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Endotoxin-Binding Substances from Human Leukocytes and Platelets

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We have found whole human platelets, granulocytes, and mononuclear leukocytes to possess high affinity for the toxic lipopolysaccharide from all gram-negative bacteria tested. We have extracted these cells and platelets with *n*-butanol–water; all endotoxin-binding activity resided in the organic phase. These endotoxin-binding extracts did not block serologically active groupings on endotoxins or receptors on the erythrocytes. The specificity of these still crude materials was less than that of the highly purified erythrocyte lipopolysaccharide receptor previously described by us, since they bound some bacterial antigens not related to endotoxins. Depending on source, the *n*-butanol extracts contained 40 to 52% glycerophosphatides (most active), 15 to 22% sphingomyelin, 17% cholesterol, <2 to 5% triglycerides, and 7 to 13% inactive peptide. The most active substances in the *n*-butanol extract were soluble in petroleum ether, whereas the peptide and sphingomyelin were not. Thus, no constituent protein, carbohydrate, or nucleic acid was present in the most highly active material. Polyacrylamide gel electrophoresis of the petroleum ether-soluble material showed for each extract one lipid band only, which was well defined and migrated similarly to phosphatidyllipids. Because of the lipidic nature of the inhibitory substances from leukocytes and platelets we also tested the lipid A component of bacterial endotoxins and some of its derivatives. Lipid A inhibited endotoxin coating of erythrocytes. De-*O*-acylation of lipid A left amide-linked 3-D-hydroxymyristic acid intact and increased the inhibitory activity of lipid A 20-fold. Complete de-*O*- and de-*N*-acylation destroyed its inhibitory effect.

Man is highly sensitive to bacterial endotoxin (lipopolysaccharide [LPS]) which is thought to be responsible for the high mortality rate from systemic gram-negative infections despite antibiotic treatment (8, 17, 37). Before LPS can begin its noxious action, it has to attach to tissue-bound or soluble receptors. Erythrocytes fix LPS of gram-negative bacteria to their surfaces in vitro (21) and under extreme conditions in vivo (2, 29). They may transport LPS to reticuloendothelium-rich organs for detoxification. We have isolated a physicochemically homogeneous lipoglycoprotein (LPS receptor) from human erythrocyte membranes which reversibly bound LPS as well as bacterial protein-LPS complexes from all gram-negative bacteria but none of the other bacterial antigens tested (27, 28, 31). Only the protein component of this receptor was involved in LPS fixation (27). Other cellular blood elements and tissue components may play at least as important a role in LPS fixation as erythrocytes. Thus, it was reported that platelets of patients with sepsis due to gram-negative bacteria contained LPS (3), and complement has been strongly implicated in mediation of LPS toxicity (cf. 19).

We report here that human granulocytes, mononuclear leukocytes, and platelets all possessed LPS-binding structures on their surfaces as well as intracellularly. We demonstrated that in contrast to the erythrocyte LPS receptor, the LPS-binding activity of leukocytes and platelets was confined to their lipid moiety and predominantly to the phospholipids.

Because of the lipidic nature and the apparent importance of hydrophobic binding sites of the active material of the leukocytes and platelets, we also determined the LPS-binding effect of lipid A from gram-negative bacteria and some of its derivatives.

(A preliminary report of this work was given at the Annual Meeting of the American Society of Biological Chemists, 14 April 1975.)

MATERIALS AND METHODS

Human blood was collected into citrate-phosphate-dextrose anticoagulant solution USP (63 ml/pint; ca. 134 ml/liter), and single units were used

within 24 h. Procedures and yields are given per pint (ca. 0.47 liter) of blood. White cells and platelets were isolated in siliconized glassware or in plastic tubes. All initial steps were performed at 23 to 25 C.

Plasma contains substances which inhibit coating of erythrocytes by endotoxin, so far as investigated, unspecifically (21, 29, 35). In the assessment of inhibitory activity of any cellular blood element it must be considered that active plasma substances may be adsorbed onto leukocytes and platelets. We therefore suspended them after separation on a Macrodex-hypaque gradient (see below) with a Vortex mixer, and washed the cellular elements with 100 volumes of 0.15 M aqueous NaCl (saline) at 5 C. Granulocytes and mononuclear leukocytes were first sedimented twice at 600 × *g* and platelets at 5,000 × *g* in 100 volumes of saline; this was followed by one wash with 2 volumes of saline. This procedure was repeated for a total of 12 washes. Throughout, all leukocytes retained unchanged inhibitory activity which was not increased when exhaustively washed granulocytes were exposed to 50 volumes of plasma followed by three more washings.

