Mechanism of Human Platelet Activation by Endotoxic Glycolipid-Bearing Mutant Re595 of Salmonella minnesota

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The mechanism through which human blood platelets interact with gram-negative bacteria with well-defined structural variations in endotoxic lipopolysaccharide was studied. Secretion of 14C-serotonin and aggregation of platelets separated from plasma proteins were observed on challenge with rough mutant Re595 of Salmonella minnesota possessing a glycolipid outer layer composed of Lipid A and 2-keto-3-deoxyoctonate (KDO) but lacking heptose phosphate in the core and O-polsaccharides in its outer portion. Both 14C-serotonin secretion and platelet aggregation were concentration-dependent, with a half-maximum response at the ratio of one bacterial colony-forming unit (CFU) to two platelets. The aggregation of human platelets induced by mutant Re595 was divalent cation-dependent and required secretion of ADP and fibrinogen from platelet storage granules because it was inhibited by chelators, by the ADP-splitting enzyme apyrase, and by monospecific antifibrinogen Fab fragments. The synthetic peptide analog of the platelet receptor recognition site on the γ chain of fibrinogen, γ 400–411, inhibited platelet aggregation induced by mutant Re595 (IC50 160 μmol/L), whereas serotonin secretion was unaffected. Tetrabeptide, RGDS, analogous to human fibrinogen α chain (α 572–575) and to the cell adhesion site of fibronectin, also inhibited aggregation induced by mutant Re595 (IC50 60 μmol/L). Secretion of 14C-serotonin was preceded by a very rapid phosphorylation of a platelet protein of mol wt 47,000, which is associated with protein kinase C.

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RAM-NEGATIVE bacteria have many cellular targets, including blood platelets, granulocytes, monocytes, lymphocytes, erythrocytes, and endothelial cells. Some of the effects of gram-negative bacteria contribute to the development of intravascular coagulation and septic shock, and it is estimated that ~71,000 patients per year in the United States develop gram-negative bacteremia, resulting in 18,000 deaths. Bacteremia due to endotoxin-producing gram-negative rods is frequently complicated by thrombocytopenia. Therefore, several attempts have been undertaken during the past years to establish in vitro evidence that human platelets interact with isolated endotoxic lipopolysaccharide. In contrast to rabbit platelets, which uniformly respond in vitro to isolated bacterial endotoxin, human platelets have shown varying responses. Although studies have shown that secretion of serotonin from human platelets was induced by endotoxic lipopolysaccharide extracted by Boivin’s method and copper salt-treated endotoxin caused aggregation of human platelets, other studies were unable to demonstrate an interaction between isolated endotoxin and human platelets. Most of the previous experiments with human platelets used endotoxic lipopolysaccharides extracted from gram-negative bacteria. These cell-free preparations may have different properties than the “native” form of cell-wall-bound endotoxic lipopolysaccharide. Moreover, it is unclear which part of endotoxic lipopolysaccharide is responsible for the interaction of gram-negative bacteria with human platelets.

Endotoxic lipopolysaccharide of Salmonella and other gram-negative bacteria is composed of complex polysaccharide subunits covalently linked to Lipid A. The polysaccharide is typically composed of two distinct parts, the core and the chains of O-polysaccharides. The core contains an unusual sugar, 2-keto-3-deoxyoctonate (KDO), in addition to N-acetylgalactosamine, glucose, galactose, heptose phosphate, and ethanolamine. Whereas O-polysaccharide subunits...
determine the immunologic specificity of endotoxin, Lipid A appears to be responsible for many of its biologic effects, most notably, pyrogenicity, lethal toxicity, mitogenic stimulation of lymphocytes, macrophage activation, Hageman factor and complement activation, and induction of Limulus amebocyte lysate gelation.12,13,15

