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Monocyte and Macrophage Activation by Lipoteichoic Acid Is Independent of Alanine and Is Potentiated by Hemoglobin

David L. Hasty,2*† Shiri Meron-Sudai,3 Kathy H. Cox,† Tetyana Nagorna,† Eduardo Ruiz-Bustos,3† Elena Losi,‡† Harry S. Courtney,*† Engy A. Mahrous,§ Richard Lee,§ and Itzhak Ofek¶

Lipoteichoic acids (LTAs) are Gram-positive bacterial cell wall components that elicit mononuclear cell cytokine secretion. Cytokine-stimulating activity is thought to be dependent on retaining a high level of ester-linked N-alanine residues along the polyglycerol phosphate backbone. However, Streptococcus pyogenes LTA essentially devoid of N-alanine caused human and mouse cells to secrete as much IL-6 as LTA with a much higher N-alanine content. Furthermore, hemoglobin (Hb) markedly potentiates the stimulatory effect of various LTAs on mouse macrophages or human blood cells, regardless of their N-alanine content. LTA and Hb appear to form a molecular complex, based on the ability of each to affect the other’s migration on native acrylamide gels, their comigration on these gels, and the ability of LTA to alter the absorption spectra of Hb. Because S. pyogenes is known to release LTA and secrete at least two potent hemolytic toxins, LTA-Hb interactions could occur during streptococcal infections and might result in a profound alteration of the local inflammatory response. The Journal of Immunology, 2006, 176: 5567–5576.

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This work was supported by research funds from the Department of Veterans Affairs (to D.L.H. and H.S.C.). E.R.-B. was a recipient of a postdoctoral scholarship from Consejo Nacional de Ciencia y Tecnologia (00193). Address correspondence and reprint requests to Dr. David L. Hasty, Research Service (151), Veterans Affairs Medical Center, 1030 Jefferson Avenue, Memphis, TN 38104. E-mail address: dhasty@utmem.edu

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Abbreviations used in this paper: LTA, lipoteichoic acid; PGP, polyglycerol phosphate; Hb, hemoglobin; metHb, methemoglobin; oxyHb, oxyhemoglobin; NMR, nuclear magnetic resonance; SFM, serum-free medium.

Materials and Methods

Bacterial strains

S. pyogenes M type 1 (strain 8004), an M1T1 strain obtained from a patient with streptococcal toxic shock syndrome (20), was provided by Drs. R. Kansal and M. Kob (Veterans Affairs Medical Center, Memphis, TN). It
This strain is designated *Escherichia coli* Host9, provided by Dr. /H11001.

The hemagglutination assay also confirms the presence of both peak. Fatty acid chain length, the number of glycerol units, and the percent

was previously found to be genotypically *enoml*1", *speA*+, *speB*-, *speF*-, *speG*, and *smez*". (20). The low-passage isolate was passed once and stored at −80°C.

To obtain a genetic mutant deficient in ω-α-lanlylated LTA, we inactivated the *dltA* gene in *S. pyogenes* strain 8004 using the temperature-sensitive *Escherichia coli*-streptococcus shuttle vector, pG+Host9, provided by Dr. E. Maguin (Institut de la Recherche Agronomique, Jouy-en-Josas, France (21). This strain is designated *S. pyogenes* ΔdltA. Efficient incorporation of ω-α-lan into LTA requires the *dltA-D* locus (22). Inactivation of genes within the *dltA-D* locus results in LTA that is either devoid of (23–25) or deficient in ω-α-lan (26, 27). A serotype III *Streptococcus agalactiae* strain (no. 874391) was provided by Dr. E. Adderson (St. Jude Children’s Research Hospital, Memphis, TN). Streptococci were maintained routinely on Todd-Hewitt broth supplemented with 1% yeast extract (THY) (BD Biosciences). For LTA isolation, cultures were started from frozen stocks to minimize changes that might occur during laboratory passage.

### LTA purification

Phenol-extracted *S. pyogenes*, *S. aureus*, and *Bacillus subtilis* LTAs (Sigma-Aldrich) were repurified according to ion exchange chromatography essentially following the procedures of Hashimoto et al. (2) using Macroprep High Q matrix (Bio-Rad) and a 0–2 M linear gradient of ammonium chloride. In some cases, this LTA was also fractionated over an octyl-Sepharose 4 Fast Flow (Amersham Biosciences) column exactly as described below.

LTA was also prepared from the M1 *S. pyogenes* strain 8004, *S. pyogenes* ΔdltA, a clinical *S. aureus* isolate and the type III *S. agalactiae* clinical isolate by the butanol-extraction procedure described by Morath et al. (4). Briefly, the bacteria were resuspended in 0.1 M sodium citrate buffer (pH 4.0) and mechanically disrupted (Bead-Beater; Biospec Products). The disrupted bacteria were shaken for 30 min with an equal volume of n-butanol. The aqueous phase was dialyzed extensively against distilled water, lyophilized, and resuspended in 0.1 M ammonium acetate buffer containing 15% 1-propanol. The materials were fractionated by hydrophobic interaction on an octyl-Sepharose column, eluting with a linear 15–80% propanol gradient. Phosphorus-containing fractions were pooled, dialyzed extensively against distilled water, lyophilized, and resuspended in pyrogen-free water.

