A lipoteichoic acid fraction of Enterococcus hirae activates cultured human monocytic cells via a CD14-independent pathway to promote cytokine production, and the activity is inhibited by serum components

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1. Introduction

Lipoteichoic acids (LTAs) are cell surface amphiphiles widely distributed in Gram-positive bacteria [1]. Several studies of the immunobiological activities of LTA have indicated that LTA shares various bioactivities with endotoxic lipopolysaccharide (LPS) from Gram-negative bacteria (reviewed in [1–3]). More recent evidence has suggested that LTA and LPS share CD14 as a common receptor system. Cleveland et al. [4] demonstrated that LTA preparations from Staphylococcus aureus and Streptococcus pyogenes induced interleukin (IL)-12 through the
CD14-dependent pathway in human monocytic THP-1 cell cultures. Hattor et al. [5] also reported that the induction of nitric oxide (NO) synthesis and an isoform of NO synthase gene expression by a mouse macrophage cell line (J774) in response to LTA was significantly inhibited by an anti-mouse CD14 monoclonal antibody (MAb). In addition, Renzi and Lee [6] reported that LTA from various bacterial species stimulated ICAM-1 expression by human umbilical vein endothelial cells in a serum-dependent manner, as does LPS, and that an anti-CD14 MAb inhibited the ICAM-1 expression by the cells in response to LTA as well as LPS.

In a previous study [7], we prepared two LTA fractions, LTA-1 and -2, from Enterococcus hirae ATCC 9790 according to the method of Fischer et al. [8]. The quantitatively minor fraction LTA-2 was bioactive, mainly in murine in vitro and in vivo assay systems, showing the ability to induce a variety of cytokines and an antitumor effect. In contrast, the quantitatively major fraction LTA-1 was scarcely active in the same assays. We also found that LTA-related compounds which were chemically synthesized by mimicking the fundamental structures of LTA-1 and -2 [9,10] were bio-inactive as far as tested, except for antigenicity [7]. The bioactive LTA-2 fraction was not satisfactorily homogeneous, and thus Suda et al. [11] tried to isolate the bioactive component of LTA-2 in terms of the IL-6- and tumor necrosis factor (TNF)-α-inducing activities of LTA-2 observed in a human whole blood culture system. Although they obtained five biologically active, high molecular mass glycolipids at a total yield of 6% of LTA-2, the chemical analytical results led them to suggest that the component responsible for the observed bioactivities might be a novel compound different from those of conventional LTAs in chemical characteristics [11]. A later study by Hashimoto et al. [12] further showed that a major LTA fraction prepared from E. hirae ATCC 9790, which shared the chemical structure with that reported by Fischer [1], was devoid of cytokine-inducing activities in a human whole blood culture system. A view similar to that described by Suda et al. was advanced by Kusunoki et al. [13], who attempted to isolate a bioactive component from a commercial LTA preparation derived from S. aureus, since a purified LTA fraction separated by hydrophobic chromatography lacked the capacity to induce cytokines in human whole blood, and the bioactive compound was recovered in a fraction other than the above LTA fraction.

These studies suggest that LTA structures, in a narrow sense (such as those described by Fischer [1]), might be incapable of exhibiting various bioactivities in human whole blood culture systems via a CD14- and serum-dependent pathway. Nevertheless, the above findings do not necessarily exclude the possibility that the quantitatively major E. hirae LTA fraction exhibits various bioactivities via a serum- and CD14-independent pathway. The objective of this study was to examine the possible activation of human monocytic cells via a serum- and CD14-independent pathway by a quantitatively major LTA preparation from E. hirae, which was reported to be inactive in the human whole blood assay system used by Suda et al. [11] and Hashimoto et al. [12].

2. Materials and methods

2.1. Major E. hirae LTA fraction, QM-1M

A test specimen of a quantitatively major LTA fraction, QM-1M, from E. hirae ATCC 9790 prepared principally according to the method of Hashimoto et al. [12] was supplied by Y. Suda and S. Kusumoto (Graduate School of Science, Osaka University, Osaka, Japan). Briefly, delipidated cells of E. hirae were extracted with hot phenol-water. The extract was digested with DNase/RNase to give a crude LTA fraction. The crude fraction was fractionated with Octyl-Sepharose (Pharmacia, Uppsala, Sweden) by serial elution with 0.1 M acetate buffer (pH 4.5) containing 15 and 60% 1-propanol to give fractions BOS15 and 60, respectively (the yield of both was about 30% of the crude LTA). A specimen of BOS60 dissolved in 0.01 N acetate buffer containing 35% 1-propanol was applied to an ion-exchange membrane, QMA-Mem Sep 1010 (PerSeptive Biosystems, Framingham, MA, USA). Unbound (pass-through) material was recovered by washing out the membrane with the above buffer to give a fraction capable of inducing IL-6 in human whole blood cultures. The materials bound to the membrane were
eluted with the buffer containing 35% 1-propanol and 1 M NaCl to give a quantitatively major LTA fraction, QM-1M, which was incapable of inducing IL-6 in human peripheral blood cultures. The recovery rate of the QM-1M fraction was 44% of BOS60. The chemical composition of the QM-1M preparation which served as a test stimulant in the present study was essentially the same as that of QM-I reported by Hashimoto et al. [12] (Table 1).

