Molecules from *Staphylococcus aureus* that Bind CD14 and Stimulate Innate Immune Responses

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Summary

Mammals mount a rapid inflammatory response to gram-negative bacteria by recognizing lipopolysaccharide (LPS, endotoxin). LPS binds to CD14, and the resulting LPS-CD14 complex induces synthesis of cytokines and up-regulation of adhesion molecules in a variety of cell types. Gram-positive bacteria provoke a very similar inflammatory response, but the molecules that provoke innate responses to these bacteria have not been defined. Here we show that protein-free, phenol extracts of *Staphylococcus aureus* contain a minor component that stimulates adhesion of neutrophils and cytokine production in monocytes and in the astrocytoma cell line, U373. Responses to this component do not absolutely require CD14, but addition of soluble CD14 enhances sensitivity of U373 cells by up to 100-fold, and blocking CD14 on monocytes decreases sensitivity nearly 1,000-fold. Deletion of residues 57–64 of CD14, which are required for responses to LPS, also eliminates CD14-dependent responses to *S. aureus* molecules. The stimulatory component of *S. aureus* binds CD14 and blocks binding of radioactive LPS. Unlike LPS, the activity of *S. aureus* molecules was neither enhanced by LPS binding protein nor inhibited by bactericidal/permeability increasing protein. The active factor in extracts of *S. aureus* is also structurally and functionally distinct from the abundant species known as lipoteichoic acid (LTA). Cell-stimulating activity fractionates differently from LTA on a reverse-phase column, pure LTA fails to stimulate cells, and LTA antagonizes the action of LPS in assays of IL-6 production. These studies suggest that mammals may use CD14 in innate responses to both gram-negative and gram-positive bacteria, and that gram-positive bacteria may contain an apparently unique, CD14-binding species that initiates cellular responses.

Gram-positive and gram-negative bacteria provoke similar inflammatory responses, yielding equivalent cardiovascular abnormalities (1), similar TNF and IL-1 production, similar tissue injury (2, 3) and death (1, 3). In baboons, an equivalent number of *Staphylococcus aureus* and *Escherichia coli* are required for an LD50 (3), and in vitro, equivalent numbers of *S. aureus* and *E. coli* are required to initiate TNF release from monocytes (4) or macrophages (5). The similarity of the responses of cells to gram-positive and gram-negative bacteria suggests a common intermediate.

The principal inflammatory component of gram-negative bacteria is LPS (endotoxin), the dominant lipid on the outer leaflet of the outer membrane. Recent studies have described an innate immune mechanism for responding to LPS that involves the proteins CD14 and LPS-binding protein (LBP)† (see reference 6 for review). LBP (7) is a lipid transfer protein that facilitates movement of LPS from microsomes to CD14 (8). CD14 expresses a binding site for one to two LPS molecules, and LPS-CD14 complexes potently stimulate cellular responses (8–10). CD14 is found both as a glycosylphosphatidylinositol-linked membrane protein on monocytes and PMN (mCD14) and as a soluble form (sCD14) in plasma, and both forms of CD14 stimulate cellular responses to LPS (7, 8, 11, 12).

In contrast with gram-negative bacteria, our understanding of the mechanism by which gram-positive bacteria induce inflammatory changes is far less developed. Most work on inflammatory molecules of gram-positive bacteria has centered on the cell wall and membrane because these portions of the bacterium are exposed and because heat-killed organisms and purified walls actively induce inflammation in animal models (13). Gram-positive bacterial walls are composed of highly cross-linked peptidoglycan (PG) decorated binding protein; LTA, lipoteichoic acid; NHP, normal human plasma; PG, peptidoglycan; sCD14, recombinant soluble CD14; SACE, *Staphylococcus aureus* crude extracts; sCD14, soluble CD14.

