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Lipopolysaccharide Interaction with Rabbit Erythrocyte Membranes

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In this study we have characterized the association of a polysaccharide-deficient, lipid-rich lipopolysaccharide (LPS) with rabbit erythrocytes (RaRBC). With polymyxin B sulfate-mediated hemolysis as a probe, we have shown that *Salmonella minnesota* R595 LPS interacts with RaRBC in two distinguishable steps. The first step whereby RaRBC exposed to LPS are rendered sensitive to polymyxin B-initiated lysis probably represents absorption of LPS to the RaRBC membrane. We investigated two possible mechanisms for the subsequent time-dependent decrease in response of LPS-treated RaRBC to polymyxin B. We found that the decay in polymyxin B susceptibility of LPS-treated RaRBC cannot be attributed to a decrease in binding of LPS to the RaRBC. On the other hand, our results are consistent with a time-dependent rearrangement of the amphipathic LPS within the lipid bilayer of the RaRBC membrane. In particular, at lower incubation temperatures of RaRBC and LPS, the decay in polymyxin B-induced hemolysis is slower, presumably, because the increased membrane viscosity allows less rapid rearrangement of LPS within the lipid bilayer. A putative hydrophobic intercalation of LPS into a mammalian cell membrane may be of importance in LPS stimulation of responsive cells.

Endotoxin, derived from the outer membrane of gram-negative bacteria, is responsible for a plethora of pathophysiological effects in humans and experimental animals. The protein-free lipopolysaccharide (LPS) component of endotoxin has been demonstrated to cause pyrogenicity, hypotension, leukopenia, thrombocytopenia, disseminated intravascular coagulation, and death when administered in an appropriate regimen to experimental animals (20). Numerous studies have implicated endotoxins as a major contributing factor in clinical cases of gram-negative septicemia (34).

The precise LPS-host interactions responsible for these varied pathophysiological responses remain to be fully elucidated, but may, in part, result from the capacity of LPS to activate numerous host cell types. Macrophage activation by LPS results in the generation of procoagulant activity (21), interferon production (12), and generation of monokines (3, 10) as well as a spectrum of additional responses (19, 20). LPS is recognized as a potent stimulus for polyclonal B-lymphocyte activation, leading to proliferation and differentiation into antibody-secreting plasma cells (19). Recent evidence has also suggested multiple effects of LPS on T-lymphocytes (1, 11). Platelets, polymorphonuclear leukocytes, and fibroblasts are also affected in their normal cellular function by LPS (19, 20).

Although these varied biological responses to LPS have been well characterized, the biochemical mechanism(s) by which LPS alters cellular function is not currently understood (18). The LPS molecule consists of polysaccharide covalently bound via a 2-keto-3-deoxyoctulosonate linkage to a unique lipid structure, termed lipid A. Of importance in this respect is the fact that the lipid A region is the component responsible for virtually all of the cellular responses to LPS cited above (33). Although the precise mechanism by which lipid A initiates a direct stimulatory response of these cells is currently not clear, interaction with the cell membrane must be the initial event. There is both biochemical and serological evidence to suggest the presence of an "LPS receptor" on LPS-responsive cells (4, 7, 27), and such LPS ligand-receptor interactions may be responsible for cell triggering. Alternatively, there is evidence documenting the high affinity of LPS for phospholipid monolayers and bilayers (2, 8, 23, 26), and it is equally conceivable that such nonspecific hydrophobic lipid-lipid interaction of LPS with cell membranes may be sufficient to initiate cell triggering (18). In this respect, Warren's finding of an LPS-induced Na^+ influx in rabbit erythrocytes (RaRBC) may represent a mechanism by which LPS enhances membrane permeability and thereby activates cells (31).

In this report, we describe a model system that permits the investigation of the interaction of LPS with cell membranes. Our data provide evidence that the interaction of LPS with erythrocyte membranes takes place in two distinguishable steps. The first involves binding of LPS to the erythrocyte membrane. The second event, which is clearly dissociable from binding, appears to be a molecular rearrangement of LPS within the membrane bilayer by a mechanism that is critically dependent on both the time and temperature of the interaction. These observations provide a foundation for analysis of mammalian cell interactions with LPS that may lead to cell activation and triggering.

MATERIALS AND METHODS

LPS extracted from the *Salmonella minnesota* R595 mutant by the phenol-chloroform-petroleum-ether method of Galanos et al. (9) was used for all experiments reported here. This LPS lacks the O-antigen polysaccharide moiety of LPS and consists of lipid A and a trisaccharide of 2-keto-3-deoxyoctulosonate. The extracted and purified LPS was pretreated for 1 h at 37°C with 0.1 M EDTA in phosphate-buffered saline at pH 7.4 to remove residual traces of LPS-bound divalent cations. We have found (unpublished observations) that this procedure optimizes the reproducibility of the interaction of LPS with polymyxin B sulfate (see below). The LPS was then dialyzed extensively against phosphate-buffered saline and sonicated for 2.5 min with a model W375 sonicator from Heat Systems Ultrasonics at maximal output. The LPS was stored frozen in working samples at −20°C. On the day of use, the LPS was thawed, adjusted to the desired final concentration in phosphate-buffered saline, and briefly

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sonicated. LPS labeled with ^{125}I by the procedure of Ulevitch (29) was generously provided by James Killion in our laboratory. Ulevitch demonstrated that the biological activity of LPS was not altered by this procedure.

