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Specific Inhibition of Endotoxin Coating of Red Cells by a Human Erythrocyte Membrane Component

GEORG F. SPRINGER, SHANKAR V. HUPRIKAR, AND ERWIN NETER

Department of Immunochemistry Research, Evanston Hospital and Department of Microbiology, Northwestern University, Evanston, Illinois 60201, and Departments of Pediatrics and Microbiology, State University of New York at Buffalo, and Children's Hospital, Buffalo, New York 14222

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We have isolated from human erythrocyte ghosts a fraction which prevents the attachment of unheated as well as heated lipopolysaccharides of gram-negative bacteria to red cells. This material has no significant inhibitory effect either toward the Vi antigen of gram-negative bacteria or towards the group and common antigens of the gram-positive bacteria investigated. We, therefore, named this fraction "lipopolysaccharide receptor." The receptor interacts with lipopolysaccharides and not with erythrocytes, it forms complexes with and blocks those groupings of lipopolysaccharides which attach to red cells. The effect of the receptor is physical and not enzymatic. The interaction of the receptor with the lipopolysaccharides is reversible, and the receptor removes lipopolysaccharides fixed to red cells. An equilibrium of lipopolysaccharide distribution between cells and receptor is established when receptor-lipopolysaccharide complexes are incubated with red cells. The receptor is labile toward heat and deviation of the hydrogen ion concentration from neutrality; aldehydes destroy its inhibitory activity.

Various bacterial substances readily become attached in vitro to erythrocytes of numerous animal species, including man. Those which are antigenic can be detected on the red cells by their corresponding antibodies; clinical as well as investigative serology make extensive use of this coating phenomenon (8, 9, 12, 16, 18, 20). Microbial antigens are also able to coat erythrocytes in vivo under extreme conditions (3, 5, 22). This finding is of importance in considerations of the pathogenesis of some immune hemolytic conditions in man. The antigens whose ability to coat mammalian cells has been most frequently studied are the O somatic antigens (endotoxins) or lipopolysaccharides, acidic polymers such as the Vi antigens, and the common (Kunin) antigen (8, 9, 12, 16, 18) of gram-negative bacteria, the polysaccharidic antigens of mycobacteria (4), and, among gram-positive bacteria, group antigens and the teichoic acid-related antigens (9, 20). It has not been determined, however, whether these various antigens attach to the same or to different receptor sites on cells, nor has any such site been defined. As part of our effort to characterize the receptor substances of human cells (21, 23–25), we have attempted to obtain material from human erythrocytes which combines with lipopolysaccharides of gram-negative bacteria, since these are among the most powerful toxins known (1). Like any other toxin or drug, the lipopolysaccharides must first combine with host receptors before they can exert their biological action.

We have preliminarily reported the isolation of material from human erythrocyte ghosts which prevents coating of human erythrocytes by the human blood group B-specific lipopolysaccharide from one bacterium, *Escherichia coli* O_{86}, as demonstrated both by serological means (25) and by radioactive tracers (J. C. Adye and G. F. Springer, Fed. Proc., p. 267, 1968). We now report the activity range and on the mode of action of this material, which we have tentatively named "lipopolysaccharide receptor." This receptor is a potent inhibitor of lipopolysaccharide attachment to red blood cells and possesses a high degree of specificity, since it has virtually no inhibitory effect on the coating by the Vi antigens and by the antigens of the gram-positive bacteria investigated. We also describe the effect of some physical and chemical agents on the receptor.

MATERIALS AND METHODS

Lipopolysaccharide receptor. Stroma was prepared from human erythrocytes as described previously (23). Washed red cells, regardless of blood group and types, which had been stored for less than 24 days, were used. Aqueous stroma suspensions were ho-

mogenized in a Waring blendor and extracted with *n*-butyl alcohol at *p*H 8.2. Purification was by fractional centrifugation and dialysis as described recently (25). The material which sedimented between about 33,000 and 151,000 × *g* either as a tight button or as a semiliquid layer possessed the highest activity; after exhaustive dialysis, this readily water-soluble material, designated as receptor Ca 1262, was used. It had no blood group M, N; Rh_o(D); or A_1, B or H(O) blood group activities but had traces of A_2 activities in that 2.5 mg/ml inhibited hemagglutination as determined in standard inhibition assays (cf. 23).

For comparison, ganglioside (type 1 Kuhn nomenclature, type A_2 Klenk nomenclature; cf. 28), kindly given by E. Klenk, L-α-phosphatidyl-L-serine [87078] (General Biochemicals, Chagrin Falls, Ohio) and twice crystallized human hemoglobin [H072262] (Pentex, Kankakee, Ill.) were included.

Antigens. All preparations were dialyzed through Union Carbide casing at 4 C for ca. 48 hr against three changes of 20 volumes of distilled water. Toluene and chloroform served as preservatives. The samples were freeze-dried and dried to constant weight at 23 to 25 C at 10^{-1} to 10^{-2} mm of Hg over P_2O_5. Dissolved antigens were stored at −20 C. Immediately before use traces of insoluble material were removed by centrifugation at 2,800 × *g* for 10 min, and the solutions were adjusted to the desired concentrations by dilution with buffered saline (*see below*).

Gram-negative bacteria; O somatic antigens of smooth colonies. Whole bacteria or isolated lipopolysaccharides were employed after heating 500 μg/ml in buffered saline in marble-covered test tubes with occasional agitation in a boiling water bath for 3 hr and adjusting to original volume with distilled water. Such heated O antigen preparations are referred to as LPS. They were used throughout unless stated otherwise.