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Abbreviations used in this paper: AF, active fraction; BPI, bactericidal/permeability increasing protein; HSA, human serum albumin; LBP, LPS-binding protein; LTA, lipoteichoic acid; NHP, normal human plasma; PG, peptidoglycan; sCD14, recombinant soluble CD14; SACE, *Staphylococcus aureus* crude extracts; sCD14, soluble CD14.
to a variable extent with teichoic acid polymers. Teichoic acid polymers are also linked to plasma membrane phospholipids, and these lipoteichoic acids (LTA) comprise another major surface component (14, 15). Gram-positive bacteria contain no LPS, and LTA is the major macroamphiphile of gram-positive bacteria. The physiochemical properties of LTA are similar to those of LPS, and it can be extracted with phenol by procedures similar to those used for purification of LPS (16).

Published work has ascribed inflammatory activity to each of the surface components described above. PG and PG fragments have been shown to stimulate production of TNF, IL-1, IL-6, and IL-8 in monocytes and macrophages (4, 17–20). Similarly, LTA has been shown to provoke secretion of TNF, IL-1, IL-8, and NO from monocytes or macrophages (2, 21–23). While these data suggest a role for the numerically dominant envelope molecules in provoking inflammation, in nearly all cases very large amounts of PG or LTA are required to stimulate responses of cells in vitro: 1–10 μg/ml of LTA (2, 21–23) and 10–100 μg/ml PG (4, 18, 20) are necessary to elicit cellular responses while LPS elicits responses in the picogram to nanogram per milliliter range. The low sensitivity to the purified gram-positive products raises the possibility that a trace contaminant, even if present as 1 part in 10,000, may be responsible for the activity observed.

Here we show that <10 ng/ml of a trace constituent of S. aureus strongly stimulates cytokine production in both monocytes and the astrocytoma cell line, U373. The response of monocytes to extracts of S. aureus was strongly blocked by anti–CD14. Moreover, the response of U373 cells, which do not express CD14, was strongly enhanced by addition of sCD14. Finally, we show that the active molecule from S. aureus binds sCD14 and blocks binding of LPS to sCD14. These data suggest the presence of a novel, proinflammatory, CD14-binding molecule in S. aureus.

Materials and Methods

S. aureus Crude Extracts (SACE). Phenol extracts of S. aureus suspensions, which are commercially available as LTA from S. aureus, were purchased from Sigma Chemical Co. (St. Louis, MO). They were prepared by the method of Fischer et al. (16). Briefly, mechanically broken S. aureus were extracted with phenol at 65°C and the aqueous portion dialyzed. Nucleic acids were removed by digestion with nuclease followed by reextraction, dialysis, and lyophilization. This procedure, which is similar to the Westphal procedure for purification of LPS from gram-negative bacteria, yields not only LTA, but also some polysaccharides and other soluble components of the cell. The material had no optical density at either 280 or 214 nm, indicating the absence of protein. We designated this product SACE and used it as our starting material. It yielded clear or slightly opalescent solutions in physiologic saline, and hydrolysis of 1 mg/ml of SACE (see below) yielded 4 mM of phosphate. An abbreviated protocol was used in one experiment (Fig. 4). Tryptic soy broth was inoculated and incubated overnight at 37°C with vigorous shaking with S. aureus strain DSM20233 (a gift of Dr. Seva Polotsky, Yale University). Cultures were centrifuged, mechanically broken, and extracted with phenol at 65°C as described above. The aqueous portion was collected, dried down in a SpeedVac concentrator (AS160; Savant Instruments, Inc., Farmingdale, NY), and resuspended in H2O.

Other Reagents. LPS from Salmonella minnesota strain R595 (Re) and H-labeled LPS from E. coli K12 strain LDC25 were purchased from List Biologicals (Campbell, CA). Recombinant human soluble CD14 (rsCD14) and LBP were expressed and purified as described (8). Mutant rsCD14 terminating at position 152 of the mature protein (rsCD14–152) and a second CD14 mutant lacking amino acids 57–64 (rsCD1457–64) were expressed and purified as described (24, 25). Bacterial/permeability increasing protein 21 (BPlp) (26) was a gift of Dr. J. Weiss, New York University. Anti-CD14 mAb 60b (27) and anti-CD18 mAb IB4 (28) were purified from ascites fluid by chromatography on protein G-Sepharose. Fresh frozen normal human plasma (NHP) was supplied by the New York Blood Center. Limulus amoebocyte reagent reagents were from BioWhittaker, Inc. (Walkersville, MD), and human serum albumin was from Armour Pharmaceutical Co. (Kankakee, IL).