Polymyxin B sulfate was obtained from Sigma Chemical Co. (St. Louis, Mo.). It was stored at 4°C and prepared at the desired concentration, usually 50 μg/ml, in phosphate-buffered saline on the day of use.

New Zealand White rabbits obtained from Gilreys Rabbitry (Lawrenceville, Ga.) were bled from the middle ear artery into a volume of acid citrate dextrose approximately one-sixth that of the total blood drawn. The blood was centrifuged at room temperature for 20 min at 300 × *g*, after which the plasma and buffy coat were aspirated and discarded. The pelleted RaRBC were washed three times with saline by centrifugation at 4°C for 15 min at 700 × *g*. RaRBC were stored at 4°C at a concentration of 10% (vol/vol) and were used within 1 week of preparation. On the day of an experiment, the RaRBC were washed three times by centrifugation as described above and suspended at an approximate concentration of 10% (vol/vol) in saline.

Two methods were used to monitor erythrocyte lysis. In the hemoglobin release assay, 200-μl samples of the RaRBC suspension were centrifuged in a Beckman microfuge for 30 s. A 100-μl sample of the supernatant was added to 1.0 ml of saline, and the absorbance was determined at 412 nm. Hemoglobin release was expressed as a percentage of the total hemoglobin of the erythrocytes releasable after osmotic lysis in distilled water. With a Sienco (Morrison, Colo.) Dual Aggregation Meter, turbidity measurements were also employed. In initial experiments, turbidity changes were assessed relative to distilled water lysis of erythrocytes (100%). In later experiments a 1:80 dilution of the untreated erythrocytes was used to adjust maximal transmission. This latter procedure allowed optimal determination of rates of change of hemolysis of erythrocytes. Only hemolytic rates obtained in the same experiment were compared. All data are presented as the change of absorbance per unit time and define relative rates of hemolysis (i.e., the slopes of the curves shown in Fig. 2).

To determine LPS binding to erythrocytes, a 10% (vol/vol) suspension of RaRBC was incubated with a 10 times greater volume of ^{125}I-LPS (0.02 μCi/μg) at a concentration of 50 μg/ml. After incubation, 100 μl of the RaRBC-LPS mixture was layered over 250 μl of a 1:4 mixture of dibutyl phthalate-dinonyl phthalate (17) and centrifuged for 30 s in a microfuge. After severing the microfuge tube below the oil-saline interphase, both the pellet (bound) and the supernatant (unbound) LPS were assayed for radioactivity in a Beckman Gamma 4000 spectrometer. The ratio of counts in the pellet to total counts in the pellet and supernatant times total micrograms of LPS was used as a measure of LPS bound to cells.

RESULTS

Our experiments reported here have been designed to examine the interaction of LPS with RaRBC. As a probe of this interaction we have taken advantage of the well-demonstrated (6, 13, 15, 25) affinity of LPS for the cationic polypeptide antibiotic polymyxin B. The experimental basis for the experiments is summarized in Table 1, which indicates the hemolytic response of LPS-pretreated RaRBC to the subsequent addition of polymyxin B. Several points are worthy of note. First, RaRBC incubated with buffer, with 50 μg of LPS per ml or with 50 μg of polymyxin B per ml at 37°C for a period of time up to 30 min, showed no detectable hemolysis. Second, when RaRBC were preincubated with LPS for short periods of time (e.g., 0.5 min) at 37°C and subsequently treated with polymyxin B, a substantial rate of hemolysis was apparent. Finally, when the preincubation of RaRBC with LPS was allowed to proceed for 5 min before the addition of polymyxin B, the hemolytic rate was considerably diminished, but still greater than that of untreated erythrocytes or erythrocytes treated only with LPS or polymyxin B; when preincubation was extended to 50 min, no hemolysis occurred upon the addition of polymyxin B. Interestingly, hemolysis was not induced when LPS and polymyxin B, at concentrations of 50 μg/ml, were premixed before the addition to RaRBC. Under such conditions, no erythrocyte lysis was detected even after periods of time up to 1 h. These observations suggest that polymyxin B-induced hemolysis of LPS treated RaRBC results from a critical interaction of polymyxin B with LPS associated with the erythrocyte and not by a modification of unbound LPS structure thereby enhancing LPS hemolytic activity. These results further indicate that polymyxin B-induced hemolysis may serve as a probe of the state of association of LPS with RaRBC, and we have utilized this probe to provide evidence for an alteration in association of LPS with erythrocytes as a function of both time and temperature of preincubation.

We employed two hemolytic assays to quantitate this time-dependent change in polymyxin B-initiated hemolysis of LPS modified RaRBC. Figure 1 portrays an initially linear increase in both hemoglobin release and in light transmittance as a function of time after polymyxin B is added to erythrocytes preincubated for a short interval with LPS. The similarity in the time course of response to polymyxin B in the two assays suggests that both assay procedures reflect cell lysis. Because continuous and reproducible increases in transmittance readings with time are more easily obtainable than changes in hemoglobin release, we utilized the former procedure to analyze LPS interaction with RaRBC in subsequent experiments. However, we confirmed that results qualitatively similar to those presented below are obtained when hemoglobin release is used to measure hemolysis.

