflammatory activities. LTA has been shown to have an antitumor effect (34, 36) and to induce inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and IL-6 (3, 31, 33). Recent research showed that such immunostimulatory activities of bacterial compounds were mediated by Toll-like receptor (TLR), a type I transmembrane receptor for innate immune activation (32). To date, more than 10 members of the TLR family have been discovered and most of their ligands were identified: TLR4 in combination with the adapter molecule MD-2 for lipopolysaccharide (LPS)/lipid A, an outer membrane component of gram-negative bacteria (21, 24); TLR9 for unmethylated CpG DNA (15); TLR3 and TLR7/8 for double- and single-stranded RNA (1, 14); TLR5 for bacterial flagellin (13); and TLR2 subfamily (TLR1, −2, and −6) for bacterial lipoprotein/lipopeptide (29, 30). LTA was also reported to be a ligand of TLR2 (22).

The structures of LTAs have been well studied and proposed as a glycoconjugates generally composed of a glycolipid anchor part, such as β-kojibiosyldiacylglycerol for *Enterococcus hirae* and *Streptococcus pyogenes* and β-gentiobiosyldiacylglycerol for *Staphylococcus aureus*, and a 1,3-linked poly(glycerophosphate) substituted by sugars and D-alanine at position 2 of the glycerol (4). Previously, we attempted to determine a structure of the LTA responsible for these activities. Fukase et al. prepared chemically synthetic glycoconjugates having fundamental structures of LTA from *E. hirae* and *S. pyogenes* and their glycolipid anchor parts (5, 6). However, these synthetic compounds exhibited no immunostimulating activities (28), suggesting that the proposed structures are not responsible for the activities. Thus, we reinvestigated the activity of LTA and found that an LTA fraction extracted from *E. hirae* by using a hot phenol (PhOH)-water method was able to be separated into two subfractions, a small amount of cytokine-inducing active fraction and an inactive major compound (27). Further, we determined that the structure of the inactive compound was identical to that of LTA (8). Those results suggested that the contaminating minor components in LTA fraction were responsible for the immunostimulation.

Recently, a structure-function relationship of LTA from *Staphylococcus aureus* has been reported. Morath et al. prepared a purified LTA by using a butanol (BuOH)-water extraction, followed by hydrophobic interaction chromatography, and showed that the LTA itself induces cytokine production (19). Further, those researchers synthesized an LTA counter-

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part and its glycolipid part and found that the counterpart displayed activity similar to that of natural LTA and even glycolipid possesses weak but distinct activity (20). From their observations, the researchers concluded that LTA itself was a potent immunostimulatory component in *S. aureus*. However, their conclusion for *S. aureus* disagreed with our data for *E. hirae*. One explanation for the contradictory results is an effect of the differences in the LTA structure, e.g., kojibiosyl is the backbone for glycolipid anchor in *E. hirae* LTA, whereas gentiobiose is the backbone in *S. aureus*, and partially alanylated oligoglucosyl is the substituent on glycerol at position 2 for *E. hirae*, but D-alanyl and glucosaminyl substitutions are made in *S. aureus*. This explanation may be supported by another report which showed that LTA exhibited from *Streptococcus pneumoniae* is 100-fold less potent than staphylococcal LTA (7). Pneumococcal LTA has been reported to be composed of a phosphocholine (PC)-linked tetraglycosylribitolphosphate polymer and a triglycosyldiacylglycerol anchor (2). Differences in extraction methods may be another possibility. Morath et al. also mentioned the critical role of D-alanine content in an LTA molecule from *S. aureus* (19), reporting that alkaline hydrolysis of the active LTA resulted in a loss of alanine substituent in LTA and reduced its activity. PhOH extraction of bacterial cells also decreased alanine, but BuOH extraction prevented alanine cleavage.

These interpretations might explain the inactivity of *E. hirae* LTA but do not clarify our minor active components. Therefore, we intended to reevaluate a principal compound responsible for the activity in *E. hirae*. In the present study, we established a mouse monoclonal antibody that neutralizes the activity of an LTA fraction from *E. hirae* and investigated its inhibitory effects for various bacterial stimuli.