2.2. Other reagents and cells

An LPS specimen prepared from Escherichia coli O111:B4 by the hot phenol-water extraction method was purchased from Difco Laboratories (Detroit, MI, USA) and used as a reference stimulant. The anti-CD14 MAb MY-4 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The human monocytic cell line THP-1 was obtained from the Health Science Research Resources Bank (Tokyo, Japan).

2.3. Cytokine induction in cell cultures

THP-1 cells (2 × 10⁶) were seeded in a 24-well culture plate and cultured in duplicate in 1 ml of RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) with or without 1% fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, USA) and in the presence or absence of test stimulants in a CO₂ incubator. After 24 h, culture supernatants were collected and stored at −80°C until use. Cytokine induction in human peripheral whole blood and peripheral blood mononuclear cell (PBMC) culture systems was performed as described previously [14]. Briefly, in the whole blood cultures heparinized human peripheral whole blood (25 ml) obtained from a healthy adult volunteer was cultured in RPMI 1640 medium (75 ml) with or without test materials in a 96-well culture plate in triplicate. In the case of PBMC cultures, human PBMC prepared from heparinized peripheral blood by Ficoll-Isopaque centrifugation were cultured at a density of 4 × 10⁵ cells per 200 ml of RPMI 1640 medium in a 96-well culture plate for 24 h with or without test materials and in the presence or absence of FCS or autologous human serum. After the cultivation, the triplicate culture supernatants were pooled and stored. Various cytokines in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) kits (Otsuka Pharmaceutical Co., Tokushima, Japan; and Bio-source International, Camarillo, CA, USA). THP-1 cell cultures were examined for the following cytokines: IL-1α; IL-1β; IL-2; IL-6; IL-8; TNF-α; macrophage colony-stimulating factor (M-CSF); granulocyte colony-stimulating factor (G-CSF); granulocyte-macrophage colony-stimulating factor.

Table 1

<table>
<thead>
<tr>
<th>Chemical compositions of QM-1M and QM-I</th>
<th>Wt%</th>
<th>Wt%</th>
<th>Wt%</th>
<th>Wt%</th>
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<tr>
<td>Phosphate</td>
<td>11.0</td>
<td>11.5</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Glycerol</td>
<td>18.6</td>
<td>18.7</td>
<td>18.7</td>
<td>18.7</td>
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<tr>
<td>Glucose</td>
<td>58.1</td>
<td>42.4</td>
<td>42.4</td>
<td>42.4</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>7.3</td>
<td>7.6</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td>16:0</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>16:1</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>18:0</td>
<td>0.01</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>18:1</td>
<td>0.17</td>
<td>0.13</td>
<td>0.13</td>
<td>0.13</td>
</tr>
</tbody>
</table>

*Analytical methods were described previously [11].
*Quoted from Hashimoto et al. [12].

Table 2

<table>
<thead>
<tr>
<th>Test material</th>
<th>IL-6 (U ml⁻¹)</th>
<th>IL-8 (pg ml⁻¹)</th>
<th>TNF-α (pg ml⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Medium alone</td>
<td>ND³</td>
<td>66 ± 10</td>
<td>ND</td>
</tr>
<tr>
<td>LPS (0.01 μg ml⁻¹)</td>
<td>11 488 ± 1785</td>
<td>1322 ± 130</td>
<td>925 ± 106</td>
</tr>
<tr>
<td>QM-1M (100 μg ml⁻¹)</td>
<td>ND</td>
<td>99 ± 18</td>
<td>ND</td>
</tr>
<tr>
<td>QM-1M (10 μg ml⁻¹)</td>
<td>ND</td>
<td>55 ± 21</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The results are representative of three different experiments.
*Not detected.
*From E. coli O111:B4.
(GM-CSF); interferon (IFN)-α; and IFN-γ. For technical reasons, only selected cytokines were measured in the human peripheral whole blood and PBMC cultures.

