Biochemical and Biophysical Research Communications **273,** 164–169 (2000)
doi:10.1006/bbrc.2000.2921, available online at http://www.idealibrary.com on IDEAL®

Cytokine-Inducing Macromolecular Glycolipids from *Enterococcus hirae:* Improved Method for Separation and Analysis of Its Effects on Cellular Activation

Masahito Hashimoto,* Yoshimasa Imamura,* Toshiyuki Morichika,* Kazumi Arimoto,* Osamu Takeuchi,† Kiyoshi Takeda,† Shizuo Akira,† Kazue Aoyama,‡ Toshihide Tamura,‡,§ Shozo Kotani,‡ Yasuo Suda,*,[1] and Shoichi Kusumoto*

**Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan; †Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan; and ‡Department of Bacteriology, and §Institute of Advanced Medical Sciences, Laboratory of Host Defense, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan*

Received May 16, 2000

Previously, we showed that several minor macromolecular glycolipids accounting for less than 5% of the lipoteichoic acid (LTA) fraction from *Enterococcus hirae* ATCC 9790 possess cytokine-inducing activity, whereas the purified LTA does not. In other words, the immunobiological activity of the LTA fraction reported in the 1980s was not attributable to LTA itself, but to other glycolipids coexisting in the fraction. In the present study, we improved the procedure of separation of the active glycolipids and evaluated their effects on cellular activation. The immunobiologically active glycolipids were separated from the crude glycolipid fraction obtained by hot phenol-water extraction of the cells. The total yield of active glycolipids was about fivefold higher than that separated by the previous method. Interleukin-6-inducing activities of the active glycolipids from 1,25-dihydroxy vitamin D_3-differentiated human monocytic leukemia cells, THP-1, were inhibited by anti-CD14 mAbs in a dose-dependent manner. Macrophages from Toll-like receptor (TLR)-2-deficient or -4-deficient mice completely lacked the ability to produce tumor necrosis factor-α on stimulation with active glycolipids. These observations indicated that the cellular activation by the active glycolipids from *E. hirae* is mediated by CD14 and by both TLR2 and TLR4. © 2000 Academic Press

Key Words: **CD14; *Enterococcus;* glycolipid; inflammatory cytokine; lipoteichoic acid; Toll-like receptor.**

Lipoteichoic acid (LTA) is a macroamphiphile widely distributed on the cell surface of Gram-positive bacteria. The structures of LTAs from various enterococcal and streptococcal species were studied in detail by Fischer *et al.* (1). In the 1980s, immunostimulating activities of several LTAs such as cytokine-inducing and antitumor activities were reported (2–4). Since then, the LTA molecule itself has been believed to be the main immunostimulatory component of Gram-positive bacteria. However, this may not be the case. Fukase *et al.* (5, 6) synthesized the fundamental structures proposed for LTAs of *Enterococcus hirae.* However, these synthetic compounds showed neither cytokine-inducing nor antitumor activities (7). Recently, we succeeded in separating the LTA fraction from *E. hirae* ATCC 9790 into small amounts (<5%) of cytokine-inducing macromolecular glycolipids and an inactive major component that accounted for over 90 wt% (8). The structure of the latter inactive glycolipid was identical to that proposed for LTA by Fischer (9). These results clearly indicated that the so-called LTA is not responsible for the cytokine-inducing activity of Gram-positive bacteria. In our previous study, we evaluated the structure of one of the cytokine-inducing glycolipids (10), but the essential minimal structure responsible for the activity has not yet been determined due mainly to the small amounts present. To complete their structural analysis, large amounts of the active components are required.

Since sepsis caused by Gram-positive bacteria is a serious problem (11), the mechanisms of activation of immune cells by cell-surface components of Gram-positive bacteria has been studied. Peptidoglycan (PGN) and LTA fractions were reported to stimulate cytokine production in a CD14-dependent manner (12, 13). Recently, Schwandner *et al.* (14) demonstrated

Abbreviations used: IL, interleukin; LBP, lipopolysaccharide binding protein; LTA, lipoteichoic acid; LPS, lipopolysaccharide; PGN, peptidoglycan; TLR, Toll-like receptor; TNF, tumor necrosis factor; $DHVD_3$, 1,25-dihydroxy vitamin D_3.

[1] To whom correspondence should be addressed. Fax: +81-6-6850-5419. E-mail: ysuda@chem.sci.osaka-u.ac.jp.