### LTA analyses

LTA samples were assayed for phosphorus according to Gao et al. (28). Briefly, 20 μl of sample and 20 μl of 10% magnesium nitrate were mixed in a pyrex tube and evaporated to dryness. The samples were hydrolyzed in 0.45 ml of 1.0 N HCl for 15 min at 100°C. One milliliter of fresh 10% ascorbic acid-0.43% ammonium molybdate (1:6 v/v) was added and after 60 min at 37°C, the OD was read at 820 nm. The standard curve was constructed with known amounts of sodium phosphate.

-waline concentrations were determined by a commercial vendor (AAA Service Laboratory). LTA samples were also analyzed by nuclear magnetic resonance (NMR) for purity, chain length, and alanine content. Each sample was resuspended in D2O (500 μl) and 1H NMR spectra were recorded on a Varian NOVA-500 spectrometer at 500 MHz. Chemical shifts are reported in parts per million (δ) relative to the residual HOD peak. Fatty acid chain length, the number of glycerol units, and the percent of alanine substitution were determined by integration of the peak volumes for their respective signals in the spectra (29).

LTA concentrations were determined by weighing purified samples and this was correlated with millimolar phosphorus and passive hemagglutination assays. The hemagglutination assay also confirms the presence of both PGP and fatty acid moieties (30).

The level of endotoxin contamination was determined using the QCL-1000 quantitative chromogenic *Limulus* amoebocyte lysate assay, according to the manufacturer’s directions (Bio-Whittaker).

### Anti-LTA preparation

Anti-LTA was prepared from *S. pyogenes* LTA as previously described (31). Briefly, 100 μg of LTA was mixed with 1 mg of methylated BSA in normal saline solution. HCl (1 N) was added dropwise until the solution became cloudy. This suspension was emulsified with an equal volume of CFA. Rabbits were immunized in at least three separate injections s.c. with a total of 250 μg of LTA. Booster injections were given in IFA. Ab reactivity was tested by Western blotting with purified LTA and using a passive hemagglutination assay, as described above.

### Macrophage cultures

#### Resident peritoneal macrophages

Mice were anesthetized with a ketamine-xylazine mixture (50/50 mg/kg), 5–10 ml of MEM tissue culture medium (Invitrogen Life Technologies) was injected into the peritoneal cavity and after massaging the abdomen for several minutes, the fluid was withdrawn. Cells were resuspended in macrophage serum-free medium (SFM; Invitrogen Life Technologies) and seeded into 24-well tissue culture plates at a concentration of ~5 × 10⁴ per well. After an hour incubation at 37°C, unbound cells were removed by washing the wells three times with medium.

#### Bone marrow-derived macrophages

Femurs were removed from anesthetized mice and the marrow cavity was flushed with tissue culture medium. Cells were seeded at a concentration of ~5 × 10⁴ and were grown 1 wk in the presence of 10 ng/ml M-CSF to induce macrophage differentiation.

All animal studies were conducted in accordance with National Institutes of Health guidelines for the use and care of laboratory animals and under an active protocol approved by the institutional animal care and use committee.

### Preparation of erythrocytes

Following lavage of the peritoneal cavities of mice to obtain macrophages, blood was collected into a heparinized syringe from the inferior vena cava using a 23-gauge needle. Erythrocytes (RBCs) were collected by centrifugation at room temperature. The Buffy coat was removed and the RBCs were washed three to four times with HBSS. A 2% suspension of washed RBCs was prepared in macrophage SFM.

### Whole blood assays

Blood samples were obtained from volunteers under a protocol approved by the Memphis Veterans Affairs Institutional Review Board. The response of human blood to LTA was tested in the same manner as described by Morath et al. (4). Briefly, heparinized blood was diluted 1/5 with 150 mM NaCl and incubated with or without LTA and exogenous human Hb (50 μg/ml; Sigma-Aldrich) overnight at 37°C.

### LTA test preparations

Macrophages were exposed to LTA in several different ways. In the simplest test, LTAs were diluted in macrophage SFM to the final concentrations indicated. Polymyxin B (Sigma-Aldrich) was added to a final concentration of 5 μg/ml. In an effort to present the PGP moiety of LTA to macrophages, LTA at concentrations indicated in the text was allowed to bind to RBCs for 1 h at 37°C before adding the mixture to macrophage cultures. In other instances, LTA-coated RBCs were separated from the unbound LTA in the supernatants and washed. Macrophages were then incubated with the separate components, washed LTA-coated RBCs or LTA-containing supernatant. These washed RBCs were coated with LTA, because they could be agglutinated with Abs against LTA in a standard sheep erythrocyte test (30, 32). In one experiment, RBCs coated with LTA and washed once were incubated a second hour in macrophage SFM and this second supernatant was collected for treating macrophages. Lastly, RBCs were incubated in macrophage SFM for 1 h at 37°C in the absence of LTA, the RBCs were removed, and LTA was added to the supernatant before adding to macrophage cultures.