The washes of the mononuclear cells and granulocytes usually exhibited no activity in the second 2-volume wash. Platelets contrasted; even after the 12th wash a 1:5 dilution of the 2-volume wash inhibited coating of erythrocytes by *Escherichia coli* O_{86} LPS to >95%; platelets therefore released active material continuously.

To eliminate as much inhibitory substance, presumably adsorbed from plasma, as feasible, all highly purified leukocytes and platelets were washed and carefully resuspended three to four times in 30 to 50 volumes of Hanks solution at 5 C before testing or extraction. The granulocytes, mononuclear leukocytes, and platelets were at least 95% pure, i.e., free of other blood cell elements, as judged by phase microscopy and ordinary light microscopy of Wright-stained slides.

Extraction of leukocytes. The highly purified leukocyte preparations retained their activity on desiccation. They were therefore dried to constant weight at ca. 10^{-2} mm over P_2O_5 and paraffin and extracted at 1% dry weight with *n*-butanol–water (1:1) at 4 C for 16 h at pH 8.0 as described for the isolation of human erythrocyte membrane LPS receptor (28), except that initial blending of the two phases was by vortexing and saline was used instead of water. Four phases resulted after centrifugation at 2,000 × *g* for 30 min: organic, intermediate, aqueous, and a pellet. The aqueous phases and the pellet were twice reextracted with *n*-butanol. All phases were thoroughly dialyzed, and for chemical analyses they were electrodialyzed as described earlier (32) and dried to constant weight.

For further purification the active *n*-butanol extract was dissolved in redistilled petroleum ether (boiling point 30 to 60 C), 1 ml/10 mg of dried extract. The mixture was centrifuged at 2,000 × *g* for 15 min. The supernatant was taken off, and the sediment was extracted once more. The petroleum ether was then removed from the supernatants with a stream of nitrogen, and supernates and residues were dried to constant weight over paraffin and P_2O_5.

Granulocytes. One-half volume of 3% dextran T 250 (Pharmacia, Piscataway, N.J.) in saline was mixed with 1 volume of blood and settled for 40 min in a graduate cylinder (6 by 43 cm) at 23 to 25 C, and the erythrocytes were removed. The leukocytes (ca. 6×10^9) were sedimented for 8 min at 100 × *g*, washed once in 40 ml of saline, and resuspended in 40 ml of saline. The leukocytes were allowed to settle for 90 min and erythrocytes remaining in the supernate were taken off; this process was repeated twice. The white cells were collected and washed twice with 40 ml of saline. The granulocytes were separated from other cells and platelets by suspension in 50 ml of cold Hanks solution and fractionation on 100 ml of a discontinuous double Macrodex-hypaque gradient as described by Tellez and Rubinstein (33). Granulocytes were obtained in yields up to 3×10^9 cells (ca. 400 mg dry weight).

Mononuclear leukocytes. Lymphocytes together with monocytes were isolated from 500 ml of platelet-depleted blood by a procedure similar to that of Fotino et al. (4) by centrifugation at 650 × *g* on 65 ml of 9% Ficoll-33.9% Isopaque (12:5). They were obtained in virtually pure form on 100 ml of the discontinuous Macrodex-hypaque gradient, suspended, washed, and dried. The yield of mononuclear cells was up to 80 mg (dry weight), corresponding to ca. 6.3×10^8 cells.

Platelets. Platelet concentrates were prepared by two centrifugations, first for 4 min at 4,470 × *g* to sediment the erythrocytes and then for 10 min, also at 4,470 × *g*, to concentrate the platelets. A portion (40 ml) of the concentrate was separated from plasma and cells on 100 ml of the discontinuous Macrodex-hypaque gradient, suspended in 50 ml of saline, washed three times, and dried. Up to 1.4×10^{11} essentially pure platelets (ca. 180 mg dry weight) were obtained.