0006-291X/00 $35.00

Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

that PGN and the LTA fraction induced human-derived cell activation mediated by Toll-like receptor 2 (TLR2). In these studies, however, the LTA fraction, a crude extract of bacterial cells, was used for the activation. As the LTA fraction of *E. hirae* consists of the major, but non cytokine-inducing, LTA molecule and cytokine-inducing minor glycolipids as described above, the corresponding LTA fractions from other bacteria may consist of similar components. Kusunoki *et al.* (15, 16) reported the occurrence and partial purification of a minor fraction from the LTA fraction of *Staphylococcus aureus*. They found that only the minor fraction stimulated cytokine production in the human astrocytoma cell line U373 in a CD14-dependent manner. They also reported that a purified LTA with no cytokine-inducing activity bound to CD14 and antagonized the cytokine-inducing activity of lipopolysaccharide (LPS). Our results and those reported by Kusunoki indicated that the "nonpurified" LTA fraction is not a suitable preparation for the study of cell activation.

In this paper, we describe a modified separation method for the cytokine-inducing active glycolipids from *E. hirae* and some new results regarding their effects on mammalian cells.

MATERIALS AND METHODS

Preparation of crude glycolipid fraction. *E. hirae* ATCC 9790 was grown as described (9). Hot phenol-water extraction of delipidated bacterial cells, RNase-DNase digestion, and reextraction with hot phenol-water were performed as described (8).

Column chromatography. An anion exchange chromatography with a stepwise elution was performed on a Macro-Prep High Q column (3 × 30 cm, Bio-Rad, Hercules, CA). The crude glycolipid (2.6 g) was dissolved in 0.1 M acetate buffer (pH 4.5) containing 35% 1-propanol and applied to the column equilibrated with the same buffer. The column was eluted with 300 ml of the same buffer containing stepwise increasing concentrations of NaCl (0, 0.05, 0.1 and 1 M) at a flow rate of 60 ml/h. An anion-exchange chromatography with a linear gradient was performed as described (9).

Hydrophobic interaction chromatography was performed on an Octyl-Sepharose CL-4B column (2.5 × 30 cm, Amersham-Pharmacia, Uppsala, Sweden). HQ-A (54 mg) or HQ-B (76 mg) was dissolved in 0.1 M formate buffer (pH 4.5) containing 15% 1-propanol and applied to the column equilibrated with the same buffer. The column was eluted with a linear gradient of 1-propanol (15–60%) at a flow rate of 24 ml/h. Fractions of 6 ml were collected and analyzed for phosphorus.

Gel filtration chromatography was performed on a Sephacryl S-200 HR column (1 × 23 cm, Amersham-Pharmacia). OSA-R (29 mg) or OSB-R (26 mg) was dissolved in 0.2 M formate buffer (pH 4.5) containing 30% 1-propanol and applied to the column equilibrated with the same buffer. The column was eluted with the same buffer at a flow rate of 20 ml/h. Fractions of 4 ml were collected and analyzed for phosphorus.

Analytical procedure. SDS–PAGE was performed as described (8). Phosphorus was analyzed according to the previous paper (8).

Biological assays. The induction of inflammatory cytokines, interleukin-6 (IL-6), IL-18 and tumor necrosis factor-α (TNF-α), in 1,25-dihydroxy vitamin D_3 ($DHVD_3$)-differentiated human monocytic leukemia cells, THP-1, and determination of their level using ELISA were performed as described (17). Anti-human CD14 monoclonal antibodies (anti-CD14 mAbs), MY-4 (Coulter, Miami, FL) and SH-M1 (Santa Cruz Biotechnology, Santa Cruz, CA), and a synthetic lipid A precursor (compound 406) (18) were used as inhibitors. TNF-α induction in murine peritoneal macrophages was performed as described (19). Macrophages from wild-type, TLR2-deficient ($TLR2^{-/-}$) and -4-deficient ($TLR4^{-/-}$) mice were prepared as described (19, 20). Human soluble CD14 (sCD14) was isolated according to the method of Sugawara *et al.* (21) using a monoclonal antibody (MY-4) affinity column. *Limulus* activity was measured with Endospecy Test (Seikagaku Co., Tokyo, Japan) as described (8). In all the biological assay, an LPS specimen from *Escherichia coli* O111:B4 (Sigma Co., St. Louis, MO) was used as a positive control.