Bacterial suspensions of *Salmonella* O groups A (155-132) and E [161-156], *Proteus* OXK (158-277) and *Proteus* OX19 [149-136] were purchased from Lederle Laboratories (Pearl River, N.Y.), those of *Salmonella* O groups B [479856], D [497540], and G [523485] and *E. coli* O_{128} [222487] were from Difco; whereas *E. coli* O_{86} suspensions were prepared by us (22). Isolated O antigens from the following bacteria were used: blood group-specific *E. coli* O_{86} (22) and O_{128} (Difco, W. 203600); *Salmonella senftenberg* (group E) and *Shigella dysenteriae* (D. A. L. Davies); *Shigella flexneri* serotype 1a strain NCTC 3 (D. A. R. Simmons); *E. coli* O_{111}, *Salmonella friedenau* (group G), *Salmonella godesberg*, *Salmonella minnesota*, the latter smooth and rough R_{60} (O. Lüderitz); *E. coli* K-235 (F. C. McIntire); *Serratia marcescens* [500] and *E. coli* O_8 [17] (A. Nowotny) and *S. flexneri* [W. 207313] as well as *Salmonella typhosa* group D [W. 529916] from Difco.

Other antigens of gram-negative bacteria. Vi antigens were donated by M. Webster (from *E. coli* 5396/38) and E. Baker (from *Paracolobactrum ballerup* lot H). Common antigen: preparations of purified supernatant fluid from cultures of *Salmonella typhimurium* HK-7 served as source of Kunin type antigen (26).

Gram-positive bacteria. The group antigens A and E of *Streptococcus pyogenes* and their stearoyl derivatives were given by H. D. Slade; purified culture fluids from *Bacillus subtilis* and *Staphylococcus aureus* (Difco, coagulase-positive) served as sources of Rantz antigen (27). These preparations were not heated before use.

Antisera. Unless indicated otherwise, the antisera were from rabbits and were used, after absorption with suitable human erythrocytes, for determinations of homologous antigen specificity. They were furnished by those individuals and firms who provided the antigens, except *S. dysenteriae* [184371] and *S. flexneri* type I [525351] antisera, which were purchased from Difco, and *E. coli* O_{111} [086-140] and the *Shigella* group B [158-124] antisera from Lederle; the latter serum was used with the commercial *S. flexneri* LPS. The Vi antiserum given by E. Baker was used with both Vi antigen preparations.

For antigens possessing high human blood group activity, commercial anti-human blood group B sera from Ortho Research Laboratories (Raritan, N.J.) [9219,-20,-26,-34] were used. The eel anti-human blood group H(O) serum was prepared by us (22). The common antigen of the Kunin type was identified with *E. coli* O_{14} antiserum [5247B] (26), and that of the Rantz type with anti-*Staphylococcus aureus* serum [5709/12, Dm 5/8] prepared by us (27). Titers of the appropriately diluted sera ranged from 64 to 256 with the exceptions indicated in Table 1.

Erythrocytes, solution, and glassware. Erythrocytes from three healthy adults of blood group O (D. S., H. T., and J. C.) were obtained, stored for 1 to 6 days, and washed as described previously (22). They were used in all experiments except in those with *E. coli* O_{128}, in which A_1B erythrocytes (D. K.) were employed. Aqueous 0.10 M sodium chloride containing 0.05 M sodium phosphate buffer [*p*H 7.3 to 7.4 (buffered saline)], was used throughout as diluent and solvent. Freshly sterilized Kimax glassware was used when incubation at 37 C exceeded 1 hr; thimerosal, 1:10,000 final concentration, or a small thymol crystal removed at the end of the incubation, served as preservatives, since they did not interfere with the reactions.

Coating of erythrocytes by bacterial antigens. All experiments were performed at least three times except the test with *S. marcescens* LPS and the stearoyl derivatives of streptococcal group antigens, which were tested only twice because of lack of material.

The standard procedure consisted of determination of the smallest amount of antigen which just afforded maximal agglutination by subsequently added antiserum. This quantity, defined as one coating unit (U) or one optimal coating dose was used in all tests, unless noted otherwise.

The coating procedures were similar to those employed previously (22, 25), but all volumes were scaled down; 0.05 ml of thrice washed, packed erythrocytes were incubated at 37 C for 45 min with 0.45 ml of the desired amount of antigen. The mixture was frequently agitated. The erythrocytes were then washed four times with ca. 50 volumes of buffered saline at 23 to 25 C, centrifuged for 2 min at

TABLE 1. *Receptor as inhibitor of bacterial antigen fixation to human erythrocytes*[a]

Antigen fixed	Smallest antigen amt (μg/ml) affording maximal titer (A)[b]	Smallest receptor amt (μg/ml) giving >95% inhibition of coating (B)	Ratio of B/A
Gram-negative bacteria			
Bacterial suspension			
Escherichia coli			
O_{86}	10; 120[c]	7; 50[c]	0.70; 0.42
O_{128}	60; 180[d]	105; 165[d]	1.8; 0.92
Salmonella group			
A	30	11	0.37
B	7	18	2.6
D	25	50	2.0
E	8	9	1.1
G	4	3	0.75
Proteus			
OXK	30	9	0.30
OX19	25	8	0.32
Isolated O antigen			
Escherichia coli			
O_8	0.8	22	27
O_{86}	0.6; 9[c]	4.5; 11.3[c]	7.5; 1.3
O_{111}	3	25	8.3
O_{128}	10; 35[d]	120; 160[d]	12; 4.6
K-235	0.3	7.5	25
Salmonella			
typhosa (group D)	8	43	5.4
senftenberg (group E)	6	23	3.8
friedenau (group G)	0.6	5	8.3
godesberg	0.7	11	16
minnesota	1.8	6	3.3
minnesota R_{60}	4.5	42	9.3
Shigella			
dysenteriae	0.2	2.2	11
flexneri (1a)	2.7	97	36
flexneri (Difco)	0.1	3.5	35
Serratia marcescens	2.2	5	2.3
Dialyzed, purified culture fluid, common antigen			
Salmonella typhimurium (HK-7)	87[e]	127[e]	1.5
Isolated Vi antigen			
Paracolobactrum ballerup	0.15	850	>5,000
E. coli 5396/38	1	550	550
Gram-positive bacteria			
Dialyzed, purified culture fluid, Rantz antigen			
Bacillus subtilis	120[f]	>4,500[f]	>38
Staphylococcus aureus	15	3,000	200
Streptococcus pyogenes, isolated group antigen			
A, Stearoyl derivative	4	1,500	375
E, Stearoyl derivative	4	2,000	500

[a] Activities determined with homologous rabbit antisera unless indicated otherwise.