MATERIALS AND METHODS

Bacterial compounds. *Enterococcus hirae* ATCC 9790 and *Staphylococcus aureus* DSM 20231 organisms were grown as previously described (27). The extraction of crude LTA fractions was performed using the BuOH-water method (19). The crude fractions were treated with DNase and RNase to digest contaminating nucleic acids and then subjected to hydrophobic interaction chromatography on octyl-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden), with a batchwise elution using stepwise 1-propanol concentrations (15, 40, and 60%) as described previously (8). Since LTA was eluted mainly with the 40% 1-propanol fractions, these fractions were used as LTA fractions and designated Eh-Bu (for *E. hirae*) and Sa-Bu (for *S. aureus*). The immunostimulatory active fractions HGL-A, HGL-B1, and HGL-B2, previously prepared from the *E. hirae* LTA fraction (9), were combined, and the resulting fraction (designated Eh-AF) was used for immunization. Glycolipid anchors of LTA, β-kosibiosyldipalmytoylglycerol for *E. hirae* (5) and β-gentiobiosyldipalmytoylglycerol for *S. aureus* (data not shown), were synthesized. LPS from *Escherichia coli* O:111 was obtained from Sigma-Aldrich (St. Louis, MO) and subjected to phenol reextraction by using sodium deoxycholate (16). PC, phosphatidylethanolamine, and phosphatidylinositol were also obtained from Sigma-Aldrich. Synthetic lipopeptides, Pam_3CSK_4, O,O′-diacyl-type Pam_2CSK_4, FSL-1, *N*-monoacyl-type $PamCSK_4$, and deacyl-type $dhCSK_4$ were purchased from EMC Microcollections (Tübingen, Germany). Monoclonal antibody for LTA was purchased from Biogenesis (Oxford, United Kingdom).

Establishment of MAbEh1. A monoclonal antibody, MAbEh1, was established according to standard methods. Briefly, BALB/c mice were immunized with Eh-AF (0.25 mg/mouse) with Freund's complete adjuvant (Becton Dickinson, Franklin Lakes, NJ) on days 0 and 21 and spleen cells obtained on day 24 were fused with SP2/0-Ag14 myeloma cells. The hybridoma cells were cultured in hypoxanthine-aminopterin-thymidine medium and subcloned by limiting dilution. Hybridoma-secreting antibody neutralizing Eh-AF activity was screened on the basis of the inhibitory effect against the production of IL-6 in THP-1 cells stimulated with 300 ng/ml Eh-AF, and an antibody was designated MAbEh1. The hybridoma was cultured in CD hybridoma medium (Invitrogen, Carlsbad, CA), and the culture supernatant was used for the antibody stock solution. The stock solution was then subjected to gel filtration chromatography on Bio-Gel A5m (Bio-Rad, Hercules, CA) to give purified antibody. Isotyping of the antibody was performed with a mouse monoclonal isotyping kit (Serotec, Oxford, United Kingdom). Isotype control antibody was purchased from e-Bioscience (San Diego, CA).

Cytokine assays. Human monocytic leukemia cell line THP-1 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS; MBL, Nagoya, Japan), 50 μg/ml penicillin, and 50 μg/ml streptomycin. THP-1 was differentiated with 10^{-7} M 1,25-dihydroxyvitamin D_3 for 3 days before use. Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized human peripheral blood collected from a healthy volunteer by density gradient centrifugation using Histopaque-1077 (Sigma).

The cells were plated onto 96-well microplates at 1×10^5 cells in 100 μl of RPMI 1640 with or without 10% FBS and stimulated with the indicated dose of the test specimens in the presence or absence of MAbEh1 for 24 h. Culture supernatants were collected and analyzed by using an enzyme-linked immunosorbent assay (ELISA) kit for secreted IL-6 (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. The concentration of secreted IL-6 from cells was determined using a standard curve of recombinant IL-6 prepared in each assay and presented as the means ± standard deviations (SD). Inhibitory effects of FBS and MAbEh1 were statistically evaluated by using Welch's *t* test.