LPS preparations were included as macrophage stimuli in a few experiments to compare with responses to LTA. Samples of a smooth variant and a rough variant of *E. coli* LPS from strain O26:B6 and an Rc mutant of strain 35, respectively, were obtained from Sigma-Aldrich.

### Cytokine measurements

Macrophages were exposed overnight to stimuli diluted in macrophage SFM −18−20 h. Medium from triplicate samples was removed from wells, centrifuged to remove detached cells or cellular debris, and stored at −80°C until assayed. Most of the data presented here were measured by ELISAs (BioSource International) performed on appropriately diluted medium samples according to the manufacturer’s protocol.

### Analysis of RBC supernatant

Materials present in RBC supernatants were analyzed by SDS-PAGE and by Western blots using anti-mouse Hb (Santa Cruz Biotechnology) and anti-mouse LPS-binding protein (clone big 33 monoclonal: Cell Sciences). Supernatants were fractionated according to molecular size using Centricon centrifugal filter devices (Millipore).

### Western blotting analysis

LTAs were diluted to 10 μg/ml in PBS and incubated in the presence or absence of various concentrations of human Hb Ao (ferrous; Sigma-
Aldrich) or gelatin (Sigma-Aldrich) for 30 min at room temperature. Samples were resolved on a 12% native PAGE gel and transferred to nitrocellulose membrane. The membrane was blocked, washed and incubated with a 1/5,000 dilution of anti-LTA polyclonal Ab described above. The membrane was then washed and incubated with a 1/10,000 dilution of a goat anti-rabbit IgG peroxidase-conjugate (ICN/Cappel). After washing, immunoreactive species were detected by ECL Western blotting detection reagents (Amersham Biosciences).

In comigration experiments, LTA and human Hb were mixed in a 5:1 molar ratio (10 μg/ml LTA and 20 μg/ml Hb) and electrophoresed on a 12% native PAGE gel. After blotting to nitrocellulose, the blots were reacted with a 1/500 dilution of rabbit anti-human Hb α-chain (H80) (sc-21005; Santa Cruz Biotechnology) or a 1/5000 dilution of anti-LTA polyclonal Ab, followed by the same procedures described above.

### Spectrophotometry

One milligram per milliliter of Hb in the presence or absence of 1 mg/ml LTA or 1 mg/ml E. coli O111:B4 LPS (Sigma-Aldrich) in PBS was mixed and incubated at 37°C overnight and the absorption values between 500 and 700 nm were recorded on a ThermoSpectronic Genesys 20 spectrophotometer. As a Hb standard, the absorption spectra of a 1 mg/ml freshly prepared Hb solution was also measured. The percentage of oxyhemoglobin (oxyHb) that had converted to methemoglobin (metHb) and hemichrome were calculated according to Winterbourn (33).

### Results

The LTA samples used in the experiments reported here were purified from commercial preparations or were purified from butanol extracts of strains grown in our laboratories, as described in Materials and Methods. In addition to wild-type strains, an isogenic ΦlTA mutant of the wild-type MIT1 strain was generated (S. pyogenes ΦlTA) and extracted with butanol to obtain LTA essentially free of alanine. The samples were analyzed chemically and by NMR for purity, chain length, and D-alanine content (Table I). There has been due concern that some of the proinflammatory properties attributed to LTA stem from LPS contaminants. We have addressed this issue carefully in our experiments. The maximal LPS contamination of any of the samples used in these experiments was 40 pg/ml (at 1 μg/ml LTA) to 200 pg/ml (at 5 μg/ml LTA) and in most cases was much lower. All experiments reported here were conducted in the presence of 5 μg/ml polymyxin B, which inhibited up to 5 ng/ml LPS completely in our experimental system. Because this is 25- to 125-fold more LPS than any of our reactions would contain, we believe that it is highly unlikely that any of the observed responses were due to LPS contamination.