[b] Maximal titers between 64 and 256 throughout except for *S. minnesota* R_{60}, *Shigella flexneri* Difco), *S. senftenberg* and Kunin antigens 32 to 64.

[c] Determined with heterologous human antiblood group B serum.

[d] Determined with heterologous eel antihuman blood group H(O) serum.

[e] Determined with rabbit anti *E. coli* O_{14} serum.

[f] Determined with rabbit anti *S. aureus* serum.

1,250 × *g*, and suspended in buffered saline at 0.5% final concentration.

Inhibition of erythrocyte coating. Receptor activity was determined by measuring its ability to inhibit antigen coating of red cells. In each test five different concentrations of receptor were run in parallel to obtain a dose-response curve. Complete inhibition was considered to have been reached when it exceeded 95%, since the value of 100% was approached asymptotically because of the twofold dilution technique used. The smallest amount of receptor which, under standard conditions, inhibited one coating unit of antigen by >95% was defined as one unit (U) of receptor.

The procedure in the inhibition assay differed from that in the coating test in that the desired amount of receptor was added to one optimally coating dose of antigen and incubated in a total volume of 0.45 ml for 30 min at 37 C. Thereafter, 0.05 ml of packed erythrocytes was added and the coating procedure was followed. In each test, a standard consisting of an ordinary coating assay employing the antigen to be inhibited was included.

Hemagglutination tests. These, as all other experiments to be described, were performed two to six times on all samples. Titration results were read independently by two to three individuals to one of whom the nature of the samples was unknown, and the arithmetic average of all tests on a given sample was reported. The mode of titration and interpretation of agglutination has been described previously (21). The procedure was carried out at 23 to 25 C; twofold geometrical dilutions of serum employing a fresh pipette for each tube were used with a constant volume (0.05 ml) of all reagents and a final addition of 0.05 ml of a 0.5% red cell suspension. The tube racks were agitated and incubated for ca. 90 min at 23 to 25 C; agglutination was read with the microscope. Preparations containing Vi antigen were spun for 1 min at 1,000 × *g* just prior to reading. Tests with the stearoyl derivatives were read after 3 hr of incubation. The last tube in a titration series which showed agglutination was taken as the end point, and activities were expressed on its basis. Coating activities of antigens were expressed as the smallest amount (μg/ml) of antigen which just afforded maximal agglutination titer. Controls in all tests consisted of erythrocytes coated with each antigen or mixture and suspended in buffered saline and of uncoated erythrocytes incubated with the serum. All samples belonging to one experiment were tested in parallel.

Inhibition was always measured at several receptor concentrations and was calculated as percentage decrease in agglutinating activity computed from the difference in coating titers afforded by the antigens in the presence and absence of inhibitor. When receptor inactivation was measured, the activity of treated receptor at each point of the experiment was compared, at five different concentrations, with the activity of untreated receptor at the same concentrations. The values obtained were averaged.

All the following procedures were carried out with *E. coli* O_{86} LPS and human anti-blood group B serum. The findings were analogous in all instances investigated when rabbit anti-*E. coli* O_{86} serum was used instead of human anti-blood group B serum.

Mode of receptor action. It was determined whether the receptor interfered with the active sites of the red cells or those of the LPS, or both. Washed erythrocytes were incubated for 30 min at 37 C with varying amounts of receptor. The erythrocyte-receptor mixtures were divided into two portions; one was washed, and then both portions were exposed to increasing amounts of LPS under standard conditions. Subsequently, all red cell samples were washed, and the extent of LPS fixation was measured.

The possibility of interaction of receptor with red cells was also determined by measuring the amount of receptor in solution before and after its exposure to erythrocytes. Two to three receptor units were incubated with red cells under standard conditions except that the incubation period was extended to 90 min. After centrifugation, a small amount of supernatant fluid was set aside, and the incubation was repeated thrice, by using a fresh lot of erythrocytes each time. The inhibitory activities of the supernatant solutions were compared with that of receptor incubated under the same conditions without erythrocytes. Buffered saline incubated with red cells in parallel with the sample was included as control.

To assess whether the effect of the receptor was physical or chemical, receptor (500 μg/ml, final concentration) was incubated with LPS in a ratio of 2:1 (wt/wt) at 37 C for 38 hr. One sample of this mixture was then autoclaved at 15 psi for 1 hr, since the receptor proved to be heat-labile (*see below*). The coating ability of different concentrations of both samples was then compared with the same amounts of free LPS preincubated and heated in the same way as the samples.

It was also determined whether the receptor interfered with the serologically active sites of LPS by incubating various batches of erythrocytes, each coated with one optimal dose of a different LPS preparation, with up to 5 U of receptor for 30 min to 1 hr at 37 C; the erythrocytes were then washed and their agglutinability compared with LPS-coated red cells treated the same way but not exposed to receptor.