Luciferase assays. Ba/F3 cells stably expressing p55IgκLuc, an NF-κB/DNA binding activity-dependent luciferase reporter construct (Ba/κB), murine TLR2 and the p55IgκLuc reporter construct (Ba/mTLR2), and murine TLR4/MD-2 and the p55IgκLuc reporter construct (Ba/mTLR4/mMD-2) were kindly provided by K. Miyake (Institute of Medical Science, University of Tokyo, Tokyo, Japan). NF-κB-dependent luciferase activity in these cells was determined as follows. Cells were inoculated onto each well of a 96-well, flat-bottomed plate at 1×10^5 cells in 80 μl of RPMI 1640 supplemented with 10% FBS and stimulated with the indicated concentrations of the test specimens. After 4 h of incubation at 37°C in humidified air containing 5% CO_2, 80 μl of Bright-Glo luciferase assay reagent (Promega, Madison WI) was added to each well and luminescence was quantified with a luminometer ARVO SX multilabel counter (Perkin Elmer, Wellesley, MA). Results are shown as relative luciferase activity, which was the ratio of stimulated activity to nonstimulated activity in each cell line.

Immune blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed by the Tris-glycine method using a mini-PAGE chamber AE-6530 and an AE-8450 power supply (ATTO, Tokyo, Japan) with a 15% gel. Materials in the gels were transferred to a nitrocellulose membrane (Bio-Rad) by using a semidry blotter AE-6677 (ATTO). For dot blot analysis, stimulus solution was placed on a nitrocellulose membrane and dried in the air. The membranes were incubated with blocking solution (3% nonfat milk in Tris-buffered saline containing 0.05% Tween 20) for 12 h at 4°C and then with 1/100 of MAbEh1 diluted in the diluent (1% nonfat milk in Tris-buffered saline containing 0.05% Tween 20) for 2 h at room temperature. The antibody was detected by incubation in peroxidase-labeled second antibody (KPL, Gaithersburg, MD; 1/2,000 in the diluent) for 2 h, followed by development using ECL (Amersham Bioscience). Luminescence was recorded with a LAS-1000 luminescence analyzer (Fuji Film, Kanagawa, Japan).

RESULTS

Preparation of LTA fractions. We previously separated small amounts of immunobiologically active fractions from *E. hirae* LTA fractions prepared by hot PhOH-water extraction (27). However, the structural elucidation of an essential compound(s) responsible for the activity was incomplete because of the difficulty of further purification based on its small amount. Recently, Morath et al. reported that an LTA fraction obtained from *S. aureus* by using BuOH-water extraction, followed by hydrophobic interaction chromatography, exhibited higher activity than that obtained by the PhOH method (19). Thus, we prepared LTA fractions by the BuOH method. *E. hirae* and *S. aureus* bacteria were subjected to BuOH extraction to give crude LTA fractions in yields of 1.5 to 2.1% and 1.6 to 2.6%, respectively. The crude extracts were digested with nu-