### Stimulation of whole human blood by LTA

Previous studies have suggested that a high degree of D-alanine substitution is essential for the stimulation of whole human blood cells by S. aureus LTA (4, 5). We used a similar whole human blood system for the analysis of S. pyogenes LTA. We found that, unlike with S. aureus LTA, D-alanine substitution of S. pyogenes LTA was not essential for stimulation of cytokine secretion by the cells (Fig. 1A). The magnitude of the blood cell IL-6 response to butanol-extracted LTA of S. pyogenes ΦlTA that is virtually devoid of D-alanine was equal to the response to the richly substituted butanol-extracted wild-type S. pyogenes LTA. The blood cell IL-6 response to phenol-extracted S. pyogenes LTA, which contains only half as much D-alanine as the wild-type butanol extract, was even higher at the same LTA concentration (Fig. 1A). Consistent

![Table 1](http://www.jimmunol.org/)  

<table>
<thead>
<tr>
<th>LTA Preparation</th>
<th>Percent Alaninationa</th>
<th>PGP Lengthb</th>
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<tbody>
<tr>
<td>Butanol-extracted S. pyogenes LTA</td>
<td>65</td>
<td>23</td>
</tr>
<tr>
<td>Butanol-extracted LTA of dltA mutant</td>
<td>≤7</td>
<td>24</td>
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<tr>
<td>Phenol-extracted S. pyogenes LTA</td>
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<td>23</td>
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<tr>
<td>Butanol-extracted S. aureus LTA</td>
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<td>45</td>
</tr>
<tr>
<td>Phenol-extracted S. aureus LTA</td>
<td>19</td>
<td>ND</td>
</tr>
<tr>
<td>Phenol-extracted B. subtilis LTA</td>
<td>7</td>
<td>ND</td>
</tr>
<tr>
<td>Butanol-extracted S. agalactiae LTA</td>
<td>68</td>
<td>19</td>
</tr>
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</table>

a Phosphorus and D-alanine millimolars determined as described in Materials and Methods and expressed as percent of glycerol phosphate units bearing a D-alanine residue.

b PGP length determined from NMR.

![FIGURE 1](http://www.jimmunol.org/)  

**FIGURE 1.** IL-6 response of human blood cells (A) and mouse peritoneal macrophages (B) to LTA. A, Heparinized human blood from one volunteer was diluted in saline and exposed to 5.0 μg/ml butanol-extracted S. pyogenes wild-type LTA (bSp), butanol-extracted S. pyogenes ΦlTA LTA (bSp(ΦlTA)), phenol-extracted S. pyogenes LTA (pSp), phenol-extracted S. aureus LTA (pSa), and butanol-extracted S. aureus LTA (bSa). After overnight incubation (18–20 h), supernatants were removed, clarified by centrifugation, and assayed for IL-6 levels. Values given are the mean ng/ml ± SD (n = 3). The differences between bSp and bSp(ΦlTA) were not statistically significant, but the response to pSp was statistically significant, p ≤ 0.004. The differences between pSa and bSa were also statistically different, p ≤ 0.001. B. Resident peritoneal macrophages from ICR/Swiss mice were seeded in 24-well plates at 5 × 10⁴/ml. After 1 h, unbound cells were washed away and indicated LTA preparations were added at the indicated concentration (micrograms per milliliter). After overnight incubation (~18–20 h), supernatants were removed, clarified by centrifugation, and assayed for IL-6 levels. Values given are the mean nanograms per milliliter of IL-6 ± SD (n = 3, except for the pSp(ΦlTA) sample where n = 6). The response of macrophages to very low concentrations of bSp or bSp(ΦlTA) was tested in a separate experiment (inset). Values given are the mean nanograms per milliliter of IL-6 of duplicate determinations; the variation did not exceed 10% of the mean.
with previous reports on staphylococcal LTA (4–6), the blood cell IL-6 response to richly alanylated butanol-extracted S. aureus LTA was much greater than the response to the poorly alanylated phenol-extracted S. aureus LTA. Whole blood samples from each of three other individuals showed qualitatively similar responses to the streptococcal LTA preparations (i.e., IL-6 response to butanol-extracted LTA from S. pyogenes ΔdltA was greater than or equal to butanol-extracted wild-type S. pyogenes LTA), but the magnitude of the responses varied among the individuals by up to 10-fold (our unpublished data).

Stimulation of mouse macrophages by LTA

The results above suggested that a high degree of α-alanine substitution of S. pyogenes LTA is not essential in the stimulation of human blood cells. Because there were significant, ill-defined, individual-to-individual differences in the magnitude of stimulation using human blood samples, further studies were performed with mouse blood, assuming that it would be a more uniform system. Whole mouse blood, however, responded very poorly to LTA at either 1 or 10 μg/ml (<20 pg/ml IL-6) and was not used further. In contrast, peritoneal macrophages gave a strong response to LTA preparations. The butanol-extracted S. pyogenes wild-type and ΔdltA mutant LTA preparations, as well as butanol-extracted S. aureus LTA, stimulated mouse macrophages to secrete IL-6 in a dose-dependent fashion (Fig. 1B). That alanine is not essential for S. pyogenes LTA activity is again underscored by the observation that LTA from S. pyogenes ΔdltA mutant stimulated macrophages as well as or better than butanol-extracted wild-type LTA over a wide range of LTA concentrations (Fig. 1B, inset). At a relatively high streptococcal LTA concentration (5 μg/ml), the poorly alanylated phenol-extracted S. pyogenes LTA preparation stimulated the macrophages as well as or better than the butanol-extracted LTAs, consistent with the results obtained with human blood. At lower concentrations, however, the LTAs obtained by butanol extraction were more active, regardless of alanine content. For example, the minimal concentration needed for butanol-extracted wild-type or S. pyogenes ΔdltA LTA to induce >10 ng/ml IL-6 was <0.1 μg/ml, whereas that for phenol-extracted S. pyogenes LTA was >1 μg/ml.