As indicated in Table 1, hemolysis occurring upon addition of polymyxin B to LPS-pretreated RaRBC is critically dependent on the time of preincubation. The data shown in Fig. 2 and 3 indicate more quantitatively the dependence of hemolytic rate on the time of incubation of RaRBC with LPS. RaRBC were treated with LPS for various periods of

TABLE 1. LPS- and polymyxin B-initiated erythrocyte hemolysis[a]

Initial reactants	Time (min)	Secondary addition	Relative hemolysis (absorbance units/min)
RaRBC + buffer	0.5–30	Buffer	<0.05
RaRBC + LPS	0.5–30	Buffer	<0.05
RaRBC + PB	0.5–30	Buffer	<0.05
RaRBC + LPS	0.5	Polymyxin B	10.5
RaRBC + LPS	4.0	Polymyxin B	0.8
RaRBC + LPS	50	Polymyxin B	<0.05
LPS + polymyxin B[b]	0.5–30	RaRBC	<0.05

[a] RaBRC were incubated at 37°C with either buffer, R595 LPS (50 μg/ml), or polymyxin B (50 μg/ml) for the various times indicated, after which buffer or polymyxin B was added (secondary addition). Relative erythrocyte lysis was then noted after additional 30 min at 37°C.

[b] R595 LPS and polymyxin B (50 μg/ml each) were incubated before the addition of RaRBC.

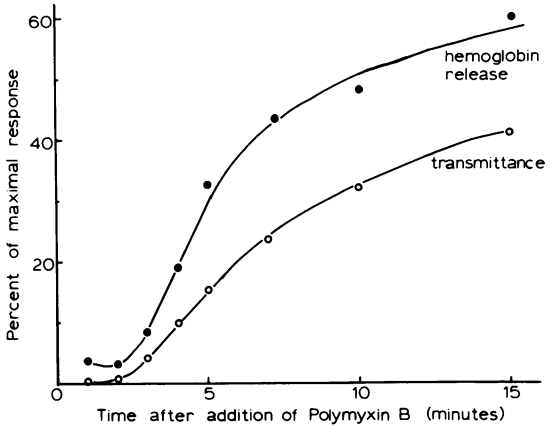

FIG. 1. RaRBC (10%) at room temperature were added to a 10 times greater volume of LPS (50 μg/ml) at 37°C. After 2.5 min, the LPS-RaRBC mixture was added to an equal volume of polymyxin B (50 μg/ml) at 37°C; at various times hemolysis was assayed either turbimetrically or by hemoglobin release as described in the text.

time up to 4.0 min. Polymyxin B was then added, and hemolysis was followed turbimetrically as shown in Fig. 2. We have calculated the slopes of these hemolytic curves from Fig. 2, and we have depicted these rates as a function of time of incubation of RaRBC with LPS in Fig. 3. Of interest is that the rate of hemolysis decreased logarithymically with increasing LPS-RaRBC incubation times. Thus, the polymyxin B-induced hemolytic rate is quantitatively dependent on the time that LPS is allowed to interact with RaRBC, and the hemolysis rate decays with a half life ($t_{1/2}$) of approximately 1.0 min at 37°C.

To facilitate manipulations of our experimental system, we compared two methods to terminate the time-dependent interaction of LPS with RaRBC. In the first, samples of the LPS-RaRBC mixture were added to polymyxin B at 37°C to initiate an immediate hemolysis. Alternatively, the LPS-RaRBC mixture was added to polymyxin B at room temperature. Interestingly, no hemolysis of RaRBC preincubated with LPS occurred upon addition to polymyxin B at room temperature. However, rates of erythrocyte lysis equivalent to those occurring when LPS-preincubated RaRBC were added directly to polymyxin B at 37°C could be demonstrated when the room-temperature mixture composed of LPS-

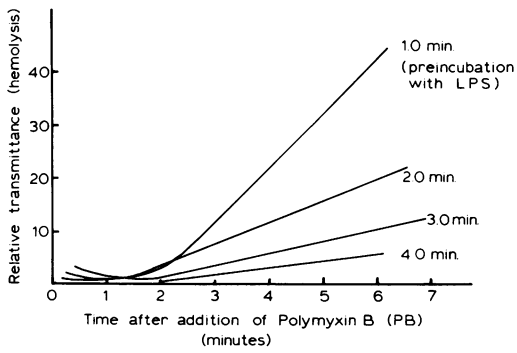

FIG. 2. RaRBC (10%) at room temperature were added to a 10 times greater volume of LPS (50 μg/ml) at 37°C. After 1.0, 2.0, 3.0, and 4.0 min, a sample of the LPS-RaRBC mixture was added to an equal volume of polymyxin B (50 μg/ml) at 37°C. Continuous hemolysis was monitored turbimetrically.

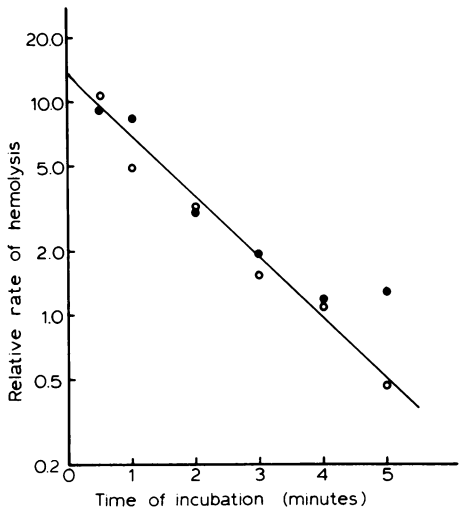

FIG. 3. Slopes of rate of hemolysis curves obtained as shown in Fig. 2 plotted as a function of the time of incubation of RaRBC with LPS. The interaction of LPS with RaRBC was terminated by adding the LPS-RaRBC suspension to polymyxin B at 37°C to initiate hemolysis immediately (○) or by adding the LPS-RaRBC sample to polymyxin B at room temperature (●). When convenient, the room temperature suspension was raised to 37°C, and hemolysis was monitored.

pretreated RaRBC and polymyxin B was raised to 37°C. For some experiments described below, therefore, we have utilized this latter procedure to terminate LPS-RaRBC interactions after various times of incubation.