2.4. CD14 expression by THP-1 cells

The expression of membrane CD14 (mCD14) on THP-1 cells was analyzed by flow cytometry (Nippon Becton Dickinson Co., Tokyo). THP-1 cells (2×10⁶) were seeded in a 24-well culture plate, and cultured in 1 ml of RPMI 1640 medium without FCS in the presence of 10 μg ml⁻¹ of QM-1M or the reference LPS. After a 36-h cultivation, cells were incubated with the MAb MY-4 at 4°C for 1 h, washed three times with phosphate-buffered saline (PBS), pH 7.4, stained with FITC-conjugated antitomouse immunoglobulin, and then analyzed by flow cytometry.

2.5. Miscellaneous

All test specimens for cytokine assays were stored at −80°C until tested. Most of the assays were carried out in duplicate or triplicate. The range of the cytokine levels induced in triplicate assays was within 20% of the respective mean value. In some experiments, the statistical significance of the differences between each test and the respective control was determined by Student’s t-test. Most experiments were carried out more than twice, and representative results are presented.

3. Result

3.1. Inability of QM-1M to induce cytokines in human whole blood cultures

As shown in Table 2, QM-1M was scarcely capable of inducing TNF-α and IL-8 as well as IL-6 in human whole blood cultures even at a high concentration (100 μg ml⁻¹), under the experimental conditions in which the reference LPS markedly induced these cytokines at a low concentration (0.01 μg ml⁻¹). These data strongly suggest that the test QM-1M preparation was not significantly contaminated with exogenous endotoxins or other bioactive materials such as those described by Suda et al. [11] and Kusunoki et al. [13].

![Fig. 1. IL-1β-, IL-8-, and TNF-α-inducing activity of QM-1M (A) and LPS (B) in THP-1 cell cultures (2×10⁶ ml⁻¹) in the presence of FCS (representative of five different experiments).](https://academic.oup.com/femspd/article-abstract/22/4/283/580784)
3.2. QM-1M induces various cytokines in THP-1 cell cultures, and serum components inhibit the cytokine induction by QM-1M

We examined the cytokine-inducing abilities of QM-1M in a human monocytic cell line, THP-1 cultures. In the absence of FCS, QM-1M was capable of inducing IL-1β, TNF-α and IL-8; the IL-8 induction was the most marked (Fig. 1A). The dose-response curve of IL-8 induction by QM-1M is shown in Fig. 2. QM-1M induced IL-8 at 0.1 μg ml⁻¹, and the activity increased dose-dependently to 10 μg ml⁻¹. An overdose depression was noted at 100 μg ml⁻¹ due to unidentified causes. QM-1M also induced IL-1α, IL-6, G-CSF and M-CSF, but the extent of induction was less than those of the other cytokines and showed fluctuations from one assay to another. IL-2, GM-CSF, IFN-α and IFN-γ were scarcely induced by QM-1M in this system (data not shown). The comparison of the data of QM-1M and LPS in Fig. 1 illustrates that in the absence of serum, QM-1M generally exhibited stronger cytokine-inducing activities than the reference LPS at comparable test dose levels (1.0 and 0.1 μg ml⁻¹). In the presence of 1% FCS, the cytokine-inducing activities of LPS were markedly increased, presumably because of the existence of cofactors such as LPS-binding protein (LBP) in the serum (Fig. 1B). The cytokine-inducing activities of QM-1M decreased (Fig. 1A). Similar inhibitory effects of FCS on the cytokine-inducing activities of QM-1M were observed in an assay using the murine macrophage cell line J774.1 (data not shown). The findings described above strongly suggest that QM-1M activates human and murine monocytic cells in a manner different from LPS.