Stimulation and Assay of IL-6 Production. The human astrocytoma cell line, U373, was obtained from the American Type Culture Collection (HTB 17; Rockville, MD) and maintained in RPMI 1640 medium (BioWhittaker, Inc.) supplemented with 10% FCS. U373 cells were plated in 72-well terasaki plates (Robbins Scientific Corp., Sunnyvale, CA) at a density of 10,000 cells/well 24 h before stimulation. In some experiments, PBMC were purified from the blood of healthy volunteers as described (29), and plated in the same way 2 h before stimulation. The cells were washed extensively with AIM-V serum-free medium (GIBCO BRL, Grand Island, NY) and then incubated with various stimuli in AIM-V medium containing 0.5 mg/ml of human serum albumin (AIM-V–HSA). After 24 h, the supernatants were collected, diluted five times in PBS containing 1 mg/ml of HSA, and IL-6 levels were quantitated using a human IL-6 ELISA (Biosource International, Camarillo, CA) with slight modification. Briefly, terasaki plates were coated with 5 μl/well of anti–IL-6 mAb B-E8, 40 μg/ml, overnight at 4°C. After blocking with 0.1% gelatin, 5 μl of sample and 5 μl of biotin-labeled anti–human IL-6 were added per well and incubated for 2 h at 37°C. After washing, bound antibodies were detected by adding 10 μl/well of streptavidin-conjugated alkaline phosphate (1:1,000; Calbiochem Corp., La Jolla, CA), and incubating for 1 h at room temperature. A fluorescent signal was generated using the fluorogenic substrate Amplex Red (JBL Scientific, San Luis Obispo, CA) and was measured using a Cytofluor 2300 (Millipore Corp., Bedford, MA).

In some experiments, heparinized whole peripheral blood from healthy volunteers was used for the assay. 90 μl of the whole blood was added to plastic tubes (Sarstedt, Inc., Newton, NC), 10 μl of various stimuli was added, and the tubes were incubated for 5 h at 37°C. After centrifugation, the supernatant was collected, diluted five times in PBS containing 1 mg/ml of HSA, and IL-6 levels were quantitated as described above.

Assay of PMN Adhesion to Fibrinogen. Adhesion of human PMN to fibrinogen-coated surfaces in response to LPS or SACE fractions was measured as described (8, 30). In this assay, adhesion of PMN in response to LPS is mediated by the leukocyte integrin CD11b/CD18 (CR3, Mac1) (30) and requires either the presence of LBP to hasten interaction of LPS with mCD14 on the PMN or preformed LPS–sCD14 complexes made by prolonged incubation of LPS with sCD14 (8). Briefly, mixtures containing LPS or SACE fractions, prepared as described in the legend to Fig. 12, were diluted to 125% of their final concentrations in 40 μl...
HAP (Dulbecco's PBS with 0.5 U/ml aprotinin, 0.05% HSA, 3 mM Mg- and glucose). 10 μl of freshly isolated PMN (2 x 10^6 cells/ml in HAP) fluorescently labeled with 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester as described (30) were added and incubated for 10 min at 37°C to stimulate the cells. PMN were then washed into HAP and added to a 72-well terasaki plate precoated with fibrinogen. After 15 min at 37°C, adherence of PMN to the plate was quantitated: The fluorescence in each well was determined before and after washing using a Cytofluor 2300, and the percent adhesion was calculated from triplicate wells as fluorescence after washing/fluorescence before washing) X 100. Donor-to-donor variation in maximal responses prohibited averaging results of separate experiments, but the pattern of responses was highly reproducible.