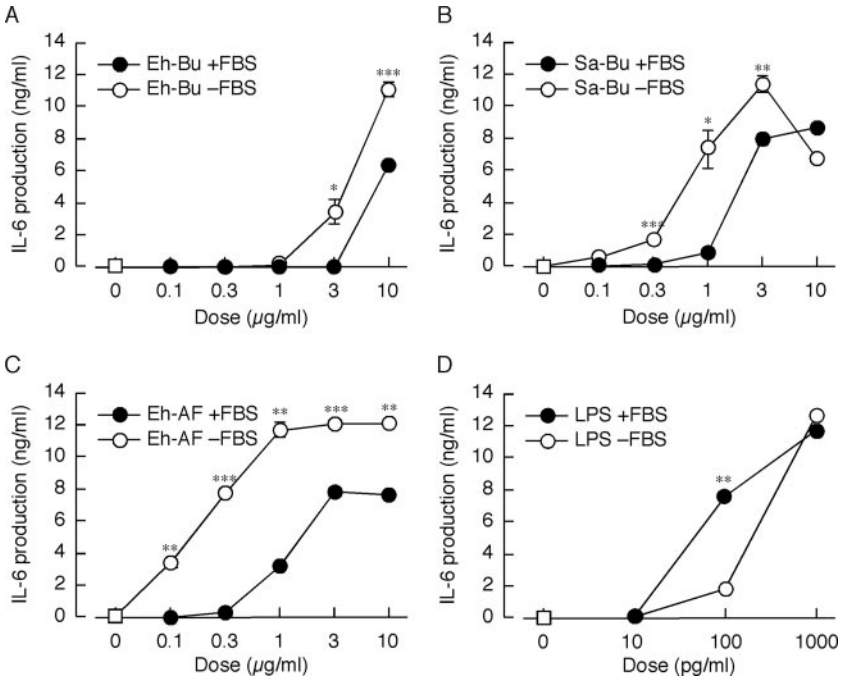

FIG. 1. IL-6 production in human peripheral blood mononuclear cells induced by (A) Eh-Bu, (B) Sa-Bu, (C) Eh-AF, or (D) LPS in the presence or absence of 10% FBS. Cells were stimulated with the indicated doses of stimuli for 24 h, and IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without FBS are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

clease and then subjected to hydrophobic interaction chromatography to obtain LTA fractions Eh-Bu (15 to 28%, yield from crude LTA fraction) and Sa-Bu (12 to 27%). Both fractions induced IL-6 production in PBMCs (Fig. 1). We previously demonstrated that IL-6 production in THP-1 cells stimulated with the active fraction was suppressed in the presence of FBS (9). Thus, the effect of serum was investigated, and the activities of Eh-Bu and Sa-Bu were found to decrease in the presence of FBS in a manner similar to that with Eh-AF, an active fraction previously prepared from *E. hirae* LTA fraction obtained by the PhOH method (Fig. 1). All of the fractions activated Ba/mTLR2 cells, but Ba/mTLR4/mMD-2 and negative control Ba/κB were not activated significantly (Fig. 2), indicating no endotoxin contamination.

Establishment of MAbEh1. We constructed an antibody that neutralized the activity of the fractions to evaluate a principal compound responsible for the activity. Mouse hybridoma cells were established by the immunization of BALB/c mice with Eh-AF. A hybridoma-secreting monoclonal antibody was screened for a neutralizing effect against the IL-6-inducing activity of Eh-AF in THP-1 cells. One hybridoma was found to secrete a neutralizing antibody named MAbEh1. The culture supernatant of the hybridoma cells in serum-free medium was subjected to gel filtration chromatography, and a fraction containing antibody was used for the solution of MAbEh1 (1.06 mg protein/ml). The isotype of the antibody was immunoglobulin M (IgM). MAbEh1 suppressed the activity of up to 1 μg/ml of Eh-AF dose dependently (Fig. 3). The antibody, in contrast, showed no inhibitory effect on the activity of LPS (Fig. 3). These results showed that MAbEh1 specifically suppresses the activity of the components in Eh-AF.

Neutralizing effects of MAbEh1. We next investigated the neutralizing effect of MAbEh1. The antibody also inhibited the activity of Eh-Bu and Sa-Bu (Fig. 4). These results suggest that structures of active components in Eh-Bu and Sa-Bu are common ones in LTA fractions and are similar to those in Eh-AF. Morath et al. reported that the glycolipid anchor in *S. aureus* LTA induced the production of TNF-α in human whole blood (19). Thus, we investigated the inhibitory effect on synthetic glycolipid anchors of *E. hirae* and *S. aureus*. However, in our assay system, neither glycolipid stimulated IL-6 production in human PBMCs (Fig. 5A and B). We recently showed that lipoproteins are predominant TLR2-activating ligands in *S. aureus* cell wall components (11). Thus, the inhibitory effects of MAbEh1 on the synthetic lipopeptides Pam_3CSK_4 and FSL-1 were studied. The activities of both synthetic counterparts were suppressed by the addition of the antibody dose dependently (Fig. 6A). The activities were also decreased in the presence of serum in a manner similar to those of Eh-AF, Eh-Bu, and Sa-Bu (Fig. 6B and C). Further, MAbEh1 bound lipopeptides Pam_3CSK_4, Pam_2CSK_4, $PamCSK_4$, and FSL-1 in dot blot analysis, but not other lipids (Fig. 7A). The antibody for LTA also failed to recognize these lipopeptides (Fig. 7B). These results suggested that MAbEh1 inhibits the activity of a compound containing lipopeptide structure.