Table 3

<table>
<thead>
<tr>
<th>Test material</th>
<th>Conc (μg ml⁻¹)</th>
<th>Serum-free</th>
<th>1% AS</th>
<th>10% AS</th>
<th>1% FCS</th>
<th>10% FCS</th>
</tr>
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<tbody>
<tr>
<td><strong>IL-8 response (ng ml⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium alone</td>
<td>57 ± 2.2</td>
<td>107 ± 11.8</td>
<td>165 ± 53</td>
<td>85 ± 9</td>
<td>63 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>QM-1M</td>
<td>1</td>
<td>120 ± 8.3**</td>
<td>81 ± 18.0</td>
<td>164 ± 1.9</td>
<td>109 ± 3.5</td>
<td>41 ± 0.5</td>
</tr>
<tr>
<td>LPS</td>
<td>0.1</td>
<td>98 ± 5.8*</td>
<td>220 ± 10.9*</td>
<td>209 ± 6.1</td>
<td>212 ± 14.7**</td>
<td>144 ± 2.2**</td>
</tr>
<tr>
<td><strong>TNF-α response (pg ml⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium alone</td>
<td>86.3 ± 5.1</td>
<td>34.8 ± 3.9</td>
<td>3.9 ± 0.9 NT</td>
<td>23.7 ± 4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QM-1M</td>
<td>1</td>
<td>149.5 ± 0.3**</td>
<td>27.3 ± 1.0</td>
<td>5.9 ± 0.4 NT</td>
<td>22.7 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>0.1</td>
<td>577 ± 139*</td>
<td>185 ± 28*</td>
<td>16.1 ± 1.9* NT</td>
<td>118 ± 0.6**</td>
<td></td>
</tr>
</tbody>
</table>

*Representative of three different experiments. Significance with Student’s t-test: *P ≤ 0.05, **P ≤ 0.01.

† Autologous serum.

‡ From E. coli O55:B5.

§ Not tested.
3.3. QM-1M induced IL-8 and TNF-α in human PBMC cultures in the absence of serum, but not in the presence of serum

We then examined the possible cytokine-inducing ability of QM-1M in human PBMC cultures. Table 3 shows that QM-1M was capable of inducing IL-8 and TNF-α by the stimulation of human PBMC cultured without serum. The extent of stimulation in terms of the net increase in the levels of either IL-8 or TNF-α was small, but the increase was significant. The reliability of data was further supported by the finding that the addition of either autologous human serum or FCS to PBMC cultures abolished the cytokine-inducing activity of QM-1M, but this was not the case with the reference LPS.

3.4. QM-1M activates THP-1 cells through a CD14-independent manner

Cleveland et al. [4] reported that LTA preparations from S. aureus and S. pyogenes induced IL-12 in THP-1 cultures through a CD14-dependent pathway. Thus, we examined the possible involvement of mCD14 in the activation of THP-1 cells by QM-1M. We first analyzed the CD14 expression on THP-1 cells by flow cytometry using the anti-CD14 MAb MY-4. Fig. 3 shows that although the THP-1 cells spontaneously expressed mCD14 to a considerable extent, LPS treatment for 36 h powerfully upregulated the expression of mCD14 by the cells. In contrast to this control experiment, QM-1M only slightly upregulated the mCD14 expression by the cells. We then examined the effects of the anti-CD14 MAb on IL-8 induction by QM-1M and LPS (Table 4). Under the experimental conditions where the MAb MY-4 definitely inhibited IL-8 induction by LPS in the presence of 1% FCS (such inhibitory effects were marginal in serum-free conditions), no significant inhibiting effect of the MAb on

![Fig. 3. Effects of QM-1M (A) and LPS (B) on mCD14 expression by THP-1 cells. Cells stained with second antibody only (dotted line) or the anti-CD14 MAb MY-4 plus the second antibody (solid line). Open area bounded by hatched line, cells cultured in medium alone; closed area, cells cultured with a stimulant. The results are representative of two different experiments.]

Table 4

<table>
<thead>
<tr>
<th>Test material</th>
<th>Conc (μg/ml)</th>
<th>IL-8 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serum-free</td>
</tr>
<tr>
<td>Medium alone</td>
<td></td>
<td>31 ± 11</td>
</tr>
<tr>
<td>QM-1M</td>
<td>1</td>
<td>1,707 ± 52**</td>
</tr>
<tr>
<td>LPS</td>
<td>1</td>
<td>304 ± 73*</td>
</tr>
<tr>
<td>MY-4</td>
<td>10</td>
<td>74 ± 18 ns</td>
</tr>
<tr>
<td>QM-1M+MY-4</td>
<td>1 + 10</td>
<td>2,012 ± 163**</td>
</tr>
<tr>
<td>LPS+MY-4</td>
<td>1 + 10</td>
<td>228 ± 18**</td>
</tr>
</tbody>
</table>

*Representative of three different experiments. Significant differences from the respective medium alone control (*,**) and from the respective serum-free culture (+,**) by Student’s t-test (*, P ≤ 0.05, ***, P ≤ 0.01). The differences were statistically significant (s) and not significant (ns) by Student’s t-test (P ≥ 0.05).
IL-8 induction by QM-1M was seen in the presence or absence of FCS.