The rough mutant Re595 of S minnesota is known to possess a glycolipid composed of Lipid A and KDO but to lack core oligosaccharides and O-poly saccharide side chains due to a deficiency of specific enzymes present in smooth organisms.14 The smooth strains of gram-negative bacteria undergo spontaneous transformation into polysaccharide-deficient rough variants in culture, especially when antibodies directed against somatic O antigens are present in the medium.16 A Lipid A-like substance reappears in the circulation 24 hours after injection of endotoxin into baboons, and significant amounts of material cross-reacting with Lipid A-antiserum appear in the urine.17 An endotoxin-like material has been detected by the Limulus amebocyte lysate assay in the serum of patients with gram-negative bacterial urinary tract infections whose blood cultures were negative.18 It is likely that these measurements detected bacterial cell wall fragments bearing Lipid A-containing material which may also be reactive with platelets. In our study, the whole cells of rough mutant Re595 were compared with the smooth organism, strain S218. These bacteria, with a well-defined endotoxin composition, allowed us to establish the structural requirements for endotoxin's interaction with human platelets and the pathways of activation of human platelets by a cell wall-associated glycolipid composed of a Lipid A–KDO complex.

MATERIALS AND METHODS

Preparation of human platelets separated from plasma proteins for secretion and aggregation studies. Blood was obtained from healthy volunteers who had not taken aspirin or any other medication for the preceding 8 days. Platelet-rich plasma was loaded with [3H]-serotonin (0.1 μCi/2mL, New England Nuclear, Boston) and platelets, labeled with [3H]-serotonin, were then separated from free amine and from plasma by combined albumin gradient centrifugation and Sepharose 2B gel filtration using modified Tyrode's buffer without phosphate and calcium, pH 7.35.18 Secretion of serotonin was measured in the presence of imipramine (1.5 μ g/mL) with continuous stirring at 900 rpm at 37 °C according to a previously described procedure,19 except that the reaction was stopped by chilling the platelets and adding 0.3% p-formaldehyde and 2 mmol/L of EDTA.20

Aggregation studies were done at 37 °C with stirring according to the method of Born,21 using a Payton Dual-Channel Aggregometer (Payton Association, Buffalo). The aggregometer baseline (10%) was set using Tyrode's buffer and bacterial suspension as described previously.19 Aggregation was measured using percentage of maximum transmission (Tmax) and slope value which represented the change in 1 minute along a tangent line to the steepest increase in light transmission.19

Preparation of anti-human fibrinogen Fab fragments. The antiseraum prepared from rabbits immunized with human fibrinogen (Kabi, Sweden) was passed through a fibrinogen-Sepharose 4B affinity column as previously described.22 The Fab fragments were prepared according to the procedure of Porter23 and purified as previously described.22

Preparation of synthetic peptides. Synthetic peptide analogs of the platelet receptor recognition site on human fibrinogen γ chain, NH2-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Asp-Val-COOH (γ 400–411), and of the cell adhesion site on fibronectin and the α chain of human fibrinogen, NH2-Arg-Gly-Asp-Ser-COOH (α 572–575)24 were prepared as previously described.25 Acetylation of the synthetic peptide γ 400–411 was done as described elsewhere.25

Phosphorylation of human platelet proteins. Phosphorylation of human platelet proteins was studied according to the method of Lyons and co-workers26 with the following modifications. Human platelets, separated from plasma proteins as above, were incubated with 32 PO4 (50 μCi/mL) for 60 minutes at room temperature without stirring. After removal of free 32 PO4 by Sepharose 2B gel filtration, the platelets were challenged with bacteria for various incubation times at 37 °C with stirring. Control samples were incubated without buffer. The incubation mixture was immediately placed in an ice bath to stop the reaction and spun down in a microcentrifuge. Platelet pellets were solubilized in 20 μL of 5% sodium dodecyl sulfate (SDS) in 0.15 mol/L of NaCl and an equal volume of Laemmli solubilizing solution27 and 2-mercaptoethanol (final concentration 10%). Samples were boiled for 2 minutes and applied to SDS-containing polyacrylamide gradient (5% to 15%) slab gels for electrophoresis according to the method of Laemmli.27 Molecular weight markers (BioRad, Richmond, Calif) were run simultaneously. The gels were stained with 0.25% Coomassie brilliant blue R, 45% methanol in 10% acetic acid, and subsequently destained using 7% methanol in 10% acetic acid. The gels were dried on BioRad filter paper, exposed to Kodak (Rochester, NY) X-Omat-AR film using a Cronex lighting plus intensifying screen (Dupont, Wilmington, Del), and developed in a Kodak RPX-Omat processor. The autoradiographs were scanned at 600 nm using a selecting spectrophotometer (Gifford, Oberlin, Ohio) equipped with gel scan accessories and software that allowed quantitation of the integrated area under the peak.28