Complexing of receptor with LPS. One coating unit of LPS was mixed with 0.41 to 10.4 parts by weight of receptor, corresponding to 0.3 to 8.3 U, and incubated for 30 min at 37 C. These mixtures were designated as complexes, although the existence of either free LPS or free receptor in them was not ruled out. Red cells were exposed to the complexes as in ordinary coating assays. Thereafter, the mixtures were divided in two samples, one of which was first washed. To both the washed erythrocyte suspensions and those still exposed to receptor complexes two-thirds to three-fourths of an optimal coating unit of LPS was added, and the erythrocyte suspensions were then treated as in an ordinary coating assay.

Competition between receptor and erythrocytes for LPS. (i) Complexes of 1 U of LPS with 1.13, 1.6 or 3 inhibiting doses of receptor were incubated with

erythrocytes under standard conditions, and duplicate samples of the mixtures were removed at intervals up to 6 hr. Thereafter, the erythrocytes were washed and the degree of coating was assessed by averaging the readings from duplicate samples. Controls consisted of LPS-receptor complexes which had been incubated without erythrocytes and whose coating ability was determined, and a coating test in which erythrocytes had been preincubated with LPS for 6 hr.

(ii) A 2% suspension of erythrocytes was sensitized with 1 unit of LPS, washed, and exposed to 10 inhibiting units of receptor at 37 C for 6 hr; samples were removed at intervals. Alternatively, such sensitized erythrocyte suspension was divided into several samples. One sample was kept as standard, and the others were added to several tubes, each containing 10 inhibiting doses of receptor. Incubation was for 90 min at 37 C. After washing as usual, one erythrocyte sample was kept for testing. The others were reincubated with 10 doses of fresh receptor. This procedure was repeated twice more. Red cells coated, incubated in buffered saline only, and then washed as in the experiments proper served as control. After washing, all red cell samples were tested for their extent of coating.

Stability of receptor. The effect of incubation up to 72 hr under standard conditions and the resistance towards heat were determined on receptor alone in buffered saline as well as on receptor preparations which had been preincubated for 45 min at 37 C with an equivalent number of units of LPS. The activities of the solutions containing only receptor were determined in inhibition assays and compared with those of untreated receptor. The receptor-LPS mixtures were tested in ordinary coating assays as if they were coating antigens; LPS preparations incubated for the same time and heated under the same conditions as the samples proper served as standards.

Stability towards acids and bases was determined by incubating 0.2% solutions of the receptor at various temperatures and times in the following buffers: McIlvaine citrate-phosphate 0.2 M, *p*H 2.2, *p*H 3.0, *p*H 4.0, *p*H 5.0, and *p*H 6.0; Soerensen phosphate 0.067 M, *p*H 7.0 and *p*H 8.0; glycine-NaOH 0.1 M, *p*H 9.0, *p*H 10.0, and *p*H 11.0 (cf. 24). The *p*H values remained constant within ± 0.1 throughout incubation. All samples were dialyzed and dried before testing as described above.

Blocking of the active receptor sites by aldehydes. Crotonaldehyde [1878] or acrolein stabilized with 0.1% hydroquinone [2037], both from Eastman Organic Chemicals (Rochester, N.Y.), from freshly opened bottles was mixed in various proportions with receptor (0.2%, final concentration). Hydroquinone alone from Fisher Scientific Co. (Pittsburgh, Pa.) [784424] was similarly tested. The mixtures were incubated for 2 hr at 37 C, dialyzed with continuous agitation for a total of 60 hr against 50 and twice 25 volumes of deionized water at 4 C, and dried as described above (*see* Antigens).

Controls consisted of two sets of each of the aldehydes alone at the highest concentrations used and receptor alone, both dialyzed and dried in the same way as the samples proper. Completion of dialysis of the aldehydes was determined by weight and by inhibition tests (cf. 25). An additional control consisted of red cells exposed to a dialyzed receptor-aldehyde mixture, containing the highest aldehyde concentration employed.

RESULTS

Table 1 summarizes the results obtained in the coating assays with the antigens (column 2) as well as those obtained in the inhibition experiments with the receptor (column 3).

Coating; gram-negative bacteria. Maximal titers, as determined with homologous sera, resulted when the erythrocytes had been exposed to 4 to 60 μg/ml of bacterial suspensions. The most potent coating suspensions were found among the salmonellae. The isolated O somatic antigens were on the average eight to nine times as potent coating agents but showed a similarly wide range of effectiveness as did the bacterial suspensions. Some lipopolysaccharides from escherichiae, salmonellae, and shigellae gave optimal coating at <2 μg/ml (Table 1). In no instance was >10 μg of LPS/ml necessary for optimal coating. Larger amounts of antigen or bacterial suspension were needed to reach optimal coating when heterologous instead of homologous antisera were used as for *E. coli* O_{86} and *E. coli*$_{128}$.

E. coli O_{86} lipopolysaccharide, which had not been boiled but was used after incubation for 64 hr at 37 C in buffered saline, had from 15 to 30% of the coating activity of boiled LPS, i.e., ca. three to six times the quantity of LPS was needed for 1 coating unit. Freshly dissolved *E. coli* O_{86} LPS possessed $<3\%$ of the activity of boiled LPS as determined with both heterologous and homologous sera.

Of the two other kinds of antigens from gram-negative bacteria, the highly purified Vi antigens afforded optimal coating at 0.15 μg/ml and 1 μg/ml, whereas 87 μg/ml of the crude Kunin antigen preparations gave this effect (Table 1). The results were the same whether or not these antigens had been boiled before coating.

Among the antigens of the gram-positive bacteria tested, the stearoyl derivatives of the streptococcal group antigens were of high coating potency; 4 μg of antigen per ml gave maximal agglutination; ordinary streptococcal group antigens coated insignificantly. Concentrations of 15 and 120 μg/ml of the common antigen (Rantz) preparations from the two gram-positive bacteria *S. aureus* and *B. subtilis*, respectively, had to be used to achieve maximal agglutination (Table 1).