DISCUSSION

LPS is a potent immunostimulatory compound in gram-negative bacteria. Although LPS is known to activate cells through TLR4, LPSs from some bacterial species have been reported to exhibit activity via TLR2 in addition to TLR4 (17,

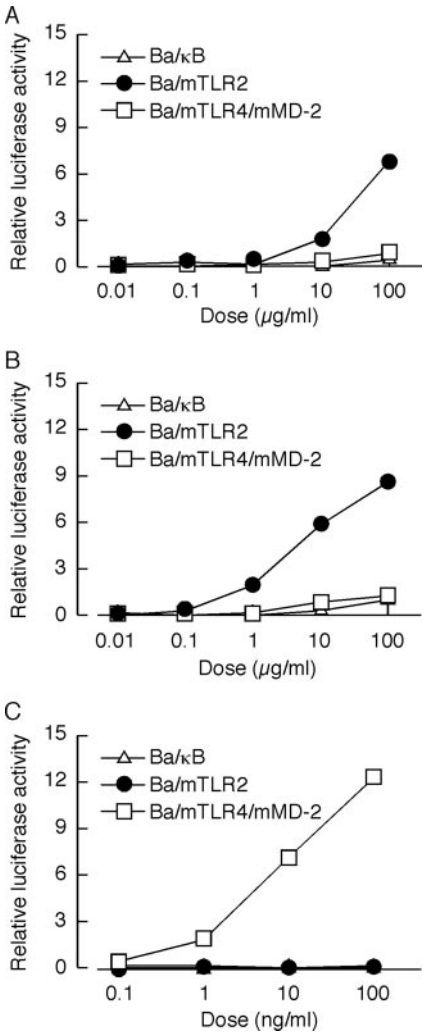

FIG. 2. NF-κB activation in Ba/κB, Ba/mTLR2, or Ba/mTLR4/mMD-2 cells induced by Eh-Bu (A), Sa-Bu (B), and LPS (C). Cells were incubated with the indicated doses of stimuli for 4 h. NF-κB activation was measured with a luciferase assay. Results are shown as relative luciferase activity, which was determined as the ratio of stimulated to nonstimulated activity.

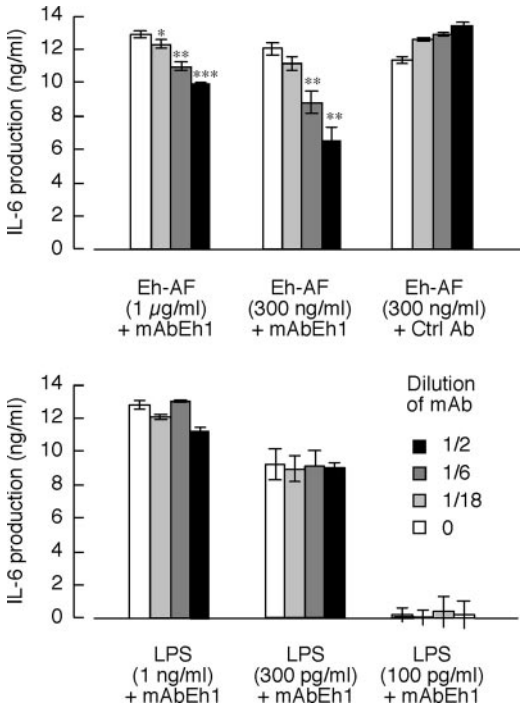

FIG. 3. Inhibitory effects of MAbEh1 or an isotype control antibody on IL-6 production in human peripheral blood mononuclear cells stimulated with Eh-AF or LPS. Cells were stimulated with the indicated doses of stimuli and antibody for 24 h in the absence of FBS, and IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without antibody are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

