Mechanism of Polymyxin B-Mediated Lysis of Lipopolysaccharide-Treated Erythrocytes

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Received 10 December 1984/Accepted 26 March 1985

A novel system was used previously to characterize the dynamic interaction of a polysaccharide-deficient, lipid-rich lipopolysaccharide (LPS) with rabbit erythrocytes (RaRBC). Exposure of the RaRBC to the LPS rendered them sensitive to induction of hemolysis by the cationic antibiotic polymyxin B (PB) in a time- and temperature-independent manner. Subsequent decay in the response of LPS-sensitized cells to PB was shown to be critically dependent on both the time and temperature of incubation of RaRBC with LPS and to be independent of a change in LPS binding (Carr and Morrison, *Infect. Immun.* 43:600-606, 1984). In the present study, we performed experiments designed to define the mechanism by which PB mediates hemolysis of LPS-sensitized RaRBC. Experiments were performed to examine the molecular requirements of the LPS and the PB that were essential for hemolytic activity. The capacity of various cations to mediate hemolysis of LPS-sensitized RaRBC or to block PB-mediated hemolysis and the temperature dependence of the PB lytic reaction were investigated. The results of these experiments suggest that PB-mediated hemolysis of LPS-treated erythrocytes is dependent upon an initial ionic association of PB with erythrocyte membrane-bound LPS, followed by hydrophobic insertion of the PB fatty acid into the erythrocyte membrane lipid bilayer.

Bacterial endotoxins have long been recognized for their capacity to bind to the membranes of a variety of cells of mammalian origin. The now classic studies of Neter and colleagues (11) established that endotoxins may be adsorbed onto erythrocytes and that such interactions probably involve the lipid A component of the lipopolysaccharide (LPS) part of the endotoxin. Neter et al. showed that procedures which modify primarily the lipid A component of LPS also alter the binding of the molecules to erythrocytes. Since these studies, other investigators have demonstrated binding of LPS to mammalian cells as diverse as polymorphonuclear leukocytes, macrophages, lymphocytes, platelets, and endothelial cells (reviewed in reference 10).

The mechanisms by which such interactions between lipid A LPS and cell membranes may occur include both non-specific hydrophobic interactions and specific LPS receptors. There is good evidence that in most instances, LPS binding to mammalian cells is nonsaturable, suggesting that, if specific LPS receptors do exist, they may not be readily detectable. On the other hand, biochemical evidence for the presence of lipoglycoprotein LPS receptor on human erythrocytes has been reported (18). In addition, immunoserological evidence has suggested the presence of defined antigens on LPS-responsive cells tentatively identified as LPS receptors (2, 4). The failure of other investigators to reproduce these latter data (30) suggests that such receptors, if they do in fact exist, are not easily identifiable. Independent of the nature of the initial interaction of LPS with mammalian cell membranes, the biochemical fate of these molecules after binding is even less well understood. There is experimental evidence in support of rapid shedding of these molecules (19), cyclical desorption and readsorption (3), capping (F. Swartzwelder and D. M. Jacobs, *Fed. Proc.* 42:413, 1983), and internalization and biochemical processing (23).

We recently described an experimental system which allows us to examine the events surrounding the interaction of LPS with mammalian erythrocytes which occur after an

initial binding interaction (1). For these studies, we used the well-documented capacity of LPS to interact with the cationic polypeptide antibiotic polymyxin B (PB) (9, 15). In these experiments, we showed that LPS from the Re mutant of *Salmonella minnesota* R595, when initially bound to rabbit erythrocytes (RaRBC), promotes hemolysis upon subsequent addition of PB. However, the rates of hemolysis decrease semilogarithmically as the time of interaction between LPS and the erythrocytes increases before addition of PB. Importantly, the decrease in the hemolytic potential of the LPS-treated erythrocytes subsequently exposed to PB is critically dependent upon temperature and is completely abrogated at temperatures below 22°C. The results of these combined studies suggested that there is a dynamic interaction of LPS with the erythrocyte membrane after binding and that the molecular state of the LPS on or in the erythrocyte membrane is the critical factor which determines whether lysis by PB can occur.

Although the parameters of this experimental system which define hemolysis, as well as those which characterize the loss of hemolytic potential, were comprehensively investigated in previous studies (1), the mechanism by which hemolysis occurred was not explored. Therefore, we performed experiments to examine the molecular requirements of the LPS and the PB which are essential for the manifestation of hemolytic activity. The results of these experiments are reported here. Our data suggest that PB-initiated hemolysis of LPS-treated erythrocytes depends upon an initial ionic binding of PB to LPS associated with the erythrocyte membrane. This is followed by a hydrophobic insertion of the PB terminal fatty acid into the membrane, which then results in membrane disruption and subsequent lysis.