Potentiation of LTA activity by RBC supernatant

Having determined that the role of α-alanylation of S. pyogenes LTA appeared not to be essential for its activity, we next examined the role of the PGP moiety in generating the macrophage response. To do this, we exposed macrophages to erythrocytes (RBCs) that had first been sensitized with phenol-extracted S. pyogenes LTA, thus anchoring LTA to the RBC plasma membrane via its lipid and presenting the PGP at the RBC surface (34). The results showed that there was a marked potentiation of the stimulatory activity of phenol-extracted S. pyogenes LTA in macrophages exposed to mixtures of erythrocytes and LTA, especially at low concentrations of LTA (Fig. 2A). The stimulatory activity of butanol-extracted S. pyogenes LTA was also potentiated by the presence of RBCs (our unpublished data), but the relative increase was less than that obtained with the phenol-extracted LTA, probably because the butanol-extracted LTA alone stimulated more IL-6 release than the phenol-extracted LTA at low concentrations. The IL-6 response of bone marrow-derived macrophages to LTA also exhibited a 10- to 20-fold increase when phenol-extracted S. pyogenes LTA was first incubated with RBCs before adding to macrophage cultures (our unpublished data).

Surprisingly, when the LTA-RBC mixtures were washed free of unbound LTA, the LTA coated RBCs did not elicit a response from macrophages above the background response elicited by untreated RBCs (Fig. 2A). The LTA-coated RBC, however, could be agglutinated by anti-LTA Abs to a titer of 1:64, indicating that the PGP moiety was, indeed, exposed at the RBC surface. The increased macrophage secretion of IL-6 in response to LTA-RBC
mixtures was paralleled by a striking increase in phagocytosis of RBCs (Fig. 2B), but LTA-coated and washed RBCs were not phagocytosed. Thirty-three percent of macrophages incubated with LTA-RBC mixtures contained one or more RBCs, while only 10% of macrophages incubated with LTA-treated and washed RBCs contained an RBC.

The supernatant obtained from the LTA-RBC mixture retained a potent ability to enhance cytokine release from peritoneal macrophages, almost equal to that of the LTA-RBC mixture (Fig. 2C). When supernatant was obtained from a second 1-h incubation of the LTA-sensitized RBCs, macrophage-stimulating activity was not detected, presumably due to the removal of soluble LTA. However, following addition of 1 \( \mu \)g/ml phenol-extracted \( S. \) pyogenes LTA to the second, inactive supernatant sample, the response observed suggested that the added LTA synergized with material contained in the supernatant (Fig. 2C).

Macrophages that were incubated with LTA for 1 h, followed by washing and overnight incubation with RBC supernatant, secreted IL-6 at essentially background levels (Fig. 2C). Conversely, macrophages that were first incubated with RBC supernatant for 1 h, followed by washing and overnight incubation with LTA, secreted IL-6 roughly equivalent to that secreted in response to LTA (35). Resident peritoneal macrophages from ICR/Swiss mice responded to 1 \( \mu \)g/ml LTA alone. Further studies demonstrated that the mouse macrophage cell line secreted much lower levels of IL-6 in response to phenol-extracted \( S. \) pyogenes LTA alone (4.7 ± 0.13 pg/ml), but these cells, too, showed a significantly increased response to LTA plus RBC supernatant (14.13 ± 1.29 pg/ml; \( p \leq 0.02 \)).

Characterization of the potentiating factor from RBCs

The requirement for the simultaneous presence of RBC supernatant and LTA in the reaction mixture to obtain maximal IL-6 secretion suggests that material released from RBCs interacted with LTA to cause this effect. To begin to define the RBC potentiating factor, the RBC supernatant was either freeze-thawed, boiled, or fractionated by ultrafiltration (Fig. 4). There was no statistical difference in IL-6 secretion from macrophages stimulated with 1 \( \mu \)g/ml phenol-extracted \( S. \) pyogenes LTA in the presence of fresh or freeze-thawed mouse RBC supernatant. In contrast, the IL-6 response to boiled supernatant and LTA was dramatically reduced and not significantly different from the response to LTA alone. Macrophages exposed to LTA and a 30- to 100-kDa fraction of RBC supernatant obtained using Centricon ultrafiltration devices secreted large amounts of IL-6, but fractions <30 kDa or >100 kDa did not enhance the level of IL-6 secreted in response to LTA alone. Further studies demonstrated that the mouse macrophage IL-6 response to 1 \( \mu \)g/ml phenol-extracted \( S. \) pyogenes LTA was potentiated by either purified mouse or human Hb in a dose-dependent fashion (Fig. 5A). SDS-PAGE of RBC supernatants indicated that the primary component present was Hb (inset, Fig. 5A) and Hb has a native molecular mass within the range of the active supernatant fraction obtained by gel filtration (Hb ≈64 kDa; monomer ≈16 kDa). LPS-binding protein, which is known to interact with LTA (10), could be demonstrated in mouse serum by