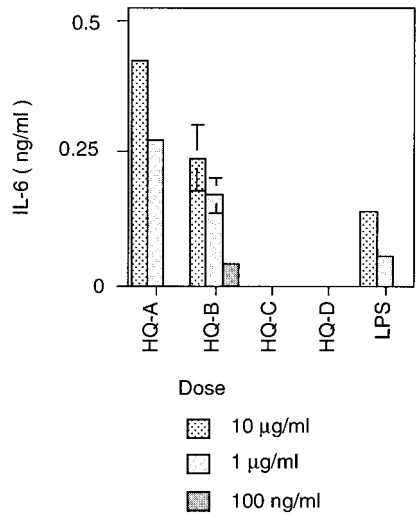

FIG. 1. IL-6 induction by HQ fractions, HQ-A (eluted with 0 M NaCl), -B (0.05 M), -C (0.1 M), and -D (1 M), from $DHVD_3$-differentiated THP-1 cells in the absence of serum. The $DHVD_3$-differentiated cells (1×10^6 cells/ml) were incubated in triplicate with test samples at 37°C for 24 h. The levels of IL-6 in the culture supernatant were measured by ELISA. Data are means ± SD of two experiments.

RESULTS

The crude glycolipid fraction was obtained from dried *E. hirae* ATCC 9790 cells according to the method described (8). The crude glycolipid was subjected to anion-exchange chromatography with stepwise elution to give HQ-A, HQ-B, HQ-C and HQ-D in yields of 10, 10, 26 and 36%, respectively. As shown in Fig. 1, IL-6-inducing activity was found only in the low anionic fractions, HQ-A and HQ-B. Further fractionation of one of the cytokine-inducing fraction, HQ-A, was performed by hydrophobic interaction chromatography. Based on the elution profile and IL-6-inducing activity (Fig. 2a), eluates were divided into the non-cytokine-inducing pass-through fraction (OSA-P) and the cytokine-inducing retained fraction (OSA-R). The yields of these fractions were 87 and 6.4%, respectively. OSA-R was then subjected to gel filtration chromatography. Based on the elution profile (Fig. 3a), eluates were collected to give a glycolipid, named HGL-A,

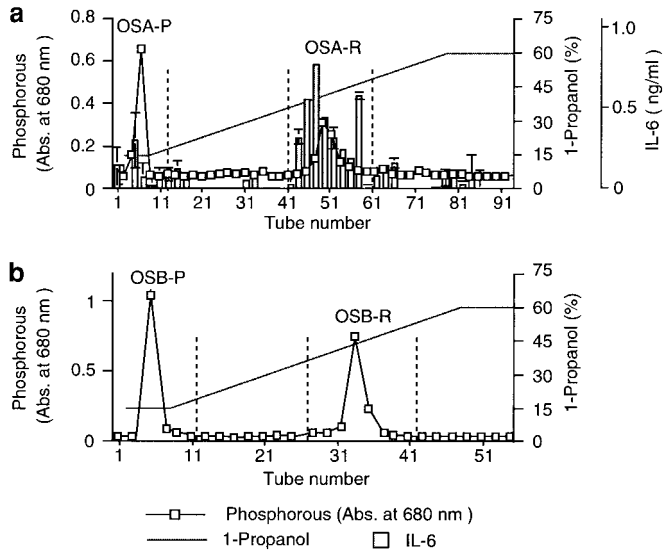

FIG. 2. The elution profiles of HQ-A (a) and HQ-B (b) on an Octyl-Sepharose CL-4B column (2.5 × 30 cm). The sample (HQ-A, 54 mg; HQ-B, 76 mg) was dissolved in 0.1 M formate buffer (pH 4.5) containing 15% 1-propanol, and applied to the column. The column was eluted with a linear gradient of 1-propanol (15–60%) at a flow rate of 24 ml/h. Aliquots of each fraction (6 ml) were analyzed for phosphorus. IL-6-inducing activity of each fraction was tested using $DHVD_3$-differentiated THP-1 cells in the absence of serum. The $DHVD_3$-differentiated cells (1×10^6 cells/ml) were incubated in duplicate with test samples at 37°C for 24 h. The levels of IL-6 in the culture supernatant were measured by ELISA. Data of IL-6 induction are means ± SD of two experiments.

quantitatively. Another cytokine-inducing fraction, HQ-B, was subjected to hydrophobic interaction chromatography (Fig. 2b) to give a path through (OSB-P, 84%) and retained (OSB-R, 14%) fractions. OSB-R was then subjected to gel filtration chromatography (Fig. 3b) to give two glycolipids, named HGL-B1 (66%) and HGL-B2 (16%). The total yield of the active glycolipids was *ca.* 0.1% based on dried bacterial cells.