25, 35). Recent research proved that some of the TLR2-activating components were contaminated with small amounts of lipoproteins (10, 18). Lipoproteins are usually extracted from bacterial cells by surfactants such as Triton X-114 (23). LPS, which consists of a long hydrophilic polysaccharide and a hydrophobic lipid A anchor, may act as a surfactant. Since LTA is macroamphiphile, LTA may also work as a surfactant to extract lipoproteins from bacterial cells. In fact, we previously demonstrated that the activity of an LTA fraction, a BuOH extract, of *S. aureus* was not abrogated by hydrofluoric acid (HF) hydrolysis but by the following treatment with lipoprotein lipase, which cleaved acyl groups essential for the activity of lipoprotein and reduced the activity, indicating a possibility of the existence of lipoprotein in the LTA fraction (11). This indicated that lipoprotein but not the LTA molecule is responsible for the activity of LTA fractions. In the present study, we further confirmed the evidence of lipoprotein contamination in LTA fractions of *E. hirae* in addition to those of *S. aureus*. MAbEh1, which inhibited the immunostimulating activity of lipopeptides Pam_3CSK_4 and FSL-1, decreased the activity of the LTA fractions. This showed that lipoproteins were responsible for the activity of minor compounds previously separated from the LTA fraction of *E. hirae* (27).

We also characterized the binding affinity of MAbEh1. Dot blot analysis showed that the antibody binds to the lipopeptides triacylated Pam_3CSK_4, diacylated Pam_2CSK_4 and FSL-1, and

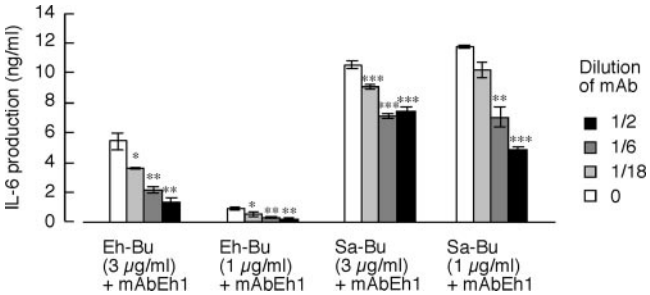

FIG. 4. Inhibitory effects of MAbEh1 on IL-6 production in human peripheral blood mononuclear cells stimulated with Eh-Bu or Sa-Bu. Cells were stimulated with the indicated doses of stimuli and antibody for 24 h in the absence of FBS, and IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without antibody are indicated. *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$.

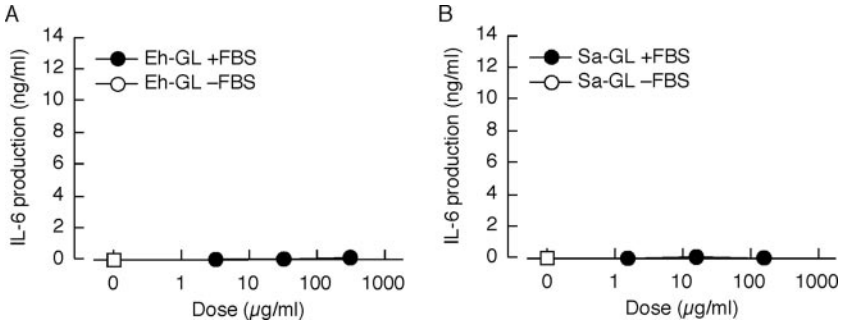

FIG. 5. IL-6 production in human peripheral blood mononuclear cells stimulated with (A) Eh-GL or (B) Sa-GL in the presence or absence of 10% FBS. Cells were stimulated with the indicated doses of stimuli for 24 h, and IL-6 production was determined by ELISA. The results are presented as the means ± SD.