Measurement of release of 14 C-arachidonic acid from platelet phospholipids. Release of arachidonic acid from platelet phospholipids was measured according to the method described by Bilis and colleagues.29 Twenty milliliters of platelets separated from plasma proteins, prepared as described above, were incubated at 37 °C for 2 minutes, and 60 μL (0.3 μCi) of 14 C-arachidonic acid was added. The sample was incubated at 37 °C for 1 hour without stirring. The reaction was stopped with EDTA, and unincorporated 14 C-arachidonic acid was removed by gel filtration. One-milliliter aliquots of 14 C-arachidonic acid-labeled platelets were incubated, either with 0.5 U thrombin or 200 μg of freeze-dried mutant Re595 of S minnesota or smooth strain S218 for 5 minutes with continuous stirring. Control samples were incubated with buffer. After incubation, 2.67 mL of EDTA (0.1 mol/L) was added to each tube, and subsamples were taken for radioactivity count. The radiolabeled phospholipids were extracted and chromatographed on a column (1 × 17 cm) containing 0.5 g of activated silicic acid on a glass wool plug.29 Release of arachidonic acid from platelet phospholipids was calculated as the difference between the radioactivity counts in control and test samples of platelet phospholipids.

Measurement of metabolites of 3H-arachidonic acid in human platelets by high-pressure liquid chromatography (HPLC). After platelets were labeled with 3H-arachidonic acid and separated from plasma proteins, they were incubated with S minnesota Re595 or with thrombin and analyzed for free arachidonic acid and its metabolites such as cyclooxygenase products (thromboxane B2, PGE2, PGD2, and PGF2α), 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12-L-hydroxy-5,8,10,14 eicosatetradecenic acid (HETE) by HPLC according to the procedure of Russell and Deykin.30 Results were expressed as 3H-arachidonic acid-derived radioactivity or as percentage of total radioactivity in the sample.
Measurement of lactate dehydrogenase leakage from human platelets. Lactate dehydrogenase (E.C.1.1.1.27) was measured according to Bergmeyer and Bernt. Results are expressed as percentage of total enzyme activity present in a platelet suspension lysed with 0.1% Triton X-100 (Sigma, St Louis).

Preparation of bacteria. The S218 strain of S minnesota and its rough mutant Re595 (kindly provided by Dr G. Schmidt, Max-Planck Institute for Immunobiology, Freiburg-Zähringer, FRG) were grown overnight in heart infusion broth. The cultures were centrifuged at 1,000 g for 15 minutes, the supernatant was discarded, and the bacteria were suspended in saline. The bacteria were incubated in a 60°C water bath for 30 minutes. These heat-killed bacteria were centrifuged at 1,200 g for 15 minutes, and the pellet was washed twice with saline and once with distilled water. Finally, bacteria were freeze-dried; fresh suspensions were prepared on the day of experiment. The viable count was determined in the bacterial suspension before it was heated at 60°C, and 1 mg of final freeze-dried bacterial preparations contained an equivalent of 10^6 colony-forming units (CFUs) of strain S218 and 3 x 10^6 CFUs of mutant Re595.

Hydrolysis and measurement of KDO associated with mutant Re595. Dry bacteria (50 mg, mutant Re595) were washed twice with 7 mL of 0.17 N acetic acid and suspended in 7 mL of 0.17 N acetic acid. The suspension was heated to 100°C in a boiling water bath for various times, up to 90 minutes. After centrifugation, the supernatant and the pellet were collected, the pellet was washed twice with distilled water, and then both the supernatant and the pellet were freeze-dried. The thiobarbituric acid method was used to measure KDO in the reconstituted supernatant and pellet.