Bacterial antigens, antisera, lipids and their preparation for assays. The antigens from gram-negative and gram-positive bacteria and the corresponding antisera have been described previously (31). Isolated O antigens from the following bacteria were used: *Shigella flexneri* and *Salmonella typhosa* (Difco, Detroit, Mich.); *E. coli* O_{111}, *S. friedenau*, *S. godesberg*, *S. milwaukee*, and *S. minnesota*, smooth and rough forms, from O. Lüderitz; *E. coli* K-235 (Abbott Laboratories, North Chicago, Ill.); *E. coli* O_8, donated by A. Nowotny; and *E. coli* O_{86} and O_{128}, prepared by us (29). A stearoyl derivative of *Streptococcus pyogenes* group A antigen was given by H. D. Slade (26). Vi antigen from *Paracolobactrum ballerup* was from E. Baker. In addition, *S. sonnei* phase I and II LPS (16) and their corresponding antisera were donated by E. Romanowska. All antigens were tested with homologous rabbit antisera except that from *E. coli* O_{86}, which was assessed with heterologous human anti-blood group B serum (31). Common (Kunin) antigen (12) from gram-negative bacteria assessed with rabbit anti-*E. coli* O_7 serum and common (Rantz) antigen (22) from gram-positive bacteria assessed with rabbit anti-*Staphylococcus aureus* serum were given by E. Neter. 2-Keto-3-deoxy-

octonate (KDO)-lipid A and lipid A isolated from *S. minnesota* R595 and some of their derivatives were obtained from E. T. Rietschel (23) and employed in alkali-activated form. Goat anti-lipid A globulin was donated by C. Galanos.

Cholesterol esters and fatty acids were purchased from Applied Science Laboratories (State College, Pa.), crystalline L-α-glycerophosphocholine and sphingomyelin from Mann Research Laboratory, (New York, N.Y.), triglycerides, phosphorylethanolamine, and ethanolamine from Sigma Chemical Co. (St. Louis, Mo.), phosphatidylethanolamine and phosphatidyl-L-serine from General Biochemical Laboratory (Chagrin Falls, Ohio), cholesterol from Eastman (Rochester, N.Y.), D,L-glyceric acid from Pierce Chemicals (Rockford, Ill.), and lecithin from Calbiochem (Palo Alto, Calif.).

Immediately prior to use for coating of erythrocytes only LPS's from gram-negative bacteria were heated in a boiling water bath in 0.1 M aqueous NaCl plus 0.05 M sodium phosphate, pH 7.4 (buffered saline), for 3 h, cooled, and centrifuged at 2,000 × *g* for 10 min to remove insoluble material.

All phases of the leukocyte and platelet extracts were tested. All aqueous and butanol phases except the pellets dissolved as opalescent solutions in buffered saline, to at least 0.1% dry weight, as did also the petroleum ether extract. To dissolve the leukocyte pellets and some commercial lipids which served as controls, they were taken up individually in a small quantity of methanol-ether (1:1), saline was added while vortexing, and the organic solvents were removed by heating under a stream of nitrogen. Fine suspensions of each resulted.

KDO-lipid A and derivatives were soluble >90% at concentrations of 0.05% in buffered saline after vortexing and boiling. Traces of insoluble material were removed by sedimentation at 2,000 × *g* immediately before experimentation.

Erythrocytes, coating, coating inhibition, blocking tests, and titrations. Erythrocytes from blood group O adults were obtained and used in the coating and coating inhibition assays which were carried out as described earlier (31). Briefly, 1 volume (0.05 ml) of washed packed erythrocytes was coated by the addition of 9 volumes of an optimal dose of LPS (i.e., the smallest amount of antigen affording a hemagglutination titer of 1:128; see [31]). The tubes were agitated in a rotary water bath at 37 C for 45 min and frequently inverted by hand. The red cells were then sedimented at 1,200 × *g* and washed three times with 50 volumes of saline before assay for serological activity with their corresponding antiserum.

The procedure in the coating inhibition assay differed from that in the coating test in that the desired amount of inhibitor was added to 1 optimal coating dose of LPS and incubated in a total volume of 0.45 ml for 45 min at 37 C. Thereafter erythrocyte addition and coating procedure were as above. In each inhibition test an ordinary coating assay using the antigen to be inhibited was included as standard.

Activities were assessed as previously described (28, 31) by hemagglutination assays. The smallest quantity (weight basis) of a given preparation which completely inhibited hemagglutination under conditions employing 1 coating dose of antigen was defined as 1 inhibitory unit. Substances with <2% of the inhibitory activity of a homogeneous erythrocyte LPS receptor preparation were considered to be inactive.