monoacylated $PamCSK_4$ but not to deacylated $dhCSK_4$ (Fig. 7A). The antibody did not bind to LPS or diacylglyceryl lipids PC, phosphatidylethanolamine, and phosphatidylinositol (Fig. 7A). The lipopeptides were not visualized by an LTA antibody which bound to Eh-Bu (Fig. 7B). These results indicated that MAbEh1 recognized the N-terminal lipid moiety of lipopeptide. Unfortunately, both dot blot and Western blotting analysis of the lipoteichoic acid fraction using MAbEh1 failed to visualize any compound (data not shown), although the contamination of lipoprotein in the fraction was expected by the inhibition assay. This might be caused by its low concentration in the fraction as suggested in our previous work (11, 27) and/or low affinity of IgM antibody. In contrast to the specific binding, the inhibitory effect of MAbEh1 against not only lipoteichoic fraction but also synthetic lipopeptides was only partial (Fig. 3, 4, and 6). One interpretation for the partial effect may be the low affinity of IgM. We also assumed another possibility, which was that the inaccessibility of antibody to the N-terminal recognition center of lipoprotein was due to the incorporation into LTA micelles. Our previous observation, that lipoprotein lipase digestion of lipoproteins existing in the *S. aureus* LTA fraction (11) or the *Porphyromonas gingivalis* LPS fraction (10; our unpublished data) is unsuccessful, supported our second assumption.

Previously, we determined that LTA from *E. hirae* was inactive for the innate immune system (8). We also investigated the effect of HF degradation of the LTA fraction derived from *S. aureus* (11). Since HF cleaves the phosphodiester bonds in

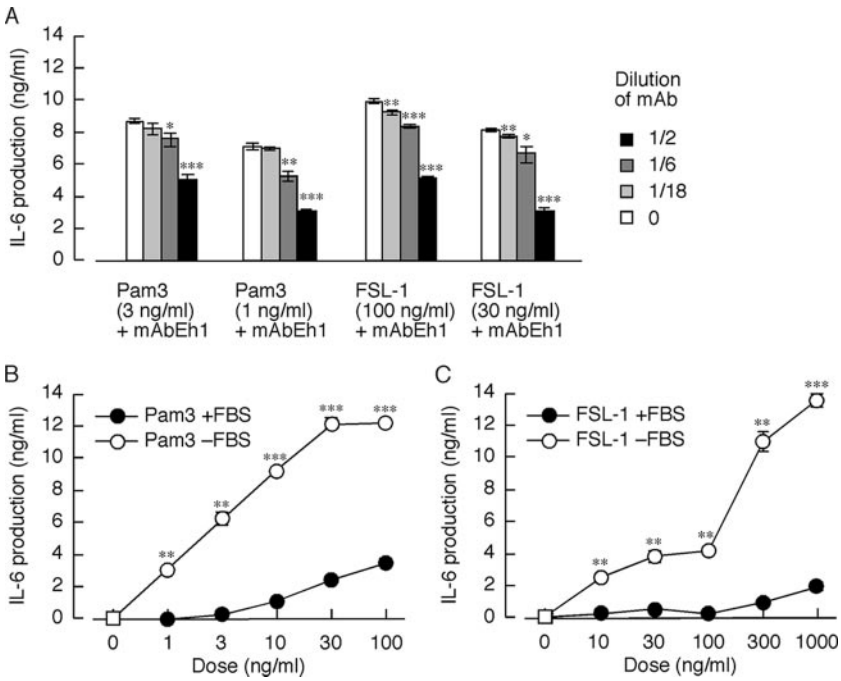

FIG. 6. (A) Inhibitory effects of MAbEh1 on IL-6 production in human peripheral blood mononuclear cells stimulated with synthetic lipopeptides Pam_3CSK_4 (Pam3) or FSL-1. Cells were stimulated with the indicated doses of stimuli and antibody for 24 h in the absence of FBS. (B and C) IL-6 production in human peripheral blood mononuclear cells stimulated with (B) Pam_3CSK_4 or (C) FSL-1 in the presence or absence of 10% FBS. IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without antibody or FBS are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