Limulus gelation assay for endotoxic lipopolysaccharide. Cell wall endotoxic lipopolysaccharide in suspensions of S minnesota was measured by a modified method of Levin. The bacterial suspensions were diluted in pyrogen-free water to obtain a concentration of 5 µg/mL and then mixed 1:10 (vol/vol) with Limulus amebocyte lysate (0.5 EU/mL). Pyrogen-free water mixed 1:10 with Limulus amebocyte lysate was used as a reference. The reaction was measured in a Gilford Response spectrophotometer at the wavelength of 360 nm at room temperature for 60 minutes. The rate of reaction was measured as a slope value representing the change in absorbance in 1 minute by drawing a tangent line to the steepest part of the absorbance tracing.

Modifications of Lipid A in mutant Re595 of S minnesota. Alkaline hydrolysis of ester-linked fatty acids of Lipid A of mutant Re595 was done according to the method of Beckman. In brief, bacterial cells (20 mg/mL) were suspended in water to which an equal volume of 0.2 N NaOH was added. The suspension was incubated at 37°C for 30 minutes and centrifuged at 1,200 g for 10 minutes, and the pellet was washed twice with distilled water and lyophilized. Treatment of mutant Re595 with polymyxin B was done according to the method of Morrison and Jacobs. Polymyxin B sulfate, 15 mg, sterile powder (Burroughs Wellcome, Research Triangle Park, NC), was added to 2 mg S minnesota Re595 suspended in 1 mL of H2O, incubated at room temperature for 30 minutes, and dialyzed against H2O for 2 hours with frequent changes of H2O. Neutralization of mutant Re595 with Limulus amebocyte lysate (Pyrotell, 0.5 EU U/mL, Cape Cod Associates, Woods Hole, Mass) was done by mixing 50 µL of suspension of mutant Re595 (2 mg/mL) in Tyrode’s buffer, with 50 µL of Limulus amebocyte lysate for 10 minutes at room temperature without stirring. The bacteria were then washed once with Tyrode’s buffer, suspended in 50 µL of Tyrode’s buffer, and then added to platelets in the aggregometer.

Reagents. Acetylsalicylic acid (aspirin) and indomethacin USP were obtained from Merck, Sharp, and Dohme (West Point, Pa); hirudin from Sigma (St Louis); bovine thrombin (topical) from Parke Davis (Morris Plains, NJ); 1^4C-serotonin, 1^4C-arachidonic acid, and 1^H-arachidonic acid were purchased from New England Nuclear; and Limulus Ameboocyte Lysate (Pyrotell, 0.5 EU/mL) from Cape Cod Associates. Apysryase (grade I, Sigma) was purified and assayed according to the procedure of Traverso-Cori and co-workers.

**RESULTS**

Activation of human platelets induced by rough mutant Re595 but not by the smooth strain S218 of S minnesota.

On addition of gram-negative bacteria to a suspension of human platelets, which were free of plasma proteins, two patterns of interaction were observed. Rough mutant Re595 induced aggregation and secretion of 1^4C-serotonin, which reached 55% at 10 minutes. When the smooth strain S218 was added, no response from platelets was observed (Fig 1). Platelet aggregation and secretion of 1^4C-serotonin induced by mutant Re595 were concentration-dependent. The effective concentration of mutant Re595 that induced a half-maximal response (EC50) was 25 µg/mL, which roughly corresponds to one bacterial CFU per two platelets (Fig 2). Smooth strain S218 remained without measurable effect over a wide range of concentrations. Secretion of serotonin induced by mutant Re595 was not accompanied by lysis of platelets because lactate dehydrogenase, a cytoplasmic marker, was not significantly increased at the end of the incubation period (9.2% ± 1.5% control 4.8% ± 0.8%).

Role of platelet endogenous ADP and fibrinogen in aggregation induced by mutant Re595. The aggregating effect of mutant Re595 on human platelets separated from plasma...
proteins raised the question of whether this process was dependent on endogenous ADP, stored in platelet dense granules and secreted along with serotonin. Addition of the ADP-splitting enzyme apyrase inhibited aggregation of platelets in a concentration-dependent manner, thus indicating the role of secreted ADP in platelet aggregation (Fig 3). The participation of platelet fibrinogen secreted from α-granules in aggregation of platelets induced by Re595 was examined using anti-human fibrinogen Fab antibody fragments. Monovalent Fab fragments were previously shown to inhibit thrombin-induced aggregation of human platelets separated from plasma proteins. Anti-fibrinogen Fab caused concentration-dependent inhibition of platelet aggregation. At 40 μmol/L, Fab fragments inhibited platelet aggregation by 60% whereas secretion of 14C-serotonin remained unaffected (90% of control).