Phosphate Assay. The absolute amount of phosphate in samples was measured by the method of Broekman et al. (31). Briefly, 25 μl of sample was mixed with 100 μl perchloric acid and digested at 180-190°C for 30 min. After cooling, 1.45 ml phosphate reagent (6 N H2SO4, 2.5% ammonium molybdate, 2.5% ascorbic acid, and water mixed in a 1:1:4:4 ratio) was added, samples were held at 50°C for 1 h, and 830 nm was read. The amount of phosphate in each sample was calculated from a standard curve prepared with KH2PO4 solutions.

Column Chromatography. A C8-HPLC column (Nova-Pak C8, 3.9 mm x 150 mm; Waters Associates, Millipore Corp., Marlborough, MA) was equilibrated in 0.05 M sodium acetate (pH 4.7) containing 5% propanol. Sample (100 μl) was applied at a flow rate of 0.5 ml/min, and the column was subsequently eluted with 5-100% propanol gradient in the same buffer. 20 fractions (1.5 ml each) were collected, dried down in a SpeedVac concentrator, and resuspended in 100 μl H2O.

Gel Electrophoresis. Tris-glycine polyacrylamide step-gradient gels (Novex, San Diego, CA) were used for native PAGE. Sample buffers and running buffers were made according to the manufacturer's suggested recipes. Samples were electrophoresed at 100-150 V for 2 h and stained with ammoniacal silver according to published procedures (32). 3H-LPS was visualized by fluorography using ENHANCE (DuPont Co., Boston, MA).

Results

Extraction of S. aureus Stimulate IL-6 Production in Monocytes. Addition of SACE to whole blood caused a strong, dose-dependent production of IL-6 (Fig. 1). High concentrations of SACE (1-10 μg/ml) alone caused production of IL-6 (Fig. 2 A). The amount of IL-6 produced in response to optimal concentrations of SACE was similar to that produced in response to LPS and sCD14 (Fig. 2 B). Addition of SACE was strongly enhanced by the addition of blocking anti-CD14 mAbs (not shown). The response to SACE was dependent on the concentration of sCD14 added, with strong potentiation of both SACE and LPS responses observed with 100 ng/ml sCD14 (Fig. 3). The CD14-dependent response of U373 cells to SACE was completely abolished by the addition of blocking anti-CD14 mAbs (not shown). These results indicate that SACE can stimulate cytokine production in U373 cells and that CD14 can strongly potentiate this response.

Cell Stimulating Activity of SACE Is Distinct from That of LPS. When cultured under aseptic conditions, strains of S. aureus release a factor that stimulates adherence of monocytes to the CD14-dependent manner.

SACE Stimulates IL-6 Secretion by U373 Astrocytoma Cell Line in a CD14-dependent and a CD14-independent Manner. The role of CD14 in responses to SACE was further explored in the U373 astrocytoma cell line which expresses no mCD14. These cells secrete IL-6 in response to LPS, but only in the presence of exogenously added sCD14 (12). Monolayers of U373 cells were incubated with dilutions of SACE for 24 h in serum-free medium in the presence or absence of rsCD14, and the supernatant was assayed for IL-6 by ELISA. Results are means ± SD of triplicate determinations of a representative experiment repeated two times.

Figure 1. SACE induces IL-6 production in whole blood in a CD14-dependent manner. Whole peripheral blood was incubated for 5 h with different concentrations of SACE in the presence or absence of 50 μg/ml of either control anti-CD18 mAb (IB4) or anti-CD14 mAb (60b), and the supernatants were assayed for IL-6 by ELISA. Results are means ± SD of triplicate determinations of a representative experiment repeated two times.
results (12, 34). In contrast, NHP did not enhance the activity of SACE (Fig. 2 C) but caused a reduction of IL-6 production at high concentrations of SACE. (d) LPS-stimulated production of IL-6 by purified PBMC in serum-free medium was strongly enhanced by LBP (7, 35, and Fig. 5 B). Concentrations of SACE of 100 ng/ml or greater also stimulated IL-6 production by monocytes (Fig. 5 A). However, LBP had no effect on this response, suggesting that LBP does not recognize the active factor in SACE. This finding is consistent with the observation of Mathison et al. (35) that LBP did not enhance the response of murine macrophage to whole heat-killed S. aureus. (e) BPI specifically binds LPS and blocks its ability to stimulate cells (26, and

Figure 2. IL-6 production by U373 cells in response to SACE or LPS. Monolayers of U373 cells were incubated for 24 h with different concentrations of (A and C) SACE or (B and D) LPS in the presence (open symbols) or absence (filled symbols) of either (A and B) rsCD14 (1 µg/ml) or (C and D) 1% NHP. The supernatant was assayed for IL-6 by ELISA. Results are means ± SD of triplicate determinations of a representative experiment repeated three times.