We found these serological procedures to be superior in speed and economy for screening and as a guide for the purification steps as compared to the strictly quantitative assays with radioactively labeled LPS which are employed once the active material is homogeneous (27, 28). The specificity of the serological procedures was monitored by the following controls. (i) The possibility of blocking LPS receptors located on the surface of erythrocytes was determined by preincubation of inhibitors with erythrocytes as described previously (31). (ii) Whether receptor-active preparations reacted with the LPS by blocking antibody-combining sites was assessed by standard hemagglutination inhibition tests. *E. coli* O_{86} LPS was exposed to receptor-active leukocyte extracts for 1 h, the mixture was titrated, and decrease in blood group or *E. coli* O_{86} activities was determined by comparison with LPS not preincubated with receptor-active substances (32).

Physical and chemical determination. The studies were restricted to the *n*-butanol phase and its petroleum ether-soluble substances, since these extracts had >95% of the total activity. The dry matter in the *n*-butanol phase amounted to ca. 35% for platelets, 26% for granulocytes, and 17% for mononuclear cells of the total dry weight of the leukocyte elements. Spectrophotometric measurements were with a Beckman DU instrument with a Gilford attachment and fluorometric determinations with a Turner model no. 111. Polyacrylamide gel electrophoresis was similar to that described by Segrest and Jackson (24) in a Canalco model 1200 apparatus on 12.5% polyacrylamide gel cross-linked with 0.5% *N,N*-methylenebisacrylamide; polyacrylamide gel electrophoresis was for homogeneity determinations rather than those of molecular weight. The "dissociating" buffer consisted of 0.1 M sodium phosphate, pH 7.1, 1.0% sodium dodecyl sulfate, 2.5% 2-mercaptoethanol, and 5 M urea; the "nondissociating" buffer differed in that the 0.1 M sodium phosphate contained only 0.1% sodium dodecyl sulfate to assure mobility and to prevent aggregation (34). Preincubation was 2 h at 37 C. Chymotrypsinogen A, ribonuclease A, insulin, glucagon, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin served as standards. Strains used were Coomassie blue (24) and Sudan black B (1), after migration at 5 mA and 3 to 4 V/gel for 4 h at 25 C.

Methods for the determination of P, N, and carbohydrates were as described previously (27). Protein was measured as by Lowry et al. (13). Sphingosine was determined as by Kisic and Rapport (11), phosphatide glycerol according to Mendelsohn and Antonis (18), triglycerides as described by Kessler and Lederer (10), and total cholesterol as by Wybenga et al. (36). Fatty acids were determined as methyl esters by gas-liquid chromatography (25). The samples were electrodialyzed to remove free fatty acids, dried, and hydrolyzed at 0.1% concentration in 1.0 ml of anhydrous 1 N methanol-hydrochloride with an "instant methanolic HCl kit" (Allied Science Lab-

oratories) at 80 C for 16 h, dried, and taken up in 1.0 ml of chloroform which was washed twice with 0.1 volume of water to remove possibly interfering materials. The preparations were redried and trimethylsilylated with 0.05 ml of Sil prep and incubated at 23 to 25 C for 15 min as described by the manufacturer (Applied Science Laboratories). A Perkin-Elmer F-30 instrument with a flame ionization detector was programmed at 100 to 220 C at 1 C/min. The glass column (180 by 0.625 cm) contained either 3% SE-30 or silicone ASI 50 phenyl 50 cyanopropyl on 100- to 120-mesh GasChrom Q. C_8- to C_{26}-saturated, monounsaturated, and hydroxy fatty acids served as standards. Free ethanolamine was determined with ninhydrin after high-voltage paper electrophoresis as described by Lugowski and Romanowska (16).

Pronase was grade B protease from Calbiochem (La Jolla, Calif.); 4.0 U/400 μg of substrate were used. Digestion and enzyme controls were described previously (32). The preservative, however, was 0.1% sodium azide. A crystallized subtilopeptidase from Sigma Chemical Co. was also used at 0.05 U/400 μg of substrate. Incubation with enzymes was for 36 h, and termination was by autoclaving at 121 C for 10 min.