The role of platelet fibrinogen in aggregation of human platelets induced by mutant Re595 was substantiated further by using synthetic peptide analogs of fibrinogen sites present on the γ and α chains of human fibrinogen and involved in interaction with platelet receptors. As shown in Fig 4, the peptide analog of the human fibrinogen γ chain (γ 400–411) blocked platelet aggregation induced by mutant Re595 with 50% inhibition (IC50) at 160 μmol/L. The acetylated form of the same peptide, which is not inhibitory toward fibrinogen receptor on human platelets, had no effect on platelet aggregation (results not shown). The synthetic peptide analog of the site on the α chain of human fibrinogen (α 572–575) and of the cell adhesion site of fibronectin also inhibited platelet aggregation with IC50 of 60 μmol/L. Neither synthetic peptide inhibited secretion of serotonin induced by mutant Re595. These experiments indicate that platelet aggregation induced by mutant Re595 involves the interaction of platelet fibrinogen with receptors that are blocked by synthetic peptides known to inhibit binding of exogenous plasma fibrinogen.

Role of divalent cations. Addition of 5 mmol/L of EDTA or 5 mmol/L of EGTA to the incubation mixture of platelets and bacteria abolished the aggregation response of platelets, whereas secretion of 14C-serotonin was 39% and 43%, respectively, as compared with a control of 57% (Fig 5). When Mg2+ EGTA (5 mmol/L) was used to chelate calcium, while magnesium was maintained at the physiologic level, aggregation was reduced (Tmax 13 ± 40 in control; slope value 2 ± 9 in control) and secretion of 14C-serotonin was 33% ± control 59%.

Because the function of platelet membrane glycoproteins IIb and IIIa depends on calcium, its chelation for 1 hour at 37 °C leads to irreversible modification of the glycoprotein heterodimer. After incubation of platelets with 5 mmol/L of EDTA or 5 mmol/L of EGTA under such conditions, the aggregation response was abolished, whereas secretion of 14C-serotonin was only moderately reduced to levels similar to those observed in the previous experiments (37% with
chelators 47% in the control). Preincubation of mutant Re595 with the same chelators did not affect the mutant's ability to induce aggregation of human platelets and secretion of 4C-serotonin. These results indicate that only the aggregation response of human platelets induced with mutant Re595 is prevented by chelation of divalent cations, primarily calcium, and that secretion of serotonin is maintained even under conditions that lead to irreversible modification of the platelet membrane complex of glycoproteins Ib and IIa.

Phosphorylation of platelet proteins induced by mutant Re595. Stimulation of platelets includes protein kinase C activation manifested by phosphorylation of a 47-kd protein in platelets, myosin light chain kinase activation resulting in phosphorylation of the 20-kd light chain of myosin, and cyclooxygenase activity resulting in transformation of arachidonic acid into thromboxane A2 through prostaglandin endoperoxides (PGG2 and PGH2). These pathways were examined in human platelets after challenge with S minnesota. Mutant Re595 induced phosphorylation of a 47-kd protein with relatively more radiolabel incorporation than in a 20-kd protein (Fig 6). The phosphorylation of both 47-kd and 20-kd proteins was rapid, reaching a peak between 60 and 120 seconds, and was followed by dephosphorylation of both proteins. During this period, secretion of 4C-serotonin was continuously increasing even at 15 minutes (Fig 7). Under the same experimental conditions, the smooth strain S218 of S minnesota did not induce phosphorylation of the 47-kd and 20-kd proteins above the level observed in control preparation with buffer.