The relative rate of polymyxin B-induced hemolysis at any specific LPS preincubation time was found to be dependent on the LPS concentration. When various concentrations of LPS were preincubated with a constant number of RaRBC for 0.5 or 2.0 min and then added to a constant amount of polymyxin B, the hemolytic rates presented in Fig. 4 were obtained. The hemolytic rate increased with increasing LPS concentration until the LPS concentration was approximately equal to that of polymyxin B. Greater increases in the amount of LPS then resulted in a decrease in hemolytic rate. The most likely interpretation for this latter observation would be that at high LPS concentrations, unbound, non-RaRBC-associated LPS in solution can compete with cell-

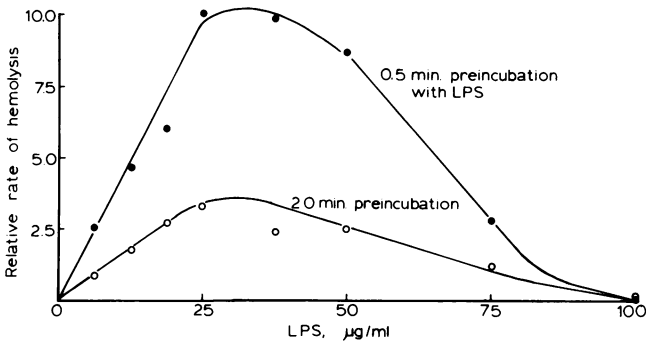

FIG. 4. RaRBC (10%) at room temperature were added to a 10 times greater volume of LPS at 37°C. The concentration of LPS varied from 6.25 to 100 μg/ml. After 0.5 or 2.0 min of incubation, a sample of the LPS-RaRBC suspension was added to an equal volume of polymyxin B (30 μg/ml) at 37°C, and the rate of hemolysis was monitored turbimetrically.

associated LPS for the limited polymyxin B and effectively reduce polymyxin B-initiated hemolysis. This interpretation is supported by experiments in which the inhibition of hemolysis noted at high LPS concentrations can be reversed by increasing the concentration of polymyxin B (data not shown). Further, the fact that, as indicated by the data shown in Table 1, polymyxin B-LPS complexes not associated with erythrocytes do not induce detectable hemolysis would also be consistent with this interpretation.

Because a decrease in hemolytic rates with increasing preincubation time of LPS with RaRBC was observed at all LPS concentrations, it was important to determine whether the rate of decrease or decay in hemolytic rate was dependent on LPS concentration. Although at any specific LPS-RaRBC preincubation time there is increasing hemolysis with increasing LPS concentration, the decay in polymyxin B-induced hemolysis with preincubation time was independent of LPS concentration. Table 2 indicates two experiments showing that at all LPS concentrations the $t_{1/2}$ for decay of hemolytic rate at 37°C was determined to be approximately 1 min. Thus, the decreases in hemolytic rates with increasing LPS-RaRBC incubation time is independent of the LPS concentration; the $t_{1/2}$ for these changes in hemolytic rate can serve as a useful comparison for LPS interactions with RaRBC under various conditions.

We have considered two possible mechanisms that would be consistent with the observed decrease in response to polymyxin B upon increasing the incubation time of LPS and RaRBC. First, there may be a time-dependent decrease in LPS binding to the erythrocyte membrane. Alternatively, the bound LPS may undergo a molecular rearrangement within the RaRBC membrane. To gain further insight into these two mechanisms we investigated the effect of temperature on the $t_{1/2}$ of the decay. The decrease in the relative rate of hemolysis with increasing LPS preincubation time is critically dependent on the temperature of preincubation (Fig. 5). At higher temperatures, the hemolytic rate decays more rapidly. Interestingly, the fact that the initial rates of polymyxin B-induced hemolysis are indistinguishable for erythrocytes treated with LPS at all temperatures examined (Fig. 5) would indicate that the initial sensitization for lysis is not dependent upon temperature.

Data from other experiments comparing the relative $t_{1/2}$ for decreases in hemolytic rates with preincubation time at 37 and 22°C is presented in Table 3. The ratios of change in hemolytic rate at 37°C to that at 22°C indicate that, independent of experimental conditions, the relative hemolytic rate decays more rapidly at 37°C than at 22°C. Figure 6 portrays two experiments in which the change in hemolytic rate with preincubation time is determined as a function of the reciprocal of the absolute temperature of preincubation. By using the standard Van d'Hoff equation, energies of activation were calculated to be approximately 49.8 kJ/mol for experiment 1 and 38.1 kJ/mol for experiment 2.