Figure 3. CD14 enhances production of IL-6 in response to SACE. Monolayers of U373 cells were incubated for 24 h with either SACE (100 ng/ml, 400 nM phosphate) or LPS (100 ng/ml) in the presence or absence of different concentrations of rsCD14. The supernatants were assayed for IL-6 by ELISA. Results are means ± SD of triplicate determinations of a representative experiment repeated three times.

Figure 4. The cell-stimulating activity of phenol extracts derived from S. aureus. Culture medium was incubated overnight at 37°C with vigorous shaking in the presence (squares) or absence (circles) of an inoculum of S. aureus strain DSM20233. Both cultures were centrifuged and extracted in an identical way with phenol as described in Materials and Methods. The extracts were diluted to the indicated concentration of phosphate and were applied to monolayers of U373 cells with or without sCD14 (1 µg/ml), and incubated for 24 h. Control extracts had no phosphate, but were diluted exactly the same way for comparison. The supernatants were assayed for IL-6 by ELISA. Results are means ± SD of triplicate determinations of a representative experiment repeated two times.

1676 S. aureus Molecules Bind CD14 and Stimulate Cellular Responses
Figure 5. Effect of SACE and LPS on IL-6 production by PBMC with or without LBP. Monolayers of PBMC were incubated for 24 h with different concentrations of (A) SACE or (B) LPS in the presence or absence of LBP (1 μg/ml), and the supernatants were assayed for IL-6 by ELISA. Results are means ± SD of triplicate determinations of a representative experiment repeated two times.

Fig. 6 B). In contrast, BPI did not significantly block CD14-dependent stimulation of U373 cells by SACE (Fig. 6 A). The above experiments indicate the existence of stimulatory molecules in SACE that are functionally distinct from LPS but which may nevertheless use a CD14-dependent mechanism of cell stimulation.

SACE Components Bind rsCD14. The above studies indicate that SACE contains a strong activator of IL-6 production and that responses of both monocytes and U373 cells to this activator are enhanced by CD14. By analogy with LPS, we hypothesized that the active component of SACE may function by binding to rsCD14. We have previously shown that binding of LPS to rsCD14 causes a characteristic increase in the mobility of rsCD14 on native gels (8) (and see Fig. 7 A). Since LPS does not cause a change in the conformation of rsCD14 (36), we believe that the shift in migration is caused by the strong negative charge of LPS. Addition of SACE also caused an increase in electrophoretic mobility of rsCD14 (Fig. 7 A), suggesting that SACE binds directly to rsCD14. The mobility shift observed upon binding of SACE to rsCD14 was greater in magnitude than that seen with any LPS, smooth or rough, previously assayed (Fig. 7 A, and not shown). This finding suggests that SACE components bind rsCD14 and are distinct from LPS. In contrast with LPS, binding of SACE to rsCD14 was not accelerated by the addition of LBP (not shown), further confirming that SACE is distinct from LPS.

We also measured the ability of SACE to compete the
Figure 7. (A) SACE causes a shift in the electrophoretic mobility of rsCD14 in native PAGE. rsCD14 (30 μg/ml) was incubated alone (lane 1) or with 10 or 100 μg/ml of LPS (lanes 2 and 3) or SACE (lanes 4 and 5) overnight at 37°C in PD-EDTA (Dulbecco’s PBS lacking divalent cations, with 1 mM EDTA). The samples were run on a 4–12% native polyacrylamide gel and stained with silver. A shift in mobility of rsCD14 by both LPS and SACE was confirmed in parallel experiments in which the location of rsCD14 was determined by Western blotting (not shown). (B) SACE competes for binding of 3H-LPS to rsCD14. 3H-LPS (2 μg/ml) and rsCD14 (50 μg/ml) were incubated overnight at 37°C in PD-EDTA alone (lane 2) or with the indicated concentrations of Re LPS (lanes 3–5), SACE (lanes 6–8), or pure LTA (lanes 12–14). 3H-LPS was incubated without rsCD14 to show the electrophoretic pattern of free (aggregated) LPS (lane 1). The samples were run on an 8–16% native polyacrylamide gel and 3H-LPS was visualized by fluorography.