Prostaglandin pathway stimulation by mutant Re595. When the involvement of the prostaglandin pathway was examined, we observed that mutant Re595 induced release of 7.7% ± 1.4 (SEM) of 4C-arachidonic acid from the prelabeled platelet phospholipid pool, whereas the smooth strain S218 caused 1.3% ± 0.4% release and control (buffer) 0.9% ± 0.2%. In subsequent experiments using HPLC for measurement of radiolabeled arachidonic acid metabolites, mutant Re595 induced release of 9% of total 3H-arachidonic acid incorporated into the platelet phospholipid pool. Release of 3H-arachidonic acid was time-dependent and was paralleled by secretion of 4C-serotonin. The arachidonic acid released from the platelet phospholipid pool constituted 53% of the total pool of released radiolabeled products. Among the remaining metabolites of 3H-arachidonic acid, products of cyclooxygenase activity (thromboxane B2, PGD2, PGE2, PGF2\alpha, and HHT) constituted 20%, malonyldialdehyde constituted 5%, products of lipoxygenase activity (HETE) constituted 10%, and an unidentified radioactive fraction eluting after the arachidonic acid peak constituted 12%. This pattern of distribution of 3H-arachidonic acid metabolites was similar to that induced by thrombin (0.5 U/mL) except that half as much HETE production was observed in platelets stimulated with mutant Re595.

When acetylsalicylic acid (10\(^{-4}\) mol/L) was added to platelets prior to challenge with mutant Re595, generation of cyclooxygenase products and malonyldialdehyde was completely suppressed. This inhibition was paralleled by the inhibition of 4C-serotonin secretion. Another nonsteroidal antiinflammatory agent, indomethacin (5 \(\times\) 10\(^{-4}\) mol/L), also reduced secretion of 4C-serotonin. Hirudin (2 U/mL), while completely blocking the thrombin-induced activation of platelets, remained without effect on changes induced by mutant Re595.

Involvement of core glycolipid in the activation of human platelets by mutant Re595. Because the structure of "incomplete" lipopolysaccharide in mutant Re595 is well established as being composed of Lipid A and KDO,\(^1\) experiments were designed to examine the role of these constituents of glycolipid in its interaction with human platelets. Lipid A is
known to form complexes with an extract of amebocytes obtained from the horseshoe crab, *Limulus polyphemus.* As shown in Table 1, mutant Re595 complexed with Limulus extract lost its proaggregatory activity toward human platelets, indicating that cell wall-bound Lipid A in mutant Re595 is involved in its interaction with the platelet membrane. Likewise, secretion of $^{14}$C-serotonin was inhibited. Furthermore, polymyxin B, another inhibitor known to form a complex with Lipid A and inhibit its toxic effect, decreased the aggregation of human platelets and secretion of $^{14}$C-serotonin by mutant Re595 (Table 1).

Mild acid hydrolysis used to modify the glycolipid markedly reduced the ability of mutant Re595 to induce secretion of serotonin and aggregation (Fig 8A and B). This was accompanied by a decrease in the concentration of KDO associated with bacteria as measured by the thiobarbituric acid assay. A corresponding increase of free KDO in the supernatant during acid hydrolysis of mutant Re595 was observed. Alkaline hydrolysis of ester-linked fatty acids in Lipid A of mutant Re595 also abolished its platelet activating effect (results not shown).

**Inhibitory effect of oligosaccharides.** Whereas the rough mutant *S. minnesota* Re595 induced secretion of $^{14}$C-serotonin and aggregation of human platelets, the smooth strain *S. minnesota* S218 was inactive (Figs 1 and 2). Because the main difference between these two strains involved the core oligosaccharide and O-poly saccharide side chains, this portion of the lipopolysaccharide structure was removed from strain S218 by acid hydrolysis. The obtained oligosaccharide fraction, when added to platelets that were subsequently challenged with mutant Re595, inhibited platelet aggregation. As shown in Fig 9, this effect was concentration-dependent, and 50% inhibition of platelet aggregation was observed at 18 μg/mL of the oligosaccharide fraction. Secretion of $^{14}$C-serotonin induced by Re595 was also inhibited by the oligosaccharide fraction but at a higher concentration (50% inhibition was observed at 240 μg/mL). Similarly, the oligosaccharide fraction obtained from isolated lipopolysaccharide *S. minnesota* 9700 (Difco) inhibited platelet aggregation and secretion of $^{14}$C-serotonin induced by mutant Re595 (results not shown). Thus, oligosaccharides derived from complete endotoxic lipopolysaccharides are capable of interfering with cell wall-bound glycolipid reactivity toward human platelets.