We have also evaluated the possible dissociation of LPS from the erythrocyte membrane, which might explain the decay in hemolytic rate. In these experiments we determined the temporal association (binding) of LPS to RaRBC and compared the LPS binding with loss of hemolytic response to polymyxin B. Our data do not support the concept that decay in hemolytic rate with increasing preincubation time

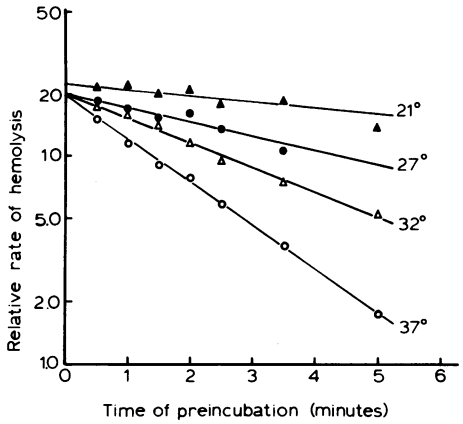

FIG. 5. RaRBC (10%) at room temperature were added to a 10 times greater volume of LPS (50 μg/ml) at 21, 27, 32, and 37°C. After various lengths of time, a sample of the RaRBC-LPS suspension was added to an equal volume of polymyxin B at room temperature to abrogate the LPS-RaRBC interaction. Hemolysis rate was determined as in Fig. 2 and 3. The hemolysis rate was plotted as function of incubation time of RaRBC with LPS at each incubation temperature.

TABLE 2. Decrease of relative rate of hemolysis with preincubation time is independent of LPS or polymyxin B concentration[a]

[LPS] (μg/ml)	[Polymyxin B] (μg/ml)	[LPS]/[PB][b]	$t_{1/2}$[c]	
			Expt 1	Expt 2
75	25	3.0	1.18	1.02
50	25	2.0	0.86	
25	25	1.0	1.02	1.00
12.5	25	0.5	1.05	1.00
6.25	25	0.25	0.96	

[a] Various concentrations of R595 LPS were preincubated with a constant percentage of RaRBC (10%) for various times at 37°C before the addition of a constant amount of polymyxin B. The rates of polymyxin B-induced hemolysis were determined.

[b] The ratio of the concentration of LPS with which RaRBC are preincubated to the constant concentration of polymyxin B employed is shown.

[c] $t_{1/2}$ values were determined from semilogarithmic plots of relative rates of hemolysis as a function of time of incubation of LPS with RaRBC before the addition of polymyxin B. Experiments 1 and 2 were performed on different days.

TABLE 3. Ratios of decay of response to polymyxin B at 37°C and room temperature[a]

Expt no.	Rate of decay of hemolysis (per min)		Rate at 37°C/ rate at 22°C[b]
	37°C	22°C	
1	0.78	0.23	3.4
2	0.52	0.14	3.7
3	0.64	0.19	3.4
4	0.92	0.27	3.4
5	0.92	0.29	3.2

[a] RaRBC were preincubated with 50 μg of LPS per ml at either 37°C or room temperature (22°C) for various periods of time. Then an equal volume of polymyxin B (50 μg/ml) at 37°C was added, and the tubes were transferred to a 37°C environment. The subsequent rate of hemolysis was then monitored turbidometrically (Fig. 2). Slopes of hemolysis rates were then determined as a function of times of pretreatment of RaRBC with LPS, and the rate of decay of hemolysis then determined from semilogarithmic plots (Fig. 3).

[b] Ratio of the rate of decay of hemolysis at 37°C to that at room temperature in experiments performed on different days. The average ± standard error of the mean was 3.6 ± 2.4.

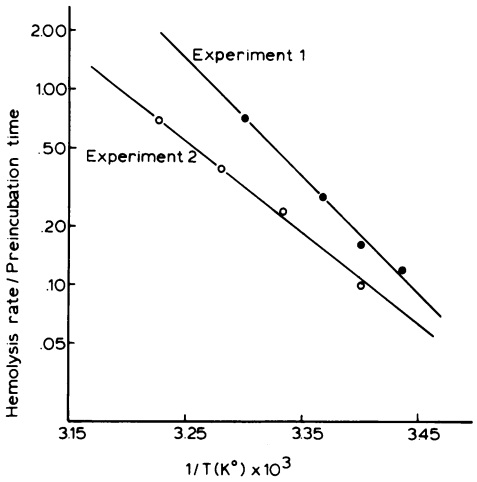

FIG. 6. Dependence of the decrease of hemolysis rate with increasing incubation time of RaRBC and LPS on the reciprocal of the absolute temperature of incubation.

of RaRBC with LPS can be attributed to changes in the amount of LPS bound to the RaRBC. The LPS bound to the RaRBC over 15.0 min of preincubation at 30°C increased gradually from approximately 5.0 to 10.0 μg (Fig. 7). Simultaneously, the relative rates of hemolysis decreased 10-fold. In this experiment we employed a 30°C incubation temperature, rather than 37°C, to decrease the rate of decay of hemolysis. These data would not be consistent with LPS dissociation from the RaRBC as being responsible for the decreased responsiveness to polymyxin B.

DISCUSSION

In the present study, we have employed polymyxin B-induced hemolysis of LPS-treated rabbit erythrocytes to define a time- and temperature-dependent alteration in the nature of the association of LPS with the erythrocyte membrane. The initial interaction of the LPS molecule with the RaRBC rendering the cell sensitive to polymyxin B-induced hemolysis appears to occur rapidly and independently of temperature. The concentrations of LPS employed in our system for sensitization of RaRBC are in the same range as concentrations used to induce other effects of LPS in vitro (19, 20). The event(s) subsequent to the initial sensitization of RaRBC with LPS, as evidenced by a decrease in hemolytic response to polymyxin B, appears to be critically dependent upon both time and temperature. Our results are not inconsistent with the postulate of a two-step process of LPS binding followed by either a molecular rearrangement of the amphipathic LPS within the erythrocyte membrane bilayer or an LPS-induced alteration of the erythrocyte membrane.