FIGURE 4. Stability and sizing of the potentiating factor. Resident peritoneal macrophages from ICR/Swiss mice responded to 1 \( \mu \)g/ml LTA alone (LTA), and the response was potentiated in the presence of RBC supernatant (LTA + Sup). The potentiating effect of RBC supernatant was not affected by freezing and thawing (LTA + F/T Sup), but it was eliminated by heating (LTA + Boiled Sup). RBC supernatants were fractionated by ultrafiltration, the fractions obtained were reconstituted to their original concentrations, and LTA was added before applying the mixtures to macrophages. Materials passing through the 100-kDa filter but retained by the 30-kDa filter potentiated the macrophage response to LTA, while the material retained by the 100-kDa filter or that passing through the 30-kDa filter exhibited no or low potentiating activity, respectively. Responses are given in nanograms of IL-6 per milliliter (mean ± SD; \( n = 3 \)).

FIGURE 3. Comparison of BALB/c and C3H/HeN macrophage response to LTA and LTA + RBCs. Peritoneal macrophage cultures were prepared as described earlier. Macrophages were incubated with 10 \( \mu \)g/ml LTA or a mixture of 10 \( \mu \)g/ml LTA and RBCs. After overnight incubation (∼18–20 h), supernatants were removed, clarified by centrifugation, and assayed for IL-6 levels. Values given are the mean nanograms per milliliter ± SD (\( n = 3 \)).
immunoblot, but was not detected in RBC supernatant (our unpublished data). As has been shown previously, the activity of both smooth and rough varieties of LPS (36, 37) was also found to be extraction method specific. Oxidized Hb (i.e., metHb) was found to potentiate the activity of phenol-extracted S. pyogenes LTA, but hemin did not potentiate the IL-6 response of macrophages to LTA (Fig. 6B). IFN-γ was not secreted in response to either stimulus. Moreover, exogenous Hb (50 μg/ml) also increased the IL-6 response of whole human blood several fold when compared with butanol-extracted S. pyogenes LTA alone (Fig. 7). Hb present in our diluted blood samples was not measured, but we estimate that in a 1/5 dilution of blood Hb would be roughly 20 μg/ml (20% of the normal serum value of ~100 μg/ml). Although some of the stimulation seen with LTA alone could be affected by serum Hb, additional Hb clearly causes an increased response. Taken together, these experiments strongly suggest that the potentiation of the activity of LTA by Hb may be a general phenomenon not limited to particular species or structural characteristics of LTA.

Interaction of LTA and Hb

The potentiation of LTA stimulation of macrophages by Hb and the requirement for their simultaneous presence suggested that the

FIGURE 5. IL-6 response of macrophages to phenol-extracted S. pyogenes LTA plus Hb and to LPS plus Hb. A, Peritoneal macrophages of ICR/Swiss mice were incubated with increasing amounts of mouse (circles) or human (triangles) Hb in the presence (closed symbols) or absence (open symbols) of 1 μg/ml LTA. Values given are the mean nanograms per milliliter IL-6 ± SD (n = 3). Inset, SDS-PAGE analysis of RBC supernatant. Samples of RBC supernatant (lane 1) and purified Hb (lane 2) were analyzed by SDS-PAGE. The gels were stained with Coomassie blue, or were blotted to nitrocellulose and reacted with anti-Hb Abs in Western blots, as indicated. In SDS-PAGE, most Hb migrates in the monomer configuration (~16 kDa), but a small fraction remains in the tetrameric form (~64 kDa). B, Macrophages were incubated with 1 or 10 ng/ml rough or smooth E. coli LPS in the presence or absence of 50 μg/ml human Hb. The amounts of LPS used were calculated to yield an IL-6 response at levels roughly comparable to the amount of IL-6 generated by 1 μg/ml LTA. Values given are the mean nanograms per milliliter of IL-6 ± SD (n = 3).