RESULTS

General and physical properties. Freshly washed intact leukocytes and platelets were as active as desiccated ones on a dry weight basis (0.1 ml of packed fresh cells had a dry weight of ca. 14 mg). The activity resided in the most lipophilic fraction of the leukocytes, the *n*-butanol extracts, which were obtained as white fluffy powders. The dry preparations retained full inhibitory activity at 2 C for at least 3 months and in solution for 3 days. Autoclaving at pH 7.0 and 121 C for 10 min did not affect activity. Electrodialyzed, desiccator-dried substances lost between 4.5 and 9.5% water when dried to constant weight at 80 C; they possessed 8.5 to 10.0% wet ash. The preparations showed no nucleic acid peak but a steady decline in the absorption range between 250 and 300 nm; the mononuclear cell material had a faint shoulder between 275 and 291 nm. Polyacrylamide gel electrophoresis revealed for each active *n*-butanol extract one band which stained with Sudan black B and one which reacted with Coomassie blue. These bands did not overlap for any of the preparations in either dissociating or nondissociating buffer. The bands were sharper in the dissociating buffer where the front edges of the lipid bands migrated like peptides with a molecular weight of 5,800 for platelets and mononuclear leukocytes and 7,400 for granulocytes. Glycerophosphatides and sphingomyelin, included as controls, migrated like peptides with a molecular weight of 5,000 to 6,000. The corresponding mobilities of the bands reacting with protein stain were like those of peptides with a molecular weight of 10,600 for platelets and mononuclear leukocytes and 8,500 for granulocytes. The peptide bands were missing in the petroleum ether extracts of *n*-butanol phases, whereas the lipid bands showed no apparent change (see below). Petroleum ether-insoluble material amounted to <25% of dry weight and <10% of the activity of the *n*-butanol extracts.

Chemical properties. The active *n*-butanol extracts from granulocytes, mononuclear leukocytes, and platelets had 2.4 to 2.6% P and 1.40 to 1.55% N, corrected for water. Analytical chemical data are listed in Table 1. Granulocyte and platelet receptor-active material had 7.5 to 7.8% sphingosine base, and the mononuclear cell preparations had ca. 5.8%. This corresponds to ca. 21 to 22% and 15 to 16% sphingomyelin, respectively (Table 1). After deduction of the glycerol in triglycerides, that in the glycerophosphatides amounted to 5.6, 4.4, and 4.6% in these leukocyte extracts. This allows for a glycerophosphatide content of 51.8% in granulocytes, 40.1% in platelets and 40.8% in mononuclear cell extracts, based on the chain lengths for the fatty acids described below.

Only fatty acids which amounted to 5% or more of total receptor-active preparations are listed in Table 1. Their values agree with the glycerol, sphingosine, and P content which we obtained for the three classes of lipids. The fatty acid present in highest concentration in all active preparations was an unidentified hydroxy acid. On gas-liquid chromatography it was retarded more than a $C_{26:0}$ fatty acid, but its

TABLE 1. *Major components[a] found in leukocyte preparations which inhibit LPS fixation to human erythrocytes*

Constituent	Granulocytes	Platelets	Mononuclear leukocytes
Lipids[b]			
Glycerophosphatides	51.8	40.1	40.8
Sphingomyelin[c]	20.8	21.6	15.6
Triglycerides	5.3	4.6	<2.0
Fatty acids (ca.)	42	38	41
Cholesterol	16.8	18.0	16.4
Peptides[c]	12.6	7.5	13.4

[a] Expressed as the percentage of total dry matter.

[b] Approximate values corrected for 5% water, assuming an average fatty acid chain length of C_{18} and disregarding minor components.

[c] Peptides were petroleum ether insoluble and inactive; sphingomyelin was poorly soluble in petroleum ether and of low activity (see text).

trimethylsilylate derivative migrated faster than a $C_{24:0}$ α-hydroxy fatty acid. The other fatty acids found in all three receptor preparations were of C_{16} and C_{18} chain length. Granulocyte and lymphocyte extracts had ca. 8% $C_{16:0}$ and $C_{18:0}$ and about 12% $C_{18:1}$. The figures for platelets were $C_{16:0}$, 6%, and ca. 8% of both $C_{18:0}$ and $C_{18:1}$ fatty acids. In addition, platelets possessed ca. 8% of a compound which on SE-30 columns migrated identically with $C_{19:0}$ but on silicone ASI columns resembled $C_{21:0}$. Trimethylsilylate treatment led to no retardation.

There was <0.3% free ethanolamine. In agreement with the N values, the peptide content ranged from 6.8 to 11.9%; some hexosamine also contributed to these (see below). Treatment with Pronase and subtilopeptidase was without effect on the activity of the preparations.

The extracts with receptor activity contained either all or the majority of the following sugars: glucose, galactose, mannose, sialic acid, hexosamine, and methylpentose; however, they all amounted to considerably <0.1 mol/mol of receptor-active substances allowing for their maximal molecular weight (see Discussion); they were thus contaminants.