Inhibition of coating. Between 2 and 120 μg/ml of receptor inhibited the fixation onto human

red blood cells of the O somatic antigens of gram-negative bacteria, both in a bacterial suspension and in isolated, highly purified form (Table 1, column 3). The range of receptor concentrations needed for this effect thus varied somewhat less than that of LPS quantities required for optimal coating. The average receptor quantity just giving >95% inhibition of coating was about 25 μg/ml both for bacterial suspensions and for highly purified lipopolysaccharides. However, optimal coating of 18 of 23 lipopolysaccharides of smooth gram-negative bacteria was inhibited to >95% by 2 to 25 μg/ml of receptor. The largest receptor amounts were needed for members of *Salmonella* group D, *S. flexneri* 1a, and *E. coli* O_{128}. Seven times as much receptor was needed to prevent attachment of LPS from a rough strain of *S. minnesota* than for the LPS from a smooth form of the same strain. More receptor was needed, per weight unit of LPS added, to prevent optimal coating when it was determined with homologous instead of heterologous serum (*see E. coli* O_{86} and *E. coli* O_{128} in Table 1).

When *E. coli* O_{86} LPS had been activated by incubation in buffered saline for 64 hr at 37 C instead of by boiling, the same to about twice the amount of receptor was required to inhibit 1 coating U as determined with both homologous and heterologous antisera.

The receptor also inhibited the attachment of the common (Kunin) antigen to erythrocytes, although, on the average, about five times that quantity was required which inhibited coating by LPS. Very large amounts of receptor were needed to inhibit coating by Vi antigen by >95%, especially in view of the small concentrations of Vi antigen which gave optimal coating.

The receptor was ineffective in preventing the attachment to red cells of the antigens of the gram-positive bacteria investigated, the Rantz antigen preparations of *S. aureus* and *B. subtilis*, and the stearoyl derivatives of the group antigens of *S. pyogenes*.

Mode of receptor action. When erythrocytes were incubated with up to 10 inhibiting doses of receptor for 30 min and then washed and exposed to 0.13 to 1.0 LPS units, they were coated at all LPS levels to the same extent as were untreated, washed red cells. In addition, exposure of a solution containing 2 to 3 U of receptor to four successive lots of red cells did not reduce its inhibitory capacity. Both these results indicate that the receptor does not interact with human erythrocytes. Buffered saline alone incubated with washed red cells did not acquire inhibitory activity.

In contrast, fixation of 1 unit of LPS onto red cells suspended in 2 units of receptor solution was inhibited by >95%. This inhibition was physical and not enzymatic since incubation of LPS-receptor mixtures for 38 hr and subsequent destruction of the receptor by autoclaving (*see below*) indicated no change in the coating activity of this LPS as compared to LPS incubated and autoclaved without receptor. The unheated LPS-receptor complex produced as little coating after 38 hr of incubation as at its outset; this demonstrated the stability of the receptor during incubation. No decrease in the serological activity of any of the LPS preparations resulted from exposure of LPS-coated red cells to receptor.

TABLE 2. *Coating of human erythrocytes by Lipopolysaccharide/Receptor complexes of various proportions*[a]

LPS/receptor (wt/wt)	Incubation period	
	45 min	90 min
1:0.41	34	83
1:1.3	4.25	22.3
1:2.6	<1	4.7
1:5.6	<1	0.76
1:10.4	<1	<1

[a] Final *E. coli* O_{86} LPS concentration in all complexes 9 μg/ml. Coating determined with human antiblood group B serum. Coating values are expressed as per cent of optimal coating.

Complexing of receptor with LPS. Table 2 summarizes the data on the stoichiometry of interaction between LPS and receptor. A large excess of LPS was present when an LPS-receptor mixture at a ratio of 1:0.41 (wt/wt) was employed since there was extensive coating of red cells. Remarkably, coating was considerably stronger after 90 min of incubation than after 45 min. With a complex of the ratio 1:1.3, only slight coating was observed after 45 min of incubation. In fact, the activity of receptor at this ratio corresponded to 1 unit as defined above. No coating was demonstrable after 45 min of incubation when the amount of receptor was doubled. After 90 min of incubation, however, coating could still be shown even at 5.6-fold receptor excess. The reason for the influence of the incubation period with complex on the extent of coating of red cells is given in the "competition" experiments below. This difference between 45 min and 90 min of incubation was corrected for in the stoichiometric experiments described in the next paragraph.

The effect of incubation with LPS-receptor complexes on coating of the erythrocytes by subsequently added LPS in presence of complex and after removal of the complex was determined.

TABLE 3. *Effect of erythrocyte incubation with Lipopolysaccharide/Receptor complexes[a] on subsequent attachment of free LPS*

LPS/receptor (U/U)	Complex removed prior to exposure to free LPS	Ratio of coating to standard coating[b] resulting from addition of free LPS	
		6[c]	7[c]
1:0.3	No[d]	2.9	3.2
	Yes[e]	3.0	3.2
1:1	No	0.46	0.42
	Yes	2.1	1.7
1:4.5	No	0.30	0.22
	Yes		1

[a] Final *E. coli* O_{86} LPS concentration in all complexes was 9 μg/ml. Coating determined with human antiblood group B serum.

[b] Standard coating = coating in absence of complex.

[c] Amount of free LPS added in micrograms per milliliter.

[d] Values corrected for migration effect (*see* Results.)

[e] Removal by washing red cell-complex mixture.