FIGURE 6. Potentiation of mouse macrophage response to LTA preparations by Hb and Hb derivatives. A, Peritoneal macrophages of ICR/Swiss mice were incubated with various LTA preparations (1 μg/ml) in the presence or absence of 50 μg/ml purified human Hb. Left panel, Phenol-extracted LTA from S. pyogenes (pSp), S. aureus (pSa), and B. subtilis (pBs). Right panel, Butanol-extracted LTA from S. pyogenes wild-type (bSp), S. pyogenes ΔdltA mutant (bSpΔdltA), S. aureus (bSa), and S. agalactiae (bSag). Values are mean nanograms per milliliter of IL-6 ± SD (n = 3). Differences between responses without and with Hb were statistically significant in each case, p ≤ 0.005. B, Macrophages were incubated with phenol-extracted S. pyogenes LTA in the absence or presence of Hb, metHb, or hemin. Values given are the mean nanograms per milliliter of IL-6 or TNF-α ± SD (n = 3).
two molecules form a biologically active complex. Complexes between LPS and Hb, as well as LTA and LPS-binding protein, have previously been demonstrated using PAGE (10, 36). Although LTA migrates on a SDS-PAGE gel at \( \approx 10 \) kDa, it migrates much slower and in a diffuse manner on a nondenaturing gel (Fig. 8). This migration pattern may be explained by the well-known ability of LTA to form micelles in an aqueous solution (38). When incubated with Hb at molar ratios \( \geq 1:1 \), LTA migrates more rapidly in a nondenaturing gel, perhaps due, at least in part, to disaggregation or dissolution of micelles (Fig. 8). A similar migration pattern was observed with phenol-extracted \( S. aureus \) LTA (Fig. 8), as well as with phenol-extracted \( B. subtilis \) LTA and butanol-extracted \( S. agalactiae \) LTA (our unpublished data). A control protein, gelatin, did not change LTAs migration in native gels (Fig. 8). In addition, Hb and some species of LTA were observed to comigrate in native gels, as evidenced by Western blotting analyses (Fig. 9A). The physical interaction of LTA and Hb can also be appreciated in the alteration of the Hb absorption spectrum following incubation with LTA. After an 18-h incubation at \( 37^\circ C \) with LTA, Hb underwent spectral changes characterized by decreases in the peaks at 540 and 577 nm indicative of the conversion of oxyHb to metHb and hemichrome (Fig. 9B). Interestingly, LPS at the same concentration resulted in less hemichrome formation (inset, Fig. 9B). The data, taken together, are consistent with the formation of LTA-Hb complexes.

**Discussion**

Ever since the pioneering studies of Fischer and coworkers (39) showing that LTA stimulated human monocytes, the role of LTA in provoking the immune system has been a controversial topic. More recent studies of Hartung and coworkers (4–6, 10, 18, 40, 41) seemed to resolve the issue by showing that LTA...
richly substituted with α-alanine can be a potent proinflammatory agent. In fact, its potency even rivaled that of LPS in some instances (41). Significantly, stimulation of blood cells from whole human blood by S. aureus LTA was shown to be dependent on the preservation of a high level of α-alanine substitutions on the PGP backbone, apparently due to the use of butanol instead of phenol for extraction (4–6). It was recently shown that LTA purified from a dltB mutant of Lactobacillus plantarum, with only 1.1% of glycers alanylated, stimulated a significantly lower proinflammatory cytokine response than did LTA purified from the wild-type strain in which 41.7% of the glycers were alanylated (26). In contrast, our results clearly indicate that a high proportion of α-alanine substitutions on S. pyogenes LTA is not essential for stimulation of either whole human blood or mouse peritoneal macrophages. These seemingly conflicting observations may be due to different structural properties of the LTAs used in the different studies. These include differences in the length of the PGP backbone, different substituents of the PGP backbone other than alanine (e.g., N-acetyl galactosamine in S. aureus), or differences in the glycolipid anchor. For example, in the studies of Morath et al. (4, 5), butanol-extracted S. aureus LTA, as well as synthetic LTA, contained both α-alanine and N-acetyl-glucosamine on its PGP backbone. The only substitution along the PGP backbone of S. pyogenes LTA is α-alanine, as it lacks N-acetyl-glucosamine or any other substituent. It is possible, therefore, that α-alanine is only required in LTAs whose PGP backbone is also substituted with certain saccharides. Recent evidence that α-alanine was not essential for activity of LTA from group B streptococci, which also does not contain saccharide substituents on its PGP, is consistent with this hypothesis (42). The PGP chain length appears not to be of key importance, since synthetic LTA with only 6 glyceral phosphate units had activity equal to native S. aureus LTA with 45 glyceral phosphate units (4, 5). One of the first studies on the ability of phenol-extracted LTAs to stimulate cytokine secretion showed that of 20 species of LTA studied, S. aureus LTA was 1 of only 4 that had no activity, but the authors concluded that there was no obvious relationship between the chemical structures and reactivity (39). We used LTA obtained from four different species of Gram-positive bacteria, including a S. pyogenes dltA mutant, which differ in their α-alanine content, their saccharide substituents, and the length of the PGP backbone. Although we noted differences in the minimal concentrations required to trigger cytokine secretion in blood cells, our results still do not allow a firm conclusion regarding the underlying structural basis for such differences, apart from the notion that a high level of α-alanine is clearly not essential for the activity of certain LTA species.