HQ-C and HQ-D were combined and subjected to hydrophobic interaction chromatography. The retained fraction (91% yield) was again subjected to anion-

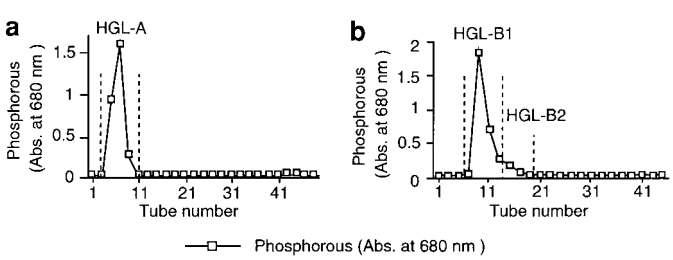

FIG. 3. The elution profiles of OSA-R (a) and OSB-R (b) on a Sephacryl S-200 HR column (1 × 25 cm). The sample (OSA-R: 29 mg, OSB-R: 26 mg) was dissolved in 0.2 M formate buffer (pH 4.5) containing 30% 1-propanol, and applied to the column. The column was eluted with the same buffer at a flow rate of 20 ml/h. Aliquots from each fraction (4 ml) were analyzed for phosphorus.

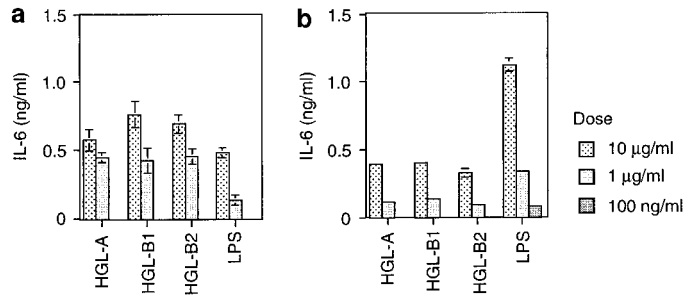

FIG. 4. IL-6 induction by the glycolipids of *E. hirae* from $DHVD_3$-differentiated THP-1 cells in the absence (a) or presence (b) of 2% fetal calf serum (FCS). The $DHVD_3$-differentiated cells (1×10^6 cells/ml) were incubated in triplicate with test samples in the absence or presence of 2% FCS at 37°C for 24 h. The levels of IL-6 in the culture supernatant were measured by ELISA. Data are means ± SD of two experiments.

exchange chromatography with a linear gradient. Elutions with high concentration of NaCl (0.6–1 M) were collected to give the high-anionic fraction in yield of 83%. From the similar properties in both hydrophobic and anion-exchange chromatographies to those of purified LTA previously determined (9), the high-anionic fraction can be thought to be purified LTA, even though the structural characterization was only partially done.

The *Limulus* activities of the purified glycolipids (HGL-A, -B1 and -B2) were at least 10^{-7} times lower than that of LPS. IL-6-inducing activity of the glycolipids in $DHVD_3$-differentiated THP-1 cells is shown in Fig. 4. No distinct difference in IL-6-inducing activity among the three glycolipids was observed. In the absence of serum, the activities of the glycolipids were stronger than that of LPS. In the presence of serum (2% FCS), IL-6 production from the cells stimulated by the glycolipids were slightly suppressed, whereas that by LPS was significantly enhanced. As shown in Fig. 5, the IL-6-inducing activity of HGL-B1 (1 μg/ml) was inhibited by at least 1 μg/ml of the anti-CD14 mAbs MY-4 and SH-M1. sCD14 (original concentration of protein was 130 μg/ml) also suppressed IL-6 production (Fig. 6). The lipid A precursor compound 406 also inhibited IL-6 induction over a dose of 10 μg/ml (Fig. 5). HGL-B1 also stimulated IL-18 and TNF-α as well as IL-6 production by THP-1 cells, but the high-anionic fraction did not (Fig. 7). TNF-α induction by HGL-B1 in macrophages from $TLR2^{-/-}$ or $TLR4^{-/-}$ mice was strongly suppressed at all doses tested compared with those from wild-type mice (Fig. 8).