**DISCUSSION**

Endotoxic lipopolysaccharide is known to have multiple interactions with the complement system, cross-reactive Re antibodies, the kinin system, and high-density lipoproteins. Therefore, our experiments were done in a plasma-free system in order to dissect the direct effects of gram-negative bacteria on the platelet membrane. Mutant Re595 was an effective inducer of platelet secretion and aggregation, which required fibrinogen secreted from platelet α-granules because anti-fibrinogen Fab antibody fragments inhibited platelet aggregation. Fibrinogen secreted from platelet α-granules or present in plasma is bound to specific platelet receptors exposed on the membrane of activated platelets, thereby linking two or more platelets together to form an aggregate. The exposure of receptors for fibrinogen by mutant Re595 required ADP since apyrase prevented platelet aggregation. Blockade of fibrinogen receptors with synthetic peptide analog corresponding to the sequence of γ chain 400–411 and with the synthetic peptide analog corresponding to the sequence of α chain 572–575 of human fibrinogen and the cell adhesion site of fibronectin prevented platelet aggregation without affecting secretion of serotonin. This indicates that fibrinogen secreted from platelet α-granules interacts with the platelet membrane receptor for fibrinogen during aggregation induced by mutant Re595.

Our results indicate that activation of human platelets by
rough mutant Re595 of S minnesota involves stimulatory pathways that are associated with protein kinase C, myosin light chain kinase, and cyclooxygenase, and resembles the pattern of response evoked by collagen.48 A hitherto unrecognized effect of gram-negative bacteria on human platelets is phosphorylation of the 47-kd protein. This effect suggests that endotoxic glycolipid activates platelet protein kinase C, an enzyme stimulated by phorbol ester, a known activator of that endotoxic glycolipid activates platelet protein kinase C, phosphorylation of the 47-kd protein. This effect suggests a pattern of response evoked by collagen.48 A hitherto unrecognized effect of gram-negative bacteria on human platelets is phosphorylation of the 47-kd protein. This effect suggests that endotoxic glycolipid activates platelet protein kinase C, an enzyme stimulated by phorbol ester, a known activator of platelets.49-51 Recently, activation of protein kinase C in a murine macrophage-like cell line by Lipid A, or its precursor Lipid X, was reported.52

The interaction of mutant Re595 with human platelets results in generation of free arachidonic acid from platelet membrane phospholipids. Conversion of newly generated arachidonic acid into endoperoxides and thromboxane A2 is essential for the functional effects of mutant Re595 because cyclooxygenase inhibitors (aspirin and indomethacin) block secretion of 14C-serotonin by mutant Re595. Apparently, stimulation of protein kinase C and myosin light chain kinase was not sufficient to cause secretion of 14C-serotonin when the cyclooxygenase pathway was blocked. In such a situation, aspirin does not appear to prevent phosphorylation of the 47-kd protein (J. Grabarek, S. Timmons, and J. Hawiger, unpublished observations). Whether dephosphorylation of 47-kd and 20-kd proteins was due to the activation of a phosphatase(s) remains to be determined. The time course indicates that progressive secretion of 14C-serotonin in this system was accompanied by dephosphorylation of 47-kd protein.

The structural integrity of cell wall-bound glycolipid is required for its activating effect on platelets. Acid hydrolysis results in a loss of reactivity toward platelets. Although KDO is dissociated from Lipid A under such conditions,52 other changes induced during acid hydrolysis cannot be discounted. Alkaline hydrolysis cleaves ester-linked lauric, palmitic, and myristic-myristic acids in Lipid A,54 resulting in the loss of reactivity of mutant Re595 toward platelets.