Polymyxin B induction of lysis of cells exposed to LPS may be an extremely useful tool for investigating the association of LPS with cell membranes. Cationic polymyxin B has been previously shown to bind either carboxylates of the 2-keto-3-deoxyoctulosonate component or phosphates on the diglucosamine region of the lipid A region of LPS (15, 25). Such an LPS-polymyxin B interaction has been shown to alter a number of biological properties of LPS including B lymphocyte proliferation (13), macrophage activation (6), and activation of the classical complement pathway (17). Polymyxin B also appears to be able to complex with LPS not bound to the RaRBC membrane and to thereby abrogate the ability of LPS to sensitize RaRBC to polymyxin B-mediated hemolysis. We have recently begun to investigate the mechanism by which polymyxin B causes hemolysis of RaRBC exposed to LPS, even though we believe that this event provides a probe to monitor the LPS interaction with membranes independent of the precise mechanisms involved. We have obtained preliminary results in support of the postulate that LPS bound to the surface of RaRBC is the specific site for polymyxin B-mediated hemolysis. LPS extracted from a polymyxin B-resistant rough mutant of *Salmonella typhimurium* does not sensitize the cells to polymyxin B-mediated hemolysis, whereas LPS extracted from the parental strain does sensitize RaRBC to polymyxin B (manuscript in preparation). It may be of relevance to note, in addition, that Vaara and Vaara (30) have recently provided evidence to suggest that polymyxin B binding to LPS localized on the outer membrane of gram-negative bacteria results in disruption of outer membrane integrity. It is not unreasonable to postulate that similar mechanisms may be operative in the LPS-erythrocyte hemolytic system we describe here.

The decrease, or decay, in polymyxin B-initiated hemolysis with increasing LPS-RaRBC preincubation time is consistent with the proposal that after binding the lipid-rich R595 LPS rearranges itself within the lipid bilayer of the membrane in a manner dependent upon hydrophobic interactions. With greater preincubation time, which may result in increased rearrangement of the bound LPS, the LPS-modified erythrocyte membrane, initially very sensitive, becomes less susceptible to polymyxin B-mediated hemolysis. The decreased decay of hemolytic rates at lower temperatures supports this model of LPS-membrane interaction. Increased membrane viscosity at lower temperatures would result in less effective rearrangement and less rapid hemolytic rate decay.

The molecular nature of this temperature-dependent rearrangement mechanism is currently not known. One possibility would be that hydrophobic R595 LPS molecules insert into the erythrocyte phospholipid bilayer and become inaccessible to polymyxin B. The initial temperature-indepen-

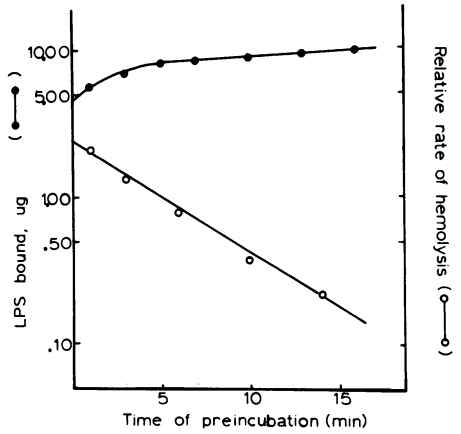

FIG. 7. RaRBC (10%) at room temperature were added to a 10 times greater volume of LPS (50 μg/ml) at 30°C. After various periods of time the amount of LPS associated with RaRBC was determined as described in the text. Bound LPS was determined in duplicate samples, and the duplicates were averaged. The hemolysis rate at various incubation times of LPS with RaRBC at 30°C was determined as in Fig. 3.

dent membrane modification may be analogous to the initial absorption of LPS to the undersurface of phospholipid monolayers before LPS penetration of the monolayers as described by Benedetto et al. (2). A related postulate is that with insertion of R595 LPS into the erythrocyte membrane, membrane fluidity is decreased, and the cell is rendered more resistant to polymyxin B lysis. Rottem has shown that lipid A decreases the fluidity of egg lecithin liposomes (24), and Liu et al. have demonstrated that R595 decreases the fluidity of phosphatidylethanolamine or phosphatidic acid dispersions (14). The inhibition of marker release from liposome preparations by various LPS preparations may constitute confirmatory evidence for increased membrane rigidity upon LPS exposure (5, 22, 24). In this respect, Warren and Kowalski's finding that base hydrolyzed LPS inhibits concanavalin A-induced agglutination of human erythrocytes may indicate a membrane-rigidifying effect of LPS (32). Experiments are currently in progress in our laboratory to explore this possibility.

A second putative mechanism for a temperature-dependent rearrangement of LPS within the lipid bilayer of the cell membrane might be by a "capping" phenomenon after the initial LPS binding. This altered state of association of LPS with the membrane may render the cell resistant to polymyxin B. Swartzwelder and Jacobs have provided data to suggest that LPS bound to murine lymphocytes caps in a manner dependent on temperature. Although these authors suggest that LPS capping is similar to that seen when antigen or antibody to surface immunoglobulin cross-links lymphocyte receptors (F. Schwartzwelder and D. M. Jacobs, Fed. Proc. **42**:413, 1983), an alternate interpretation that membrane-bound LPS molecules aggregate due to a strong self-affinity can not be dismissed. The strong self-affinity of LPS would be inconsistent with an alternate model of LPS rearrangement in which LPS initially binds the membranes as patches and then disperses in the lipid bilayer allowing polymyxin B resistance. Takeuchi and Nakaido (29) have shown that LPS molecules existing as domains in phospholipid dispersions remain tightly associated and do not undergo monomolecular dispersion even after relatively long (>72 h) periods of incubation. Work to define the nature of putative LPS rearrangement is being performed in our laboratory. Nevertheless, the energy of activation calculated in the experiments reported here for the decay in hemolytic rates (41.9 kJ/mol) suggests that the rearrangement of LPS into the lipid bilayer is thermodynamically feasible.