Biological properties. Whole leukocytes, platelets, and the organic phases of their extracts inhibited coating of erythrocytes by LPS as depicted in Tables 2 and 3. The activity of the leukocyte extracts was due to their combination with areas of LPS not related to those which carry serological specificity; the extracts did not interact demonstrably with erythrocytes. This was shown as follows. The fixation onto erythrocytes of 1 U of LPS when incubated in 1 U of active leukocyte preparation was decreased by >95%. In contrast, the inhibitory leukocyte extracts did not block receptor structures on the erythrocytes. When up to 10 inhibiting units of leukocyte receptor preparations were incubated with erythrocytes for 45 min at 37 C under standard conditions and the erythrocytes were subsequently washed and exposed to 0.5 to 1.0 LPS doses, they were coated to the same extent as were untreated, washed erythrocytes.

TABLE 2. *Inhibition of E. coli O_{86} LPS fixation to human erythrocytes by dried cellular elements of human blood*

Cellular component	Smallest quantity inhibiting optimal coating by >95% (μg/ml)
Thrombocytes	400[a] (300–500)[b]
Granulocytes	600 (400–1,000)
Mononuclear leukocytes	450 (300–600)
Erythrocyte stroma[c]	150 (100–200)

[a] Arithmetic average of seven experiments each.
[b] Numbers in parentheses indicate range of values.
[c] Approximately 12.5% of cell dry weight.

TABLE 3. *Inhibition of LPS fixation to erythrocytes cells by n-butanol extracts from blood cells*

Receptor-active substances[a] from:	Smallest avg receptor quantity inhibiting optimal coating by LPS >95%[b] (μg/ml)	Avg activity yield/500 μg of whole dry cells[c]
Thrombocytes	60 (10–100)[d]	3
Granulocytes	70 (6–100)	2
Mononuclear leukocytes	35 (10–100)	2.5
LPS receptor from human erythrocytes	22 (4–120)	0.1

[a] Butanol phase of *n*-butanol–water extractions except erythrocyte receptor, which was from aqueous phase.
[b] Twelve preparations from different *Enterobacteriaceae* tested separately.
[c] Approximately 3.6×10^6 cells except platelets (ca. 3.0×10^8). Expressed as activity units; 1 activity unit is that amount of receptor substance which inhibits coating of erythrocytes by 1 unit of LPS >95% under standard conditions (see text).
[d] Numbers in parentheses indicate range of values.

The interaction of the leukocyte extracts with LPS did not affect the serological properties of the latter. One unit of LPS, preincubated for 1 h with 10 units of inhibitor, resulted in no change of serological activity as measured in hemagglutination inhibition assays.

Table 2 shows that whole platelets, mononuclear leukocytes, and granulocytes had similar activities as inhibitors of human erythrocyte coating by LPS. The data for erythrocyte stroma are given for comparison. The stroma amounts to ca. 12.5% of the erythrocyte dry weight; it was three to four times as active as whole leukocytes and platelets.

Material on the average 6 to 13 times as active as whole leukocytes and platelets could be extracted from them in ca. 300% yield (Table 3). Whereas the average erythrocyte LPS receptor preparation was significantly more active than those from leukocytes, its yield was only ca. 5% of that of leukocyte receptors.

Inhibiting activities of all preparations from leukocyte elements were especially high towards LPS from *S. sonnei* phases I and II, *E. coli* O_{86}, O_{111}, and O_{128}, and *S. minnesota* S form. Activity units of all preparations from

leukocytes and platelets towards *S. typhosa* and *S. minnesota* R forms ranged from 75 to 400 μg/ml; they are not considered in Table 3 nor is a protein-glycolipid complex prepared from *E. coli* O_{86} whose inhibitability was very close to the averages listed in Table 3 with all three receptor-active preparations.

As with the LPS receptor from red cells (27, 28, 31), we found no significant inhibitory activity of the leukocyte preparations towards Vi antigens, but, in contrast, the *n*-butanol extracts of leukocytes at 100 to 200 μg/ml inhibited erythrocyte coating by Rantz antigen and by the stearoyl derivative of group A antigen of *S. pyogenes*.

Comparison of bacterial lipid A with lipidic extracts of leukocytes. There is strong evidence that it is the lipophilic area of LPS which interacts with cells and cell products (9, 14). Our present studies on leukocyte and platelet extracts and those on the erythrocyte LPS receptor (27) likewise point to the importance of hydrophobic binding sites for their activity. Because of this apparent similarity, enterobacterial KDO-lipid A, lipid A as defined by Rietschel et al. (23), and some of their derivatives were tested for their ability to coat erythrocytes as well as to block subsequent LPS fixation onto erythrocytes. The ability of lipid A to interact with LPS directly was also investigated.