Table 3, in which all LPS-receptor ratios are given on a unit basis, shows that LPS was present in excess in the complex mixture at an LPS-receptor ratio of 1:0.3, since there was substantially more coating of the erythrocytes which had been incubated with complex either for 45 or 90 min than of those which had only been exposed to LPS. In the area of equivalence between receptor and LPS, incubation with free LPS in presence of complex resulted in suboptimal coating (ca. 45%), whereas agglutination of cells which had been incubated with LPS after removal of the complex was around twice that obtained by free LPS. Added LPS in the presence of a 4.5-fold excess of receptor had only a faint coating effect when complex was present but coated as under ordinary conditions when the complex had first been removed.

Competition between receptor and erythrocytes for LPS. The more extensive coating of red cells which had been incubated for 90 min with complex, as compared to those incubated for 45 min (Table 2), indicated the possibility of transfer of receptor-bound LPS to the red cells. Figure 1 shows that there was indeed migration of LPS, since the erythrocytes acquired LPS and became progressively more coated with increasing incubation time. The LPS migration was most marked in presence of the smallest amount of receptor, 1.13 inhibiting U. During the first 4 hr of incubation, coating increased linearly and approximately doubled with doubling time. By 4 hr, an equilibrium was reached and coating was 68% of that amount which occurred when the erythrocytes were exposed to 1 U of LPS in the absence of receptor. Migration was less when 1.6 inhibiting U of receptor were present, but it was still linear up to 4 hr. Migration of LPS to the red cells occurred, albeit greatly diminished, even in the presence of 3 neutralizing U of receptor; the transfer was still increasing after 4 hr, and by 6 hr coating had reached only approximately 30% of the optimal level. The complexed receptor lost no activity but retained its full inhibitory capacity when it was incubated for 6 hr in absence of erythrocytes. This is further evidence that the coating effect of red cells was due to migration of the LPS.

LPS was also transferred from red cells to receptor in solution. Red cells coated with 1 U of LPS and incubated with 10 inhibiting U of receptor showed a decrease of coating by about 40% after the 6-hr incubation period, as compared to both freshly coated red cells and coated erythrocytes incubated for 6 hr at 37 C with buffered saline. To demonstrate more significantly the removal of LPS from the red cells by receptor, the coated erythrocytes were incubated with four successive lots of 10 receptor units which were changed at 90-min intervals. Figure 1 depicts the finding that, by the device of successive receptor changes, over 75% of the LPS was removed from the red cell surface. Red cells treated as in

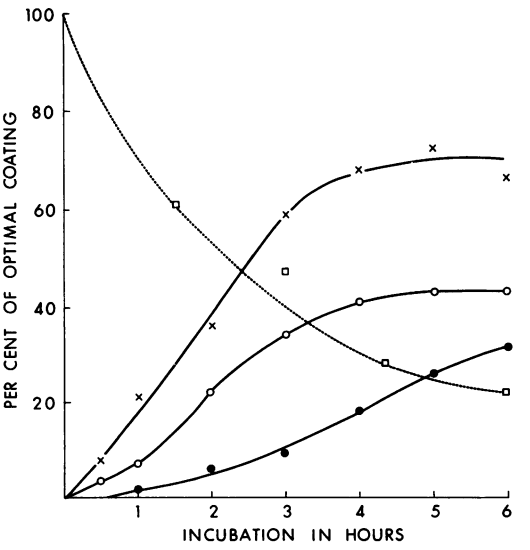

FIG. 1. *Equilibration of Escherichia coli O_{86} lipopolysaccharide between red cells and receptor. Transfer of LPS from red cells to receptor* (□). *Transfer of LPS from receptor to red cells, receptor to LPS ratio: 1.13* (×), *1.6* (○), *3.0* (●).

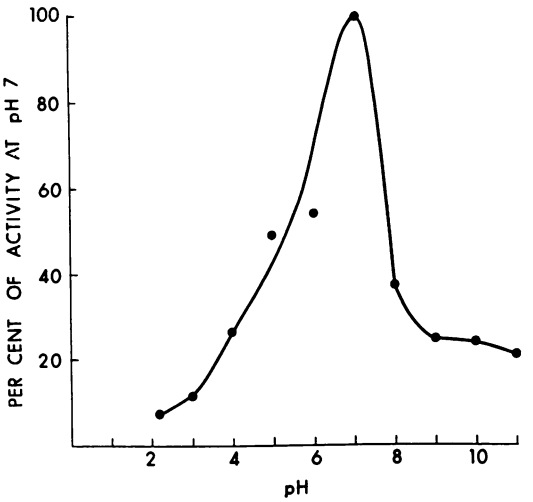

FIG. 2. *Inactivation of receptor in 0.2% concentration by incubation with acidic and basic buffers at 56 C for 6 hr. Activity at pH 7.0, 100%.*

the experiment proper, but in the absence of receptor, showed no decrease in the extent of coating.

Stability of receptor. Under standard incubation conditions, the receptor retained full activity for at least 48 hr and showed a slight decrease in potency after 72 hr. It lost about 40% of its activity upon incubation at 56 C for 6 hr. The receptor was inactivated by 80 to 90% on heating at 80 C for 90 min; the residual activity resisted additional heating at 80 C for at least another 4.5 hr. Boiling or autoclaving at 15 psi destroyed all receptor activity within 30 min. Preincubation of the receptor with LPS did not protect it from inactivation between 56 and 100 C.

The influence of varying hydrogen ion concentrations on incubation at 56 C is depicted in Fig. 2. The receptor was relatively stable only near *p*H 7, where it lost somewhat less than 40% of its potency. The activity decreased steeply in more acid and more alkaline ranges. The receptor lost activity during 6 hr when incubated under acidic or basic conditions even at 23 to 25 C.

Blocking of active receptor sites by aldehydes. The LPS-binding capacity of 10 parts of receptor was blocked, by about 90%, by 1 part of acrolein (wt/wt) and 1 part receptor was inactivated to 90% by 1 part crotonaldehyde. Hydroquinone had no effect on receptor activity. Aldehyde-treated receptor did not agglutinate red cells both in presence and in absence of serum.