DISCUSSION

In our previous studies (8–10), separation of the cytokine-inducing glycolipids was performed as follows: (i) the crude glycolipid fraction obtained from bacterial cells by hot phenol-water extraction was sub-

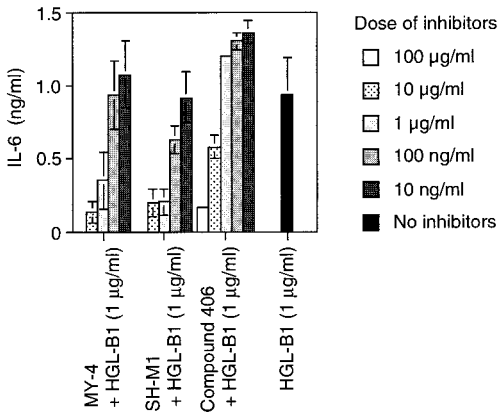

FIG. 5. Inhibitory effects of anti-CD14 mAbs, MY-4 and SH-M1, and a lipid A precursor compound 406 on IL-6 induction of HGL-B1 from $DHVD_3$-differentiated THP-1 cells in the absence of serum. The $DHVD_3$-differentiated cells (1.3×10^6 cells/ml) were incubated in triplicate with the mixtures of HGL-B1 and inhibitor at 37°C for 24 h. The levels of IL-6 in the culture supernatant were measured by ELISA. Data are means ± SD of three experiments.

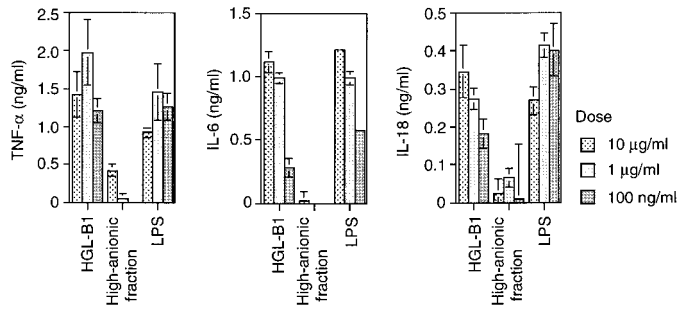

FIG. 7. Cytokine induction by HGL-B1 and the high-anionic fraction from $DHVD_3$-differentiated THP-1 cells in the absence of serum. The $DHVD_3$-differentiated cells (1.3×10^6 cells/ml) were incubated in triplicate with test samples at 37°C for 24 h. The levels of cytokines in the culture supernatant were measured by ELISA. Data are means ± SD of two experiments.

jected to hydrophobic interaction chromatography to give the LTA fraction; (ii) the LTA fraction was then subjected to anion-exchange chromatography to give a cytokine-inducing fraction and purified LTA; (iii) the cytokine-inducing fraction was again subjected to hydrophobic interaction chromatography to give cytokine-inducing glycolipids. The first step of the above protocol corresponds to the procedure described for the separation of LTA itself (22). The bioactive glycolipids were found to have much weaker anionic properties than LTA. Since a considerable amount of bioactive glycolipids seems to be lost during the first isolation step by hydrophobic interaction chromatography, this step was omitted in the present study: the crude glycolipid fraction was directly subjected to anion-exchange chromatography with a stepwise elution. As shown in Fig. 1, the IL-6-inducing activity was only present in the low-anionic fractions, HQ-A (0 M NaCl) and -B (0.05 M NaCl). HQ-A and -B were then subjected to hydrophobic interaction chromatography separately to give low-anionic and high-hydrophobic IL-6-inducing glycolipid fractions, OSA-R and OSB-R, respectively. By this modified separation method, the fractions were obtained in a yield about fivefold higher than those separated previously.

OSA-R and OSB-R contained glycolipids with wide molecular weight range (6000–15000 for OSA-R and 7000–17000 for OSB-R, estimated by SDS–PAGE) which may be attributable to the diversity of lipids and differences in numbers of repeating units and carbohydrate components. To separate the glycolipids on the basis of its molecular weight, gel filtration column chromatography was performed. OSA-R was eluted as a broad but single peak (Fig. 3a, named as HGL-A).