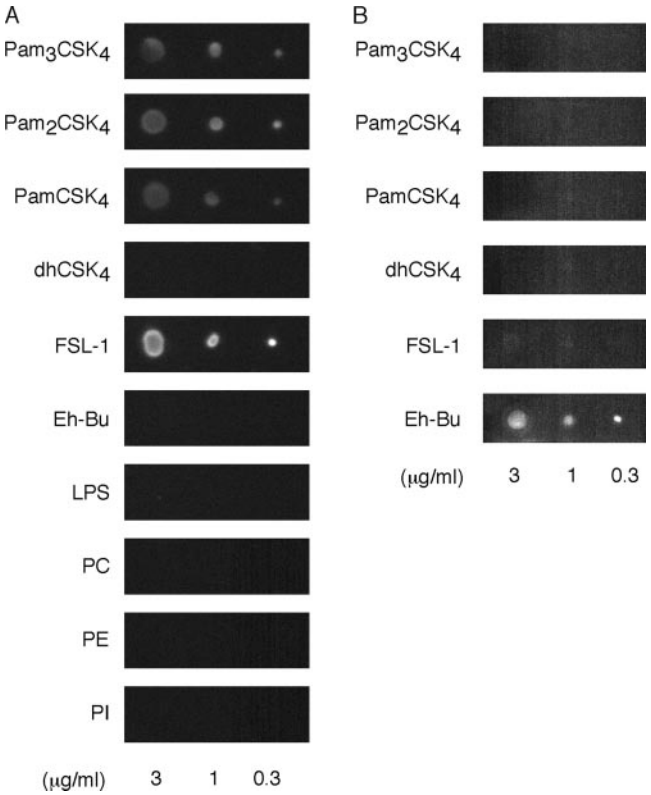

FIG. 7. Dot blot analysis against lipopeptide derivatives and other lipids with (A) MAbEh1 or (B) antibody for LTA. The indicated doses of stimuli were blotted onto a nitrocellulose membrane. The membranes were blocked with nonfat milk and incubated with MAbEh1. The bound antibody was detected with peroxidase-labeled second antibody using ECL reagents.

polyglycerophosphate, a hydrophilic part of LTA, most of the molecular mass of LTA is decomposed into small components, such as phosphate, glycerol, and phosphoglycerol (4). After HF degradation, no Alcian blue-stained band was found in the sodium dodecyl sulfate-PAGE gel, showing the complete decomposition of LTA. The treatment, however, did not abrogate the activity of the LTA fraction. Further, we showed that glycolipid parts of LTA for *E. hirae* and *S. aureus* were both inactive (Fig. 5). These results suggest that LTA itself was not an active molecule. However, we have not confirmed that natural LTA from *S. aureus* was immunobiologically inactive since a selective deletion of lipoprotein was not achieved. It was reported that *S. aureus* LTA was not separated into active and inactive fractions by the hydrophobic interaction and anion-exchange chromatographies which were used for the separation of *E. hirae* LTA (20). Direct lipoprotein lipase digestion of the LTA fraction was not successful (11), probably because contaminated lipoproteins may be incorporated into LTA micelles and the enzyme was not able to approach them. The reextraction of the natural LTA from *S. aureus* with PhOH containing deoxycholate, which was used for the extraction of contaminated lipoprotein from LPS (16), was also unsuccessful (data not shown). Since the reextraction method was also ineffective in some cases, such as for the extraction of lipoprotein from *Porphyromonas gingivalis* LPS (10, 16), it may be considered that the micellation of lipoprotein with LTA is very tight. Recently, we demonstrated that LTA from a lipoprotein diacylglycerol transferase deletion mutant of *S. aureus*, which contains no detectable lipoproteins (26), is 100-fold less active than that from the wild type (12). This result indicated that most of the activity of LTA fraction appears to be caused by lipoproteins. The identification of active lipoprotein species and the determination of chemical structure of compounds responsible for the residual activity in mutant LTA fraction are required for further understanding of biological activity of LTA molecule.

In conclusion, we established a monoclonal antibody that neutralizes the activity of natural LTA and demonstrated that the monoclonal antibody also blocked the activity of lipopeptides. These results strongly suggest that MAbEh1 neutralizes the activity of lipoprotein-like compounds existing in the natural LTA fraction from *E. hirae* and *S. aureus*.

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