We confirmed that this peak is LTA by measuring equimolar glyceral and phosphate in base hydrolyzates of the fractions (Kusunoki, T., and S.D. Wright, unpublished observations). Purified LTA competitively displaced 3H-LPS from sCD14 (Fig. 7 B) and caused a shift in mobility of sCD14 (not shown), indicating that it binds to sCD14. The purified LTA, however, did not stimulate U373 cells either in the absence (Figs. 8 and 9) or the presence (Fig. 9) of sCD14. Similarly, purified LTA failed to stimulate IL-6 production in monocytes (not shown) or adhesion in PMN (see below). The inability of LTA to stimulate cytokine production is consistent with the data of Keller et al. (23) who showed that highly purified LTA from S. aureus was incapable of stimulating NO secretion from macrophages.

The biosynthetic precursor of LPS known as lipid IVa also binds to sCD14 (8) but does not stimulate cytokine synthesis in human cells. Rather, it acts as an LPS antagonist, inhibiting responses of cells to complete LPS (37). In a similar fashion, we observed that LTA caused a dose-dependent inhibition of LPS-stimulated, CD14-dependent IL-6 re-

Figure 8. Purification of the stimulatory components of SACE with C8-HPLC. SACE (14 mM phosphate X 100 μl) was applied to a reverse-phase HPLC C8 column and eluted with a 5–100% propanol gradient. 20 fractions were collected and assayed as described in Materials and Methods. For the IL-6 assay, each fraction was further diluted 100-fold with AIM-V-HSA medium and applied to U373 monolayers. Results are means of triplicate determinations of a representative experiment repeated three times.
Figure 9. CD14-dependent and -independent activities of fractions 1 and 12 from C8 HPLC. Fractions 1 and 12 from C8 HPLC were diluted to the indicated concentrations of phosphate in AIM-V-HSA medium and applied to U373 monolayers with or without rsCD14 (1 μg/mL). After a 24-h incubation, IL-6 in the supernatant was assayed by ELISA. Results are means ± SD of triplicate determinations of a representative experiment repeated three times.

Figure 10. Pure LTA antagonizes the activity LPS. LPS (1 μg/mL), sCD14 (10 μg/mL), LBP (1 μg/mL), and different concentrations of pure LTA were mixed and incubated at 37°C overnight. The mixtures were then diluted 100-fold and applied to U373 monolayers and further incubated for 24 h. The x-axis depicts the final concentration of pure LTA present with cells. The supernatants were assayed for IL-6 by ELISA. Results are means ± SD of triplicate determinations of a representative experiment repeated two times. The inhibitory effect of LTA is likely to be due to competition with LPS binding for sCD14 since the potency of LTA as an inhibitor was reduced ~100-fold if the preincubation step with sCD14 was omitted (data not shown).

Figure 11. rsCD141-152, but not rsCD14152-346, induces a dose-dependent cell-stimulatory activity of AF. Monolayers of U373 cells were incubated for 24 h with AF (400 nM phosphate) in the presence or absence of different concentrations of either rsCD14, rsCD141-152, or rsCD14152-346. The supernatant was assayed for IL-6 by ELISA. Results are means ± SD of replicate determinations of a representative experiment repeated two times.

lease from U373 cells (Fig. 10). This observation confirms that LTA does not stimulate cells and suggests that, by binding CD14, LTA may blunt responses to LPS. More importantly, it indicates that the active factor in SACE must be distinct from LTA.