Lipid A and KDO-lipid A coated human erythrocytes weakly. Agglutinin titers with anti-lipid A globulin amounted to 2 to 6% of those of ordinary smooth or rough LPS at a concentration of 10 μg of lipid A/ml. This low activity did not increase even when the lipid A concentration was raised 120-fold. Both phthalylated and succinylated lipid A's were slightly more active; de-*O*-acylation decreased and de-*O*- and de-*N*-acylation by hydrazinolysis abolished this weak activity.

This limited interaction of lipid A with erythrocyte surface receptors was also expressed by the absence of a blocking effect of lipid A and derivatives towards subsequent LPS coating of erythrocytes. Only the rather water-soluble, dinitrophenylated, succinylated, or partially de-*O*-acylated lipid A preparations at concentrations of 1,000 μg/ml blocked subsequent coating by 1 unit of *E. coli* O_{86} LPS, but only to 75%.

On the other hand, preincubation of lipid A and derivatives with endotoxin prevented erythrocyte coating by LPS from all bacteria tested. The preparations were less active than the inhibitory leukocyte extracts. An exception was sodium methylate-treated lipid A, which was completely and selectively de-*O*-acylated (E. T. Rietschel, personal communication) and possessed as the only fatty acid amide-linked 3-D-hydroxymyristic acid. Its inhibitory activity was ca. 20 times that of lipid A and approached that of our most active leukocyte preparations. Free 3-D-hydroxymyristic acid had ca. 10% of the activity of de-*O*-acylated lipid A. Lipid A de-*O*- and de-*N*-acylated by >90% was a considerably poorer inhibitor of erythrocyte coating by LPS than ordinary lipid A (F. G. Springer, J. C. Adye, and E. T. Rietschel, to be published). 3-D-Hydroxymyristic acid and the racemic 2-hydroxymyristic, 3-hydroxypalmitic, and 3-hydroxystearic acids possessed ca. 20% of the activity of free 3-D-hydroxymyristic acid, whereas myristic, oleic, and palmitic acids as well as their methyl esters were inactive.

DISCUSSION

LPS of gram-negative bacteria are among the most potent toxins (17). Whereas much is known of the nature of the toxic part of LPS (9, 14), the grouping which fixes to tissue or its components has not been defined, and our understanding of the actions of LPS is inadequate. Even in the acknowledged interaction of LPS with complement the precise mode of action of LPS is unknown, and antibody as a prerequisite, while unlikely, has not definitely been ruled out; furthermore, activation of the complement system is only one of the many incitements of multiple effector systems of LPS (5).

We have described in the present report substances on the surfaces of leukocytes and platelets which bind endotoxin and prevent its attachment to erythrocytes. These inhibitory substances are intrinsic to the cells and platelets, with a major portion located on the surface membranes as indicated in the experiments with whole leukocytes, since even most extensive prior washing did not decrease leukocyte activity. Extraction revealed that additional active material is located within the cells (see Tables 2 and 3).

We reported earlier on a physicochemically homogeneous, macromolecular LPS receptor from erythrocytes. The latter is a lipoglycoprotein of specific binding affinity for LPS of gram-negative bacteria with activity confined to its protein moiety (27, 28). In contrast, the active substances, isolated from leukocytes and platelets as described here, differed profoundly physically from the erythrocyte receptor and belong to a different class of chemical compounds. In leukocytes and platelets, the binding affinity was confined to the lipid moiety exclusively. Neither proteins, peptides, carbohydrates, nor nucleic acids were involved. Table 3 shows that

leukocytes contain considerably more active substances per unit weight than erythrocytes; on the other hand, the mass of erythrocytes in whole blood exceeds that of leukocytes by a factor of about 1,000.

Whereas molecular weights have not been determined hydrodynamically on the active leukocyte extracts, their electrophoretic mobility was studied by polyacrylamide gel electrophoresis with known lipids as reference compounds. This procedure permits comparisons even though the migration rates cannot be interpreted in terms of molecular weight (7). The petroleum ether-soluble part of the active *n*-butanol extracts migrated as one sharp band, each similar to phosphatidyl lipids with a molecular weight of <1,000. The petroleum ether-insoluble, largely inactive residue contained all the peptide and most of the sphingomyelin (J. C. Adye and G. F. Springer, to be published). Even after peptide removal, all active leukocytes and platelet extracts were mixtures, but almost two-thirds of the petroleum ether-soluble material consisted of glycerophosphatides. The remainder was predominantly cholesterol. Triglycerides were present in significant quantities only in granulocyte and platelet preparations.