Irrespective of the structural differences among the various LTA species, the stimulation of macrophages by all of the LTA preparations tested was potentiated by the presence of Hb at 50 μg/ml, a concentration that is below physiological concentrations of serum Hb (i.e., 115–155 μg/ml). IL-6 values were the only ones reported for most of the experiments reported here, but TNF-α was also found to increase significantly in the experiments where it was measured. Our results suggest that the enhancement of stimulation appears to be due to the formation of complexes between LTA and Hb, but more work will be required to determine the precise nature of this interaction. Hb has been previously shown to form complexes with LPS and to augment its cytokine-stimulating activity (43–47). The magnitude of enhancement of the activity of LTA appears to be generally greater than that observed for LPS, although further studies will be required to substantiate this point. For example, at a 1:40 molar ratio of LPS:Hb, there was only a 4-fold increase in TNF-α production by human blood cells (47), while at a 1:40 molar ratio of LTA:Hb there was a 40-fold increase in IL-6 secretion by murine macrophages (Fig. 5). It should be pointed out, however, that the magnitude of enhancement was different among the various LTA preparations, suggesting that Hb-mediated enhancement of LTA activity is also related to structural differences that remain to be determined. Because the enhancement of LTA activity by Hb begins to plateau at roughly a 1:1 molar ratio, and this is precisely the same ratio where electrophoretic migration of LTA began to be changed by Hb, we hypothesize that a heterodimer containing one molecule of LTA and one molecule of Hb is the main active principle. This hypothesis is generally in line with one of the mechanisms proposed for the enhancement of LPS activity by Hb involving disaggregation of micelles (47). The tetrameric Hb molecule is known to form a single pocket that accommodates 2, 3-bisphosphoglycerate and it would seem possible that this pocket could be involved in the interaction of Hb with the PGP moiety of LTA.

Hb potentiates the activity of several different LTA preparations for a number of different types of cells, including those in whole human blood, peritoneal macrophages of different mouse strains, and mouse bone marrow-derived macrophages. Even the RAW 276.7 macrophage-like tissue culture cell line, which responded very poorly to LTA alone, secreted increased amounts of IL-6 in the presence of LTA-Hb complexes. In most cases, the magnitude of enhancement by Hb was in roughly the same range for the different types of cells. The latter may suggest that the primary effect of the interaction of LTA with Hb is to facilitate the interaction of LTA with its cell receptor. This suggestion is strengthened by preliminary observations indicating that LTA and LTA plus Hb stimulate the same pattern of secretion of 18 different cytokines, with the differences being quantitative and not qualitative (our unpublished data). The finding that the macrophages ingest markedly increased numbers of RBCs in the presence of soluble LTA, but did not ingest LTA-coated and washed RBCs, suggests that soluble LTA-Hb complexes prime the cells for phagocytosis. The molecular mechanism of this effect remains to be determined.

It has been suggested that Hb-mediated stimulation of macrophages to release proinflammatory cytokines may have implications in the context of hemolysis, which can occur following surgery, blood transfusions, or infections (46). Several studies have shown that LTA can be released from actively growing streptococci in vitro (48–51), and it has also been shown that individuals infected with streptococci can have LTA levels that exceed the concentrations we have used in this study, in cerebrospinal fluid, for example (52). Because S. pyogenes produces two potent hemolysins, streptolysin S and streptolysin O, local concentrations of Hb during a typical course of infection could be even higher than normal levels of free Hb in plasma. The major importance of these hemolysins in infections may be that they provide the bacteria access to a source of iron, but it is also possible that LTA-Hb complexes could be formed at the site of infection and affect macrophages in other ways as well that could either promote or impede resolution of the infection. The fact that Hb potentiates the activity of both LPS and LTA suggests that perhaps Hb should now be considered one of the soluble pathogen recognition receptors.

Excessive production of cytokines by macrophages and other mononuclear cells is thought to be a primary factor in the evolution of shock caused by Gram-positive bacteria. Because LTA alone has not been found to induce in vivo effects that are comparable to those induced by LPS, its role in septic shock and multiorgan...
failure has been questioned (53). It was suggested that serum inhibitors may be responsible for the lack of a shock-like in vivo response to LTA. Our observations demonstrate that at least one host protein, Hb, can enhance LTA-induced cytokine production in vitro, including in whole human blood, suggesting that Hb-LTA interactions could contribute to the balance of cytokines secreted during human infections. Although our study highlights the potential importance of Hb in increasing the proinflammatory potency of LTA, it will be important to search for other factors with a similar function, especially because LTA is known to interact with a number of host macromolecules.

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Disclosures

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