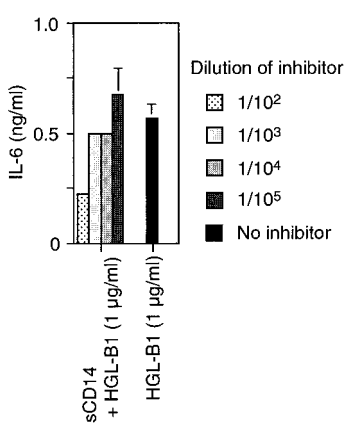

FIG. 6. Inhibitory effects of sCD14 on IL-6 induction of HGL-B1 from $DHVD_3$-differentiated THP-1 cells in the absence of serum. The original concentration of sCD14 was 130 μg/ml. The $DHVD_3$-differentiated cells (1.3×10^6 cells/ml) were incubated in triplicate with the mixture of HGL-B1 and inhibitor at 37°C for 24 h. The levels of IL-6 in the culture supernatant were measured by ELISA. Data are means ± SD of three experiments.

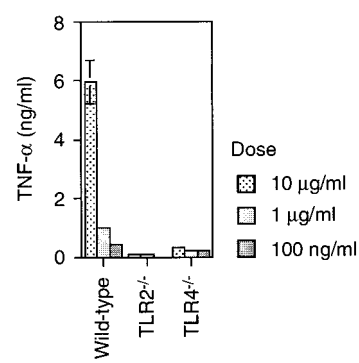

FIG. 8. TNF-α induction by HGL-B1 from murine macrophages. Peritoneal macrophages (5×10^4 cells/well) from wild-type, $TLR2^{-/-}$ or $TLR4^{-/-}$ mice were incubated in duplicate with HGL-B1 for 24 h. The levels of TNF-α were measured by ELISA. Data are means ± SD of two experiments.

Since HGL-A was purified by multi-step chromatography procedure of different principle, the sample was considered to contain a single kind of glycolipid which was hydrophobically and ionically homogeneous. OSB-R was eluted as a broad peak with tailing on the column and divided to HGL-B1 and HGL-B2 (Fig. 3b). Though the molecular weight ranges of HGL-B1 and HGL-B2 were slightly different, they were considered to be fundamentally homologous.

The cytokine-inducing activities of the glycolipids (HGL-A, -B1 and -B2) were tested in human monocytic leukemia cells, THP-1, after differentiation by $DHVD_3$ (17). In these cells, the IL-6-inducing activities of the glycolipids were almost equivalent to that of LPS (Fig. 4a). The observed IL-6-inducing activities of the glycolipids were at least 10^8 times higher than those of possible contaminating LPS based on the comparison of their *Limulus* activity. Therefore, the IL-6-inducing activities were concluded to be inherent characteristics of the glycolipids themselves. Because no distinct differences in the IL-6-inducing activities and their chemical composition (data not shown) among the three glycolipids were observed, HGL-B1 was used as a typical glycolipid for the further biological experiment.

HGL-B1 also stimulated production of other inflammatory cytokines (Fig. 7). IL-18- and TNF-α-inducing activities of HGL-B1 were nearly equivalent to those of LPS. On the contrary, the high anionic fraction, which corresponds to purified LTA, did not stimulate production of any inflammatory cytokines. These results indicated that the glycolipids from *E. hirae* act as an innate immune system stimulator.

The cellular activation induced by LPS is mediated by LPS-binding protein (LBP), CD14 and the TLR family. LBP in the serum binds to LPS and the LBP-LPS complex is recognized by CD14 (23), which may act as a helper or introducer to a signal transducing receptor (24). The TLR family was recently identified as a candidate for the signal transducing receptor of LPS (25, 26). Thus, the participation of these proteins in the cellular activation induced by HGL-B1 was examined. In the presence of serum (Fig. 4b), the activity of HGL-B1 was slightly suppressed, whereas that of LPS was strongly enhanced probably because of an effect of LBP. This suggested that LBP or LBP-like binding proteins are not involved in the cellular activation by HGL-B1. The weak suppressive effect of serum might be explained that some serum factors such as antibodies or lectins bind to HGL-B1 and inhibit further binding to the relevant factors. Anti-CD14 mAbs, MY-4 and SH-M1, inhibited IL-6 induction dose-dependently (Fig. 5). Addition of sCD14 also decreased IL-6 induction (fig. 6). These results indicated that cellular activation by HGL-B1 is mediated by CD14. This coincides with the results reported by Kusunoki *et al.* (15, 16), indicating that a minor fraction separated from the LTA fraction of *S. aureus* activates the human astrocytoma cell line U373 in a CD14-dependent manner. Lipid A precursor compound 406, an antagonist of LPS in human cells (27, 28), also inhibited the IL-6 induction by HGL-B1 at higher dose (Fig. 5). No cytotoxic effect was observed at all by the addition of compound 406. Although the mechanism responsible for this inhibition is not clear, HGL-B1 and LPS may share the same receptor protein, CD14.