DISCUSSION

It has been known since 1899 (8, 9, 12, 13, 15, 16) that many bacterial antigens readily attach to erythrocytes, where they may be detected by their corresponding antibodies. Erythrocyte coating by antigens of gram-negative bacteria has been investigated most extensively (cf. 16). Numerous but contradictory studies on the nature of the lipopolysaccharide groupings responsible for LPS fixation onto human cells have been made (cf. 6, 19). It is also uncertain whether the various lipopolysaccharides and the diverse antigens of different bacteria attach to the same receptors or to different ones (6, 9, 14, 19).

There are only two preliminary reports on the isolation of the receptor structure(s) with which antigenic products of gram-negative bacteria combine. Boyden prepared an alcohol-ether extract from horse erythrocytes and used it to inhibit horse erythrocyte coating by a filtrate of *Pfeifferella mallei* (2). From our laboratory, isolation of glycolipoprotein material from human erythrocyte stroma, which inhibited coating of human erythrocytes by highly purified LPS from *E. coli* O_{86} has been reported (25). No data on the specificity of these erythrocyte extracts were given nor any on their mode of action and their active groupings. Such studies would contribute to the understanding of the nature of fixation of endotoxins to cells. Indeed, it has been stated that there exists a direct relationship between the susceptibility of different strains of mice to the lethal effects of *Salmonella* endotoxin and the affinity of their red cells for heat-killed *Salmonellae* and the LPS derived from them (10). Another outcome of such studies may be the isolation of the active groupings of cell receptors and their use in prevention of attachment of endotoxins to cells and cell products.

We report here that the receptor isolated by us prevented the attachment to human red cells of the O somatic antigens of all gram-negative bacteria tested (*see* Table 1), and that it did so by physically blocking those sites on the LPS which attach to red cells. No blocking of the immunologically active groupings of the LPS preparations by the receptor was detectable with the serological procedure employed. The receptor had no enzymatic effect on the LPS and did not combine with the erythrocytes.

Both the amount of LPS needed to obtain optimal red cell coating and the quantity of receptor required to inhibit this coating by >95% showed considerable and frequently unrelated variations depending on the origin of the LPS. Remarkably, on the average, closely similar receptor quantities, around 25 μg/ml, were needed to inhibit coating by bacterial suspensions and isolated LPS. The relation between inhibitory unit of receptor and coating unit of LPS became more clearly recognizable when ratios between

these units were established on a weight basis [Table 1, column 4 (B/A)]. The ratio of 1 inhibiting unit of receptor to 1 coating unit of antigen measured with homologous serum averaged 1.1 when bacterial suspensions which contained much inert material were used and 14.4 when isolated LPS from smooth bacterial cultures was employed. In spite of the scatter of the individual values, the averages of these ratios were usable on a strictly quantitative basis provided antigens of similar purity were compared.

Aside from the O antigens, the common (Kunin) antigen of gram-negative bacteria was the only other antigen which was significantly inhibited by the receptor.

Very large quantities of receptor were required for the inhibition of Vi antigens and the antigens of gram-positive bacteria investigated. The receptor-antigen ratios for the highly purified Vi antigens and the stearoyl derivatives of the streptococcal group antigens were 26 to >350 times as high as those of the average of highly purified LPS; the ratios for the crude Rantz antigens were >25 to 180 times higher than those for the other crude antigens tested, the suspensions of gram-negative bacteria and the Kunin antigen. This clearly indicated the specificity of the receptor for LPS and the closely related Kunin antigen.

The influence of inert material on the ratios was confirmed when bacterial suspension and isolated LPS from the same bacterial strain were compared (Table 1), e.g., for *E. coli* O_{86} and *E. coli* O_{128}. The low ratios for the bacterial suspensions indicated that the receptor did not interact with most of their components, a finding which dovetailed with the observed specificity of the receptor. Specific receptor consumption by LPS and Kunin antigen may thus conceivably be used for quantitative measurement of the reactive form of these two antigens in bacterial preparations.

The establishment of ratios also demonstrated more clearly the relative efficiency of receptor towards the isolated lipopolysaccharides. The ratios showed the receptor to be equally potent toward *S. senftenberg* and *S. minnesota*, although nearly four times the quantity of receptor was needed to inhibit the coating by *S. senftenberg* LPS; and the efficiency of the receptor was equal towards the two *S. flexneri* LPS preparations listed in Table 1 even though in one instance 97 μg/ml was needed for neutralization and, in the other, 3.5 μg/ml.

There was no strict correlation between coating activity of a given LPS and the amount of receptor which inhibited this reaction, except that generally the receptor-LPS ratio was high for those lipopolysaccharides of which <2 μg/ml afforded optimal coating (Table 1). The reason for the lower ratio for the less efficient LPS preparations may be that they contained much material incapable of coating and of combining with the receptor. Alternatively, the more powerful lipopolysaccharides may have been of smaller molecular size and thus more particles per given weight attached to the red cell surface, which in turn would require more receptor for neutralization. Both possibilities are compatible with our observation that, for LPS which had not been boiled but had been incubated under physiological conditions for 64 hr, the ratio by weight of neutralizing receptor to optimal coating LPS was considerably smaller than for the same LPS activated by boiling.

Considerably more *E. coli* O_{86} or *E. coli* O_{128} LPS was needed to achieve optimal coating when measured with heterologous serum instead of homologous serum (*see* Table 1). Furthermore, relatively more receptor was needed in the homologous systems for prevention of red cell coating by LPS. For example, for *E. coli* O_{86} LPS, the ratio (on a weight basis) of neutralizing receptor dose to optimal coating dose was 7.5:1 in the homologous system, and 1.3:1 with heterologous human anti-blood group B serum (Table 1). Both these differences are explained by the larger amount of reactive structures detectable, per weight unit of LPS, by a homologous antiserum, with its greater variety and quantity of antibodies, as compared to a cross-reacting antiserum.