A third explanation for the decay in hemolytic rates is that the LPS bound to erythrocytes rendering them polymyxin B sensitive might become dissociated from the membrane over time. Davies et al. have demonstrated cyclical binding of an *Escherichia coli* LPS to RaRBC (5). However, in that study the first decrease in LPS-cell binding was not significant until 45.0 to 90.0 min of incubation of the LPS with rabbit erythrocytes at 37°C. Thus, the decreased LPS binding demonstrated by Davies et al. occurred at a time significantly later than the time at which we have found markedly decreased hemolytic rates. To address this question more conclusively we have shown that, rather than a decrease in binding, there is actually a modest increase coincident with a 10-fold decrease in susceptability to polymyxin B-induced hemolysis.

It is difficult to envision a mechanism by which the modest increase in LPS binding to RaRBC occurring in the first 5.0 min of incubation of RaRBC with LPS could lead to the more prolonged decrease in polymyxin B sensitivity, particularly since the hemolysis rate is decreased by more than 80% during this time period. In addition biphasic responses are only noted when LPS is in excess relative to polymyxin B. Since all of our experiments are performed in polymyxin B excess, we believe it unlikely that the decrease in polymyxin B hemolysis simply reflects an increase in LPS binding to the erythrocytes.

In this study we have most extensively characterized the interaction of a polysaccharide-deficient LPS, R595, with RaRBC. However, in other experiments we have found that LPS derived from *E. coli* J5 reacts in a qualitatively similar, although not identical, manner with RaRBC and that both sheep and human erythrocytes treated with R595 LPS are also rendered sensitive to polymyxin B-induced hemolysis (data not shown). These experiments suggest that the polysaccharide content of LPS and differences in membrane properties of cells from various species may modulate the modification of the cells by LPS. A two-step model of initial association and subsequent rearrangement to describe LPS interaction with the cell membrane is therefore not unique to RaRBC and R595 LPS.

Although, we have proposed a two-step model for the postulated nonspecific hydrophobic interaction of R595 LPS with RaRBC membranes, it would be premature to generalize these results to putative events operative in LPS activation of mammalian cells. A hydrophobic LPS rearrangement causing generalized membrane perturbation, such as intercalation into the lipid bilayer, may be the critical event in cell triggering, or it may be independent of and mask a specific, crucial binding of LPS to the putative LPS receptors. Intercalation may represent one of a series of events allowing subsequent LPS interaction with an intrinsic membrane protein or cytoplasmic factor necessary for cell stimulation. Springer's description of an erythrocyte membrane protein having specific affinity for LPS is not inconsistent with intercalation being one of a series of events leading to cell triggering (27). However, Coutinho et al. have presented serological evidence for the presence of a specific surface membrane receptor for LPS on murine B lymphocytes that is important in cell stimulation (4, 7). On the other hand, numerous investigators have proposed a nonspecific hydrophobic interaction of LPS with lipid bilayers (2, 8, 23, 26). Both Benedetto et al. (2) and Romeo et al. (23) have demonstrated increased surface pressures when LPS was allowed to interact with phospholipid monolayers, and Benedetto further showed increased surface potential of these LPS-treated monolayers (2). Those accumulated data support a mechanism of surface absorption of LPS followed by penetration into the monolayer upon LPS interaction with lipid membranes. It is attractive to consider a similar mechanism both in the system we describe here with intact erythrocyte membranes and perhaps in other mammalian cells in which LPS elicits profound and diverse cellular responses.

LITERATURE CITED

1. **Armerding, D., and D. H. Katz.** 1974. Activation of T and B lymphocytes *in vitro*. Regulatory influence of bacterial lipopolysaccharide (LPS) on splenic T-cell helper function. J. Exp. Med. **139**:24–43.
2. **Benedetto, D. A., J. W. Shands, Jr., and D. O. Shah.** 1973. The interaction of bacterial lipopolysaccharide with phospholipid bilayers and monolayers. Biochim. Biophys. Acta **298**:145–157.
3. **Bodel, P.** 1974. Studies on the mechanism of endogenous pyrogen production. III. Human Blood Monocytes. J. Exp. Med. **140**:954–965.
4. **Coutinho, A., L. Forni, and T. Watanabe.** 1978. Genetic and functional characterization of an antiserum to the lipid A-