The participation of TLRs was examined using peritoneal macrophages from $TLR2^{-/-}$ or $TLR4^{-/-}$ mice (19, 20). In mice, TNF-α induction by LPS was reported to be mediated only by TLR4 (20). In contrast, no TNF-α induction was observed by HGL-B1 stimulation in macrophages of $TLR2^{-/-}$ or $TLR4^{-/-}$ mice up to a dose of 100 μg/ml (Fig. 6). These observations indicated that the cellular activation by the glycolipids from *E. hirae* is mediated not only by TLR4 but also by TLR2 through a partially common but distinct pathway from that by LPS.

Several research groups reported a relationship between TLR family and cell components of Gram-positive bacteria in mice. Medwedev *et al.* (29) reported that LPS-hyporesponsive C3H/HeJ mice, which have a single amino acid mutation in the cytosolic domain of TLR4 (30), respond normally to stimulation with preparations from group B streptococci type III. Underhill *et al.* (31) reported that murine RAW 264.7-derived cells expressing a dominant negative mutant TLR2 did not respond to heat-killed *S. aureus,* but those expressing a dominant negative mutant TLR4 responded normally. Takeuchi *et al.* (20) reported that macrophages of $TLR4^{-/-}$ mice were stimulated by *S. aureus* PGN, whereas those of $TLR2^{-/-}$ mice were not. These observations suggested that cellular activation by some of the components from Gram-positive bacteria (mainly PGN) is mediated only by TLR2 and not by TLR4. This may not be discrepant with the present results, since no PGN was present in our glycolipid preparations.

In contrast, Takeuchi *et al.* (20) reported that commercially available LTA fractions stimulated cytokine production in macrophages of $TLR2^{-/-}$ mice, but not in $TLR4^{-/-}$ mice. Schwandner *et al.* (14) also reported that activation of NF-κB not only by LTA fractions from *S. aureus* and *Bacillus subtilis* but also LPS in human embryonic kidney HEK293 cells was dependent on the expression of human TLR2, but not TLR4. These result seemed to be contradictory to those of the present study, but the effects of contamination by other components must be taken into consideration because they used a "nonpurified" LTA fraction.

ACKNOWLEDGMENTS

This study was supported in part by "Research for the Future" Program No. 97L00502 from the Japan Society for the Promotion of Science, by Grants-in-Aid for Scientific Research (No. 05403035 to S.K.) and for Scientific Research on Priority Areas (No. 05274102 to

Y.S.), a Grant-in-Aid for JSPS Fellows (No. 4504 to M.H.) from the Ministry of Education, Science, Sports, and Culture of Japan, and by a grant from Chugai Pharmaceutical Co. Ltd., Tokyo, Japan. We are grateful to Prof. T. Kishimoto at Osaka University School of Medicine for his generous gift of rHuIL-6, Prof. S. Kuramitsu at Osaka University Graduate School of Science for invaluable help in culturing bacterial cells, and Dr. M. Okamoto at Tokushima University School of Dentistry for measuring the levels of IL-18.