Purification of the Active Factor in SACE. The chromatogram of SACE showed that all cell-stimulating capacity appeared in a single peak (Fig. 8, fraction f) that was well separated from LTA. This peak contained 20% of the total applied phosphate and caused both CD14-dependent and CD14-independent stimulation of U373 cells in a dose-dependent fashion (Fig. 9). Fraction 1 also bound sCD14 as shown by its ability to shift the mobility of sCD14 in native PAGE (not shown) and by its ability to block binding of 3H-LPS to sCD14 (Fig. 7, lanes 9–11). These results suggest that fraction 1 contains the biologically active species in SACE. Since this active fraction (AF) is distinct from LTA, we suggest that it may contain a novel gram-positive compound.

Approximately 20% of the original IL-6-inducing activity was recovered in fraction 1 as judged by dose-dependence data (not shown). We noted, however, that LTA effectively antagonized the action of AF (not shown), and thus any additional activity that may have eluted with LTA would not have been detected because of this antagonist activity.

AF Interacts with a Site in the Amino Terminus of CD14. Previous work has suggested that only the amino-terminal 152 residues of sCD14 are necessary for biological activity since residues 153–346 of rsCD14 (the carboxy-terminal two-thirds of the molecule) can be deleted without affecting its ability to mediate cellular responses to LPS (24). In a parallel fashion, we found that rsCD141-152 enabled responses of U373 cells to AF with concentration dependence similar to that of full-length rsCD14 (Fig. 11). These studies indicate that residues in the carboxy-terminal two-thirds of rsCD14 are not necessary for the interaction of AF with rsCD14. Additional recent studies have identified residues 57–64 as crucial to the function of sCD14 (25). Deletion of these residues leads to a mutant (CD14Δ57-64) that cannot mediate cellular responses to LPS, and in a parallel fashion, we found that rsCD14Δ57-64 did not enable responses of U373 cells to AF (Fig. 11). This finding indicates that AF requires residues 57–64 of CD14 to stimulate cells.

AF binds CD14 Complexes Stimulate Neutrophils. LPS causes a rapid, CD14-dependent enhancement of integrin-mediated PMN adhesion (8,11). Because adhesion is measured after exposure of PMN to LPS for only 10–20 min, LPS...
alone is ineffective and a response is only observed if LBP is added to speed movement of LPS into CD14 or if preformed LPS–sCD14 complexes are used (8). In a parallel fashion, we observed that AF alone did not stimulate PMN, but incubation of AF with sCD14 overnight to form AF–sCD14 complexes lead to strong stimulation (Fig. 12). To our knowledge, this is the first demonstration that gram-positive molecules may directly stimulate leukocyte integrin function in PMN. To determine if formation of AF–rsCD14 complexes was required for stimulation of PMN, AF and rsCD14 were mixed and added immediately to PMN, without time for binding of AF to rsCD14. Under these conditions, AF caused no PMN adhesion (Fig. 12). This observation suggests that binding of AF to sCD14 is a prerequisite for efficient PMN stimulation. We have observed in addition that pure LTA alone (not shown) or preincubated with rsCD14 (Fig. 12) failed to stimulate PMN adhesion, confirming that AF, not LTA, comprises the biologically active species in SACE.

Discussion

A large number of reports have described cytokine synthesis in response to bacterial cells or extracts (1–5, 13, 17–23, and see reference 38 for review), and several recent reports have implicated CD14 in these responses. Lipoprotein–mannnan from Mycobacterium tuberculosis stimulates TNF and IL-1 production from monocytes, and this response is blocked by anti-CD14 mAbs (39). Similarly, manuronic acid polymers from Pseudomonas (40), soluble peptidoglycan fragments from S. aureus (41), rhamnose-glucose polymers from Streptococcus mutans (42), chitosans from arthropods (43), and insoluble cell walls from several gram-positive species (44) have been shown to induce a cytokine response in monocytes, and all these responses can be blocked with anti-CD14 antibodies. The studies reported here confirm a role for CD14 in responses of monocytes, PMN, and U373 cells to S. aureus. Addition of sCD14 enhanced responses of U373 cells by up to 100-fold, and blockade of CD14 decreased sensitivity of monocytes by ∼1,000-fold. These studies suggest that the role of CD14 is to enhance the sensitivity of responses that can occur in the absence of CD14.