Previously, attempts to demonstrate aggregation of human platelets by lipopolysaccharide preparations extracted from various gram-negative bacteria produced divergent results.9,12,53,54 Our results indicate that association with the cell wall, or part of it, endows the glycolipid with the ability to exert a functional effect on the human platelet membrane. We observed an analogous phenomenon when protein A-bearing staphylococci were shown to interact with human platelets through the IgG Fc receptor to cause their aggregation, whereas isolated protein A (in fluid phase containing IgG) did not.12 Furthermore, Ginsberg and Hen-son15 observed that lipopolysaccharide extracted from mutant Re595 did not induce secretion of serotonin from washed human platelets. It significantly enhanced serotonin

Fig 9. Inhibitory effect of oligosaccharide fraction isolated from smooth strain S218 of Salmonella minnesota on platelet aggregation (O-O) and 14C-serotonin secretion (■-■) induced by rough mutant Re595. Oligosaccharide fraction obtained after acid hydrolysis of smooth strain S218 was added to human platelets (2 x 10^8/mL) challenged with rough mutant Re595 (100 µg/mL). Other conditions as in Fig 1. Cumulative data from two experiments.

Fig 8. Loss of cell wall-bound 2-keto-3-deoxyoctonate (KDO) from mutant Re595 during acid hydrolysis and decrease in its reactivity toward Limulus amebocyte lysate and toward human platelets. Mutant Re595 was subjected to acid hydrolysis for 90 minutes, during which time samples were obtained and centrifuged to determine residual KDO content of bacterial pellet, free KDO content in supernatant and reactivity of bacterial pellet toward Limulus amebocyte lysate and human platelets. Panel A: KDO content in mutant Re595 (O-O) and in free supernatant (■-■) and reactivity toward platelets as measured by secretion of 14C-serotonin (■-■). The content of KDO is expressed in microgram per milligram of dry weight of Re595 subjected to acid hydrolysis. Panel B: reactivity of mutant Re595 toward human platelets as measured by their aggregation (O-O) and 14C-serotonin secretion (■-■). Data represent one of two experiments.
secretion when combined with heat-aggregated immunoglobulin G or with immune complexes, however. The smooth strain S218 of S. minnesota with a complete core oligosaccharide structure and with O-polysaccharide side chains did not induce human platelet aggregation in these studies, although it possesses a cell wall-bound glycolipid. Oligosaccharide fraction isolated from strain S218 and from its lipopolysaccharide was inhibitory toward platelet aggregation induced by mutant Re595. Assuming that the mol wt of the oligosaccharide is ~3,500, the IC₅₀ for aggregation and secretion were 5 μmol/L and 69 μmol/L, respectively. This range of concentration is 2 to 3 orders of magnitude lower than the concentration of amino sugars reported to inhibit platelet aggregation induced by other agonists.

Saba and colleagues showed that complete endotoxic lipopolysaccharides inhibit aggregation of human platelets treated with a number of agonists with the exception of ristocetin-induced agglutination. It is apparent from the data presented in this and the preceding reports that gram-negative bacteria bear in their endotoxic lipopolysaccharide a structure that is responsible for their interaction with the human platelet membrane to induce secretion and aggregation, as exemplified by mutant Re595. Whether this structure, comprising Lipid A and KDO, is operational in vivo in patients with gram-negative bacteremia awaits direct evidence. Specific antibody against Lipid A can gain access to neutralize its toxic effects and opsonize Escherichia coli 0111 during in vivo phagocytosis. Higher titers of cross-reacting Re antibodies against rough mutants of gram-negative bacteria, such as mutant Re595 used in our study, appeared to protect patients from hemodynamic and hematologic derangements complicating gram-negative bacteremia.

In conclusion, a mutant of gram-negative bacteria, possessing cell wall-bound endotoxic glycolipid composed of Lipid A and KDO, is capable of activating human platelets through three mechanisms involving cyclooxygenase, protein kinase C, and myosin light chain kinase. The secretory response of human platelets leads to their aggregation, which can proceed in the absence of plasma fibrogen. This new attribute of endotoxic glycolipid associated with the cell wall of S. minnesota Re595 provides a useful model for further study of the structure–function relationship of the proaggregatory effect of endotoxic glycolipid on human platelets and the inhibitory effect of the adjoining oligosaccharides and O-polysaccharide side chains.

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