Since these lipidic substances were the only constituents of active leukocyte and platelet extracts, we investigated the inhibitory activities of commercial lipids. The most active compound was phosphatidylethanolamine. It had ca. 25% of the activity of the most active cell extracts, and cholesterol and sphingomyelin had ca. 5%. Inactive were L-α-glycerophosphocholine, phosphorylethanolamine, and ethanolamine; similarly, cholesteryl myristate, cholesteryl linoleate, glyceric acid, and triolein were inactive in spite of their fine dispersion. These findings indicate that the active inhibitory principle(s) of the leukocyte extracts may be glycerophosphatide. It may differ from the model compounds tested; this is indicated by the much higher activities of the leukocyte extracts. In fact, some individual extracts approached the activities of the homogeneous erythrocyte LPS receptor. This higher activity may be due to a different fatty acid and/or phosphate ester composition of the active extracts as compared to the commercial products. It should be noted that we have found major quantities of two unidentified fatty acids. One of these occurred in all active extracts, and the other occurred in extracts from platelets only.

The specificity of the receptor material was not pronounced. Of all the antigens tested, only the pure Vi and the crude Kunin antigen showed no affinity for the leukocyte extracts.

Our studies of the LPS receptor from erythrocytes have implicated hydrophobic areas on the receptor molecule as well as electrostatic interactions in the LPS-binding effect (27, 28). It is likely that hydrophobic groupings also play a preeminent role in the binding of LPS to receptor-active substances from leukocytes. Both LPS and receptor-active substances are negatively charged species, and bi- and trivalent cations may therefore also be involved in binding activity (6).

As hydrophobic binding sites on the leukocyte preparations are involved in its interaction with LPS and since similar areas on LPS have been shown to play an important role in its pyrogenic and toxic effects (14, 20, 23), it was of interest to determine the effect of lipid A and derivatives on LPS and on the intact surfaces of erythrocytes. Our findings are described above. Lipid A's predominant interaction was with LPS directly. The most remarkable finding was that, whereas the ability of lipid A to inhibit coating of erythrocytes by LPS was faint, de-*O*-acylation of lipid A, which left amide-linked 3-D-hydroxymyristic acid intact, increased this activity 20-fold and made it as powerful an inhibitor as highly active leukocyte and erythrocyte preparations. The finding that *O*-acyl groups on lipid A did not contribute to, but actually interfered with, its activity agrees with our earlier observation on *O*-esterified streptococcal group antigens, A and E, which did not interact with erythrocyte LPS receptor (31).

The least-active lipid A preparation was a completely de-*O*- and de-*N*-acylated KDO-lipid A. These findings suggest that amide-linked 3-D-hydroxymyristic acid may be the active group in coating inhibition, and that it interferes with the erythrocyte-combining site on the LPS. Indeed, free 3-D-hydroxymyristic and, less so, other hydroxy fatty acids showed some inhibitory activity. It must be considered, however, that in deacylated lipids pyrophosphate linkages may be cleaved as a result of the harsh chemical treatment and thus affect activity. Final proof of the role of hydroxymyristic acid will be furnished when selectively de-*N*-acylated lipid A and completely de-*O*- and de-*N*-acylated lipid A with intact pyrophosphate bridges become available.

Fixation of LPS to leukocytes and platelets in many instances is one of the initial steps of interaction of the host with endotoxin. This primary interaction involves two fundamental processes: the binding of endotoxin as a precondition for its noxious action and triggering the

mobilization of host defensive mechanisms in which the white cells are likely to play a paramount role.

It is important also from a clinical point of view to determine which of these two events predominates with leukocytes and platelets, and whether the fixation of LPS to the inhibitory substances of leukocytes is irreversible. It is conceivable that the active inhibitory groupings of leukocytes may find use in the amelioration of endotoxic shock. Definitive assessment of the mode of action of these substances or their active groupings will have to await their isolation in pure form. We are engaged in such studies. Once this aim is accomplished the mode of action and affinity of the inhibitory substances to the toxic component of LPS will be assessed by strictly quantitative means with radioactively labeled LPS as reported earlier for the homogeneous LPS receptor from erythrocytes (27, 28).

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