Additional experiments have suggested that stimulatory microbial substances may bind to CD14. Manuronic acid polymers compete with anti-CD14 for binding to monocytes (40), and sCD14 binds to insoluble walls of Streptococcus mitis (44). Our studies offer unequivocal evidence that molecules from S. aureus bind sCD14. They further show that binding to CD14 is necessary for enhancement of sensitivity to these molecules, and that residues 57–64 of CD14, previously defined as a binding site for LPS, are necessary for CD14 to enhance responses to S. aureus molecules.

Our studies extend previous work in that we have specifically sought the bacterial molecule that binds CD14 and stimulates cells. While prior authors have been meticulous in excluding LPS from their preparations, the whole bacteria, cell walls, or cell wall fragments used in past studies are inherently heterogeneous, and it is not possible to exclude a highly active subspecies as the source of the biological activity. We have observed that a minor component in extracts of S. aureus bears all of the cell-stimulating activity in the extract. This component has no optical density or other characteristic by which its presence could be detected. Therefore, it is possible that such a molecule may have been the source of the stimulatory activities reported for other bacterial preparations. In keeping with this hypothesis, Tsutsui et al. (45) and Kotani et al. (46) recently fractionated LTA from Enterococcus hirae and found cytokine-inducing activity only in the most lipophilic fractions. However, synthetic LTA molecules with the structure predicted from their studies did not have biological activity (47). It is thus clear that a minor, uncharacterized component of gram-positive bacteria may express the inflammatory activity. The precise structure of the active molecule is thus of considerable interest and is currently under study.

CD14 appears to serve in enhancing responses to an unexpectedly broad range of pathogens, and Pugin et al. (44) have suggested that CD14 may act as a “pattern recognition” receptor capable of binding and mediating responses to a broad range of bacterial molecules. Our results confirm a role for CD14 in binding and mediating responses to molecules other than LPS, but it is not clear that CD14 provides the specificity needed to discriminate LPS or AF from similar host molecules such as gangliosides or phospholipids. Indeed, CD14 binds inactive LTA analogues (8, 48), inactive LTA species (Fig. 5 B), and certain phospholipids (Hailman, E., and S. D. Wright, unpublished observations). Moreover, CD14 has been shown not to discriminate between LPS variants with opposite biological effects (49), and additional cellular molecules must contribute recognition specificity (48, 49). How could CD14 enhance responses to bacterial molecules without the recognition spec-

Figure 12. AF–rsCD14 complexes stimulate adhesion of PMN to fibrinogen. sCD14 (500 μg/ml) was incubated overnight at 37°C in PBS with AF (1.5 mM phosphate) ( ), pure LTA (1.5 mM phosphate) ( ), or LPS (5 μg/ml) ( ). As control, AF was preincubated without sCD14 ( ). The samples were diluted 50-, 500-, or 5,000-fold, added to PMN, and adhesion of PMN to fibrinogen was measured as described in Materials and Methods. To determine if overnight incubation of AF with rsCD14 was necessary to observe cell stimulation, AF was mixed with sCD14 and added immediately to the PMN for assay ( ).
ificity of IgG or the T cell receptor? We have recently shown that, by sequentially binding and releasing LPS, sCD14 serves to shuttle LPS from micelles into HDL particles (9) or lipid membranes (Wurfel, M.M., and S.D. Wright, manuscript in preparation). This type of shuttle action may thus concentrate LPS or AF in the membrane of a sensitive cell and facilitate recognition by additional cellular molecules. By concentrating a subset of bacterial molecules at the cell surface, CD14 may contribute both to the sensitivity and selectivity of cellular responses even in the absence of decisive recognition specificity. We have thus proposed that CD14 acts as a shuttle in the neutralization of lipopolysaccharide by LPS-binding protein and reconstituted high density lipoprotein. J. Exp. Med. 181:1743–1754.

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