REFERENCES

1. Fischer, W. (1990) Glycolipids, Phosphoglycolipids and Sulfoglycolipids (Kates, M., Ed.), pp. 123–234, Plenum Press, New York.
2. Yamamoto, A., Usami, H., Nagamuta, M., Sugawara, Y., Hamada, S., Yamamoto, T., Kato, K., Kokeguchi, S., and Kotani, S. (1985) *Br. J. Cancer* **51,** 739–742.
3. Usami, H., Yamamoto, A., Yamashita, W., Sugawara, Y., Hamada, S., Yamamoto, T., Kato, K., Kokeguchi, S., Ohokuni, H., and Kotani, S. (1988) *Br. J. Cancer* **57,** 70–73.
4. Bhakdi, S., Klonisch, T., Nuber, P., and Fischer, W. (1991) *Infect. Immun.* **59,** 4614–4620.
5. Fukase, K., Matsumoto, T., Ito, N., Yoshimura, T., Kotani, S., and Kusumoto, S. (1992) *Bull. Chem. Soc. Jpn.* **65,** 2643–2654.
6. Fukase, K., Yoshimura, T., Kotani, S., and Kusumoto, S. (1994) *Bull. Chem. Soc. Jpn.* **67,** 473–482.
7. Takada, H., Kawabata, Y., Arakaki, R., Kusumoto, S., Fukase, K., Suda, Y., Yoshimura, T., Kokeguchi, S., Kato, K., Komuro, T., Tanaka, N., Saito, M., Yoshida, T., Sato, M., and Kotani, S. (1995) *Infect. Immun.* **63,** 57–65.
8. Suda, Y., Tochio, H., Kawano, K., Takada, H., Yoshida, T., Kotani, S., and Kusumoto, S. (1995) *FEMS Immunol. Med. Microbiol.* **12,** 97–112.
9. Hashimoto, M., Yasuoka, J-i., Suda, Y., Takada, H., Yoshida, T., Kotani, S., and Kusumoto, S. (1997) *J. Biochem.* **121,** 779–786.
10. Hashimoto, M., Imamura, Y., Yasuoka, J-i., Kotani, S., Kusumoto, S., and Suda, Y. (1999) *Glycoconj. J.* **16,** 213–221.
11. Bone, R. C. (1994) *Arch. Intern. Med.* **154,** 26–34.
12. Dziarski, R., Tapping, R. I., and Tobias, P. S. (1998) *J. Biol. Chem.* **273,** 8680–8690.
13. Cleveland, M. G., Gorham, J. D., Murphy, T. L., Toumanen, E., and Murphy, K. M. (1996) *Infect. Immun.* **64,** 1906–1912.
14. Schwandner, R. R., Dziarski, R., Weesche, H., Rothe, M., and Kirschning, C. J. (1999) *J. Biol. Chem.* **274,** 17406–17409.
15. Kusunoki, T., Hailman, E., Juan, T. S.-C., Lichenstein, H. S., and Wright, S. D. (1995) *J. Exp. Med.* **182,** 1673–1682.
16. Kusunoki, T., and Wright, S. D. (1996) *J. Immunol.* **157,** 5112–5117.
17. Suda, Y., Aoyama, K., Arimoto, K., Tamira, T., and Kusumoto, S. (1999) *Biochem. Biophys. Res. Commun.* **257,** 327–332.
18. Oikawa, M., Wada, A., Yoshizaki, H., Fukase, K., and Kusumoto, S. (1997) *Bull. Chem. Soc. Jpn.* **70,** 1435–1440.
19. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999) *J. Immunol.* **162,** 3749–3752.
20. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11,** 443–451.
21. Sugawara, S., Arakaki, R., Rikiishi, H., and Takada, H. (1999) *Infect. Immun.* **67,** 1623–1632.
22. Fischer, W., Koch, H. U., and Haas, R. (1983) *Eur. J. Biochem.* **133,** 523–530.
23. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) *Science* **249,** 1431–1433.
24. Yu, B., Hailman, E., and Wright, S. D. (1997) *J. Clin. Invest.* **99,** 315–324.
25. Kirschning, C. J., Wesche, H., Ayres, M. T., and Rothe, M. (1998) *J. Exp. Med.* **188,** 2091–2097.
26. Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard, A., Wood, W. I., Gurney, A. L., and Godowski, P. J. (1998) *Nature* **395,** 284–288.
27. Golenbock, D. T., Hampton, R. Y., Qureshi, N., Takayama, K., and Raetz, C. R. (1990) *J. Biol. Chem.* **266,** 19490–19498.
28. Kovach, N. L., Yee, E., Munford, R. S., Raetz, C. R., and Harlan, J. M. (1990) *J. Exp. Med.* **172,** 77–84.
29. Medvedev, A. E., Flo, T., Ingalls, R. R., Golenbock, D. T., Teti, G., Vogel, S. N., and Espevik, T. (1998) *J. Immunol.* **160,** 4535–4542.
30. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoi, P., Layton, B., and Beutler, B. (1998) *Science* **282,** 2085–2088.
31. Underhill, D. M., Ozinsky, A., Hajjar, A. M., Stevens, A., Wilson, C. B., Bassetti, M., and Aderem, A. (1999) *Nature* **401,** 811–815.