4. Discussion

In this study, we demonstrated that a quantitatively major LTA fraction, QM-1M, was capable of exhibiting various cytokine-inducing activities in a human monocytic cell line, THP-1, in the absence of serum under experimental conditions in which the possibility that observed bioactivities are due to contamination with extraneous endotoxins can be excluded (Fig. 1). Considerable evidence indicates that LPS activates various cells mainly through CD14-dependent mechanisms (reviewed in [15]). Experimental evidence (Table 4) indicated that QM-1M stimulated THP-1 cells to produce IL-8 through a CD14-independent pathway (unlike the reference LPS), although QM-1M slightly upregulated mCD14 on THP-1 cells (Fig. 3) as suggested by Landmann et al. [16]. In contrast, there are reports of CD14-dependent activities of LTA preparations [4-6]. We also found that high mCD14-expressing human gingival fibroblasts released IL-8 in response to QM-1M and Bacillus subtilis LTA, whereas low mCD14-expressing human gingival fibroblasts did not (Sugawara, S., Arakaki, R. and Takada, H., unpublished). These findings indicate that LTA specimens exhibited activities through both CD14-dependent and -independent pathways. Whether an active structural entity responsible for both activities is the same or not is unclear at present. A possible interaction of the test LTA preparations with mCD14 was suggested by the observation of Kusunoki et al. [13] that a purified LTA obtained from S. aureus bound soluble CD14 (sCD14) and antagonized the IL-6-inducing activity of LPS in human astrocytoma U373 cultures, probably by competition with LPS binding for sCD14. We also observed the antagonistic effect of some LTA preparations against LPS in a human gingival fibroblast culture system (Sugawara, S., Arakaki, R. and Takada, H., unpublished). These observations suggest that LTA is capable of binding with CD14, but the binding may result in signal induction in some cases and may result in antagonistic effects (to agonists such as LPS) in other cases.

We also found that a marked cytokine induction by QM-1M in THP-1 cells and human PBMC was reduced by the presence of FCS or human serum, unlike LPS (Fig. 1 and Table 3). Standiford et al. [17] also reported that definite IL-8-inducing activity of LTA from S. aureus and S. pyogenes from human PBMC probably in the absence of serum, although they did not examine the influence of serum. The inability of QM-1M to induce cytokines in a human whole blood culture system might be attributable to the high concentration (more than 10%) of human serum in the assay system. One of the possible inhibitory factors is the anti-LTA antibodies which are known to be common in healthy human serum (reviewed in [1]). Serum specimens of the donors of whole blood cultures in the present and previous studies contained considerable levels of anti-LTA antibodies (Arakaki, R., Sato, M. and Takada, H., unpublished) which might influence the bioactivities of LTA preparations. Mancuso et al. [18] reported that anti-LTA antibodies enhanced the release of cytokines by human monocytes sensitized with LTA. It is well known that serum factors such as LBP [19-21], septin [22] and sCD14 [23,24] are required for efficient cellular activation. These proteins and other LPS-recognizing agents such as bactericidal permeability-increasing protein [25] and high-density lipoprotein [26,27] might also interact with LTA and interfere with the exhibition of its bioactivities. In this context, Greenberg et al. [28] reported that bovine macrophage scavenger receptor which binds LPS also recognized specified LTA structures. The following serum factors have been demonstrated to bind with LTA as well as LPS: a 28-kDa protein in murine sera [29]; albumin [30]; and sCD14 [13]. A study examining the possibility that sCD14 is involved in the inhibitory activity of serum against the cytokine-inducing activities of QM-1M is in progress at Tohoku University, using recombinant sCD14.

Our previous [12] and present chemical analyses (Table 1) suggest that the QM-1M fraction is predominantly composed of molecules whose structure in principle corresponds to that proposed for LTA-1 from Streptococcus faecalis (E. hirae) ATCC 9790 by Fisher et al. [1,8]. The bioactivities exhibited by the QM-1M fraction in this study may be attributable to a main compound with an LTA-1 structure; how-
ever, since the QM-1M fraction was not homogeneous, the possibility should be considered that the bioactivities of the QM-1M fraction are attributable to an amphiphile different in chemical structure from LTA-1 molecules. In this context, we note that a purified Streptococcus sanguis LTA, the structure of which was reported to be comparable to that of LTA-1 [31], was scarcely active in the same assay systems adopted here (Arakaki, R. and Takada, H., unpublished). Further studies are in progress at Osaka University on bioactive structure(s) in LTA preparations from Enterococcus hirae ATCC 9790. We reserve a final conclusion on the chemical entity responsible for the bioactivities exhibited by LTA preparations including the QM-1M fraction until it is completely chemically defined, preferably chemically synthesized LTA molecules.

Acknowledgments

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