The quantitative relation between coating dose of LPS and inhibiting unit of receptor was confirmed when LPS-receptor complexes of various proportions were incubated with red cells and the extent of erythrocyte coating subsequently measured (Tables 2 and 3). It is noteworthy that, in the equivalence zone (Table 3, 1:1 ratio, U/U), there was moderate inhibition of red cell coating when the complex was present during incubation with freshly added LPS, whereas, when the complex had been washed off beforehand, there was a significant increase in coating over that of the standard due to the cumulative effect of available LPS in the complex and freshly added LPS. Therefore, in this area, small amounts of both free LPS and free receptor most likely coexisted in the mixture.

The titration procedure as well as steric factors do not allow one to obtain strictly quantitative information on inhibition or lack of it by serological methods. Furthermore, an LPS quantity which affords optimal agglutination is not necessarily equal to that which is maximally taken up by erythrocytes. We found in studies with radioactive LPS that one optimal coating dose of *E. coli* O_{86} LPS, as determined with heterologous

serum, was about 15% of that quantity which human erythrocytes from healthy adults are able to fix maximally; the value for homologous serum was ca. 1% (Springer et al., *manuscript in preparation*).

These considerations, the experiments in which complexes were studied, and our finding that the receptor was able to remove LPS from red cells form the basis for an understanding of the experiments describing the competition between the receptor in solution and that on the red cell surface. LPS was transferred to erythrocytes from LPS/receptor complexes in which receptor was in excess until an equilibrium was reached. An equilibrium in LPS distribution between receptor and red cells was also approached, in accordance with the law of mass action, when LPS-coated red cells were incubated for prolonged periods with large amounts of free receptor. These findings, which we have also made in strictly quantitative studies with ^{32}P-labeled LPS (Springer et al., *manuscript in preparation*), prove the reversibility of LPS fixation to cells under physiological conditions and also indicate that the structures on red cells and isolated receptor, which combine with LPS, are probably alike.

Our studies do not permit a decision on whether the same sites on the erythrocyte surface combine with the various lipopolysaccharides. However, the similar interaction of all investigated LPS preparations with the receptor favors the possibility that there is likeness of the general area on the red cell surface to which the various lipopolysaccharides attach.

The receptor was inactivated by both heat and acids and bases (*see* Fig. 2), but 10 to 20% of the receptor activity resisted heating at 80 C for several hours. This indicates that the receptor may possess more than one kind of structure capable of binding the LPS. The lability of the receptor and our observation that aldehydes inactivated it point to the possibility of protein involvement in receptor activity. Aldehydes are known to form condensation products with the amino acids in proteins (7). Further support of this possibility was our observation that proteases inactivated the receptor (Springer et al., *manuscript in preparation*). However, these findings do not establish the proteinaceous nature of the active groupings of the receptor. Other erythrocyte structures, the blood group MN antigens and myxovirus receptors, were inactivated by proteases to >90%; even though their determinant groups are carbohydrates (23), their activities depend importantly on conformation and size of the macromolecules carrying them (11, 24).

The nature of the active groupings on the receptor must be determined by their isolation and physical and chemical characterization. We have found model compounds related to structures occurring in cell membranes which prevent coating by LPS in the same way as the receptor (16), but we do not know if the action of these models is also specific. The most active substances were the gangliosides and hemoglobin (25; Adye and Springer, Fed. Proc., 1968, p. 267) and the phosphatidyl lipids (17, 25; Adye and Springer, Fed. Proc., 1968, p. 267). Crystalline human hemoglobin, ganglioside, and L-α-phosphatidyl-L-serine were, therefore, tested in parallel with receptor Ca 1262 which contained 1.5% hemoglobin and 0.1% phosphorus (Springer et al., *manuscript in preparation*), permitting for <3% phosphatidyl lipids. The hemoglobin possessed only 10% and the two other compounds <3% of the activity of the receptor. It is, nevertheless, possible that similar substances attached to the receptor in a specific arrangement in space play a role in its activity.

Human serum and some of its fractions prevented the fixation of LPS to red cells, but earlier studies furnished no evidence that they removed LPS once it had become attached (16, 22). The red cells employed in the preparations of receptors were thoroughly washed before hemolysis, and the stroma was washed again before extraction; also the electrophoretic properties of the receptor preparations had no similarity with those of human serum (25; Springer et al., *manuscript in preparation*).

An erythrocyte stroma extract, which inhibited the attachment to sheep erythrocytes of a polysaccharide-containing fraction isolated from tuberculin, has been described previously (4). The specificity of this preparation towards various antigens was not investigated. It differed from the receptor described here by its low activity, its insolubility in water and solubility in apolar solvents, and by its heat-stability. In addition, Tween 80, cephalin, and lecithin were considerably better inhibitors of fixation of the tuberculin-derived product than was the preparation extracted from red cell stroma. These substances possessed in our system <3% of the activity of the receptor. Serum albumin did not prevent erythrocyte fixation of the tuberculin-derived product (4), but was a potent inhibitor of LPS fixation (25). The possibility that the LPS receptor contains active groupings akin to the material inhibiting the tubercle bacillus antigen cannot be excluded, in spite of the profound difference of the LPS receptor from this preparation.

The description, in this paper, of a cell-bound receptor substance which interacted specifically with the endotoxins of gram-negative bacteria

may have general importance, especially since the receptor was found to prevent also the attachment to red cells of lipopolysaccharide which had been preincubated under physiological conditions. Study of this receptor is likely to further the understanding of the mode of attachment of these and other toxic substances to cells and tissue components in addition to erythrocytes.

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