specific triggering receptor on murine B-lymphocytes. Eur. J. Immunol. **8**:63–67.
5. **Davies, M., D. E. S. Stewart-Tull, and D. M. Jackson.** 1978. The binding of LPS from *E. coli* to mammalian cell membranes and its effect on liposomes. Biochim. Biophys. Acta **508**:270–276.
6. **Doe, W. F., S. T. Yang, D. C. Morrison, S. J. Betz, and P. M. Henson.** 1978. Macrophage stimulation by bacterial lipopolysaccharides. II. Evidence for differentiation signals delivered by lipid A and by a protein rich fraction of lipopolysaccharide. J. Exp. Med. **148**:557–558.
7. **Forni, L., and A. Coutinho.** 1978. An antiserum which recognizes LPS reactive B-cells in the mouse. Eur. J. Immunol. **8**:56–62.
8. **Fried, V. A., and L. I. Rothfield.** 1978. Interactions between lipopolysaccharide and phosphatidylethanolamine in molecular monolayers. Biochim. Biophys. Acta **298**:145–147.
9. **Galanos, C., O. Luderitz, and O. Westphal.** 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. **9**:245–249.
10. **Gery, I., R. K. Gershon, and B. H. Waksman.** 1971. Potentiation of cultured mouse thymocyte responses by factors released from peripheral lymphocytes. J. Immunol. **107**:1778–1780.
11. **Hamaoka, T., and D. H. Katz.** 1973. Cellular site of action of various adjuvants in antibody responses to hapten-carrier conjugates. J. Immunol. **111**:1554–1563.
12. **Ho, M., and J. A. Armstrong.** 1975. Interferon. Annu. Rev. Microbiol. **29**:139.
13. **Jacobs, D. M., and D. C. Morrison.** 1977. Inhibition of the mitogenic response to lipopolysaccharide (LPS) in mouse spleen cells by polymyxin B. J. Immunol. **18**:21–27.
14. **Liu, M., T. Onji, and N. E. Snelgrove.** 1982. Changes in phase transition temperature of phospholipids induced by endotoxin. Biochim. Biophys. Acta **710**:248–251.
15. **Morrison, D. C., and D. M. Jacobs.** 1976. Binding of polymyxin B to the lipid A portion of bacterial LPS. Immunochemistry **13**:813–818.
16. **Morrison, D. C., and D. M. Jacobs.** 1976. Inhibition of lipopolysaccharide-initiated activation of serum complement by polymyxin B. Infect. Immun. **13**:298–301.
17. **Morrison, D. C., Z. G. Oades, and D. DiPietro.** 1980. Endotoxin induced calcium-dependent platelet secretion-implications for generalized membrane perturbations, p. 311–325. *In* M. K. Agarwal (ed.), Bacterial endotoxins and host response—1980. Elsevier-North Holland Biomedical Press, New York.
18. **Morrison, D. C., and J. A. Rudbach.** 1981. Endotoxin-cell membrane interactions leading to transmembranes signaling, p. 187–218. *In* F. P. Imman and W. J. Mandy (ed.), Contemporary topics in molecular immunology *8*—1981. Plenum Publishing Corp., New York.
19. **Morrison, D. C., and J. L. Ryan.** 1979. Bacterial endotoxins and host immune responses. Adv. Immunol. **28**:293–450.
20. **Morrison, D. C., and R. J. Ulevitch.** 1978. The effects of bacterial endotoxins on host mediation systems. Am. J. Pathol. **93**:527–618.
21. **Niemitz, J., and K. Fauni.** 1971. Role of leukocytes in blood coagulation and the generalized Shwartzmann reaction. Nature (London) New Biol. **232**:247–248.
22. **Onji, T., and M. Liu.** 1981. Effect of E. coli endotoxin on the leakage of ^{14}C-sucrose from phosphatidylcholine liposomes. Circ. Schock **8**:403–410.
23. **Romeo, D., A. Girard, and L. Rothfield.** 1970. Reconstitution of a functional membrane enzyme system in a monomolecular film. I. Formation of a mixed monolayer of LPS and PL. J. Mol. Biol. **53**:475–490.
24. **Rottem, S.** 1978. The effect of lipid A on the fluidity and permeability properties of phospholipid dispersions. FEBS Lett. **95**:121–124.
25. **Schindler, M., and M. J. Osborn.** 1979. Interaction of divalent cations and polymyxin B with LPS. Biochemistry **18**:4425–4430.
26. **Schuster, B. G., R. F. Palmer, and R. S. Aronson.** 1970. The efect of endotoxin on thin lipid bilayer membranes. J. Membr. Biol. **3**:67–72.
27. **Springer, G. F., S. V. Huprikar, and E. Neter.** 1970. Specific inhibition of endotoxin coating of red cells by a human erythrocyte membrane component. Infect. Immun. **1**:98–108.
28. **Takeuchi, Y., and H. Nakaido.** 1981. Persistence of segregated phospholipid domains in phospholipid-lipopolysaccharide mixed bilayers: studies with spin-labeled phospholipids. Biochemistry **20**:523–529.
29. **Ulevitch, R. J.** 1978. The preparation and characterization of a radioiodinated bacterial lipopolysaccharide. Immunochemistry **15**:157–164.
30. **Vaara, M., and T. Vaara.** 1981. Outer membrane permeability barrier disruption by polymyxin in polymyxin-susceptible and -resistant *Salmonella typhimurium*. Antimicrob. Agents Chemother. **19**:578–583.
31. **Warren, J. R.** 1982. Stimulation of Na^+ influx by endotoxic lipopolysaccharide and glycolipid. FEMS Microbiol. Lett. **17**:41–44.
32. **Warren, J. R., and M. M. Kowalski.** 1977. Inhibition of Con A erythroagglutination by alkali-treated lipopolysaccharide. Exp. Cell Res. **107**:462–466.
33. **Westphal, O.** 1975. Bacterial endotoxins. The Second Carl Pransnitz Memorial Lecture. Int. Arch. All. App. Immunol. **49**:1–43.
34. **Wolff, S. M.** 1973. Biological effect of bacterial endotoxins in man, p. 251. *In* E. H. Kass and S. M. Wolff (eds.), Bacterial lipopolysaccharides—1973. University of Chicago Press, Chicago.