(The results were taken from a Ph.D. thesis submitted by C.C. to the Emory University School of Medicine.)

MATERIALS AND METHODS

Bacteria. *S. minnesota* R595 and *Salmonella typhimurium* SH7426 and SH9178 are LPS core mutant strains with well-

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FIG. 1. PB and PBNP structures. PB was treated with ficin by the method of Vaara and Vaara (26) to remove the terminal diaminobutyric acid and fatty acid residues of PB and form PBNP. DAB, Diaminobutyric acid; Thr, threonine; Phe, phenylalanine; Leu, leucine.

defined LPS chemotypes. *S. minnesota* R595 is an Re mutant having only the 3-deoxy-D-mannooctulosonic acid residues of the core polysaccharide region of LPS bound to the lipid A region; strains SH7426 and SH9178 are Rb2 mutants of *S. typhimurium* LT-2 which possess all except the two terminal polysaccharide residues of the core but none of the O-antigen side chain region (24). Strain SH7426 is a *pmrA* mutant of strain SH9178 and is resistant to the bactericidal effect of PB. This phenotypic characteristic is consistent with the observation that strain SH7426 LPS binds significantly less PB than strain SH9178 LPS (29). *S. typhimurium* strains SH7426 and SH9178 were generous gifts from Martti Vaara, Helsinki, Finland.

LPS. LPS was extracted from *S. minnesota* R595, from *S. typhimurium* SH7426 and from *S. typhimurium* SH9178 by the phenol-chloroform-petroleum ether method of Galanos et al. (5). A group of LPS preparations with well-defined altered core polysaccharide structures extracted from mutant *S. minnesota* strains by the same procedure were purchased from List Laboratories, Campbell, Calif.

PB. PB sulfate and poly-L-lysine were purchased from Sigma Chemical Co., St. Louis, Mo. These chemicals were stored at 4°C and were prepared at the desired concentration in phosphate-buffered saline on the day of use. Polymyxin B nonapeptide (PBNP), a derivative of PB lacking the terminal amino acid and the fatty acid moiety, was generously provided by Martti Vaara and was prepared as described by Vaara et al. (26). The structures of PB and PBNP are shown in Fig. 1. CaCl_2 was obtained from the J. T. Baker Co., Pittsburg, Pa.

Erythrocytes. New Zealand white rabbits obtained from Gilreys Rabbitry, Lawrenceville, Ga., were bled from the middle ear artery into a volume of acid-citrate-dextrose that was approximately one-sixth the volume of the total blood drawn. The blood was centrifuged at room temperature for 20 min at $300 \times g$, 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 $700 \times g$. These RaRBC were stored at 4°C at a concentration of 10% (vol/vol) and were used within 2 weeks of preparation. On the day that RaRBC were used in an experiment, they were washed three times by centrifugation as described above and suspended at a concentration of approximately 10% (vol/vol) in saline.

Hemolysis determinations. Using a dual aggregation meter (Sienco, Morrison, Colo.), we measured turbidity to monitor erythrocyte lysis. We previously demonstrated that turbidity measurements correlate with hemoglobin release assays in determinations of erythrocyte hemolysis (1). In the present experiments a 1:80 dilution of the untreated stock suspen-

sion of erythrocytes in phosphate-buffered saline was used to adjust maximal (100%) transmission. This procedure allowed optimal determination of rates of change of hemolysis of erythrocytes with time. Some data are presented as changes in transmittance per unit time (defined as the relative rate of hemolysis). Only hemolytic rates obtained in the same experiment were compared.

RESULTS

We showed previously that RaRBC pretreated with *S. minnesota* R595 LPS lyse upon subsequent addition of PB and that the lytic response of LPS-treated RaRBC to PB "decayed" at 37°C but not at 4°C. In the majority of the experiments performed in this study to explore the mechanism of the PB-mediated lysis, we used experimental conditions in which RaRBC was added to LPS at 4°C in the absence of PB and subsequently transferred to a 37°C PB environment; that is, a sample of the LPS-RaRBC mixture at 4°C was added to an equal volume of PB at 37°C and transferred to the aggregation meter at 37°C. In the temperature dependence experiments, the RaRBC were sensitized with LPS at room temperature, and the LPS-RaRBC sample was then added to the solution of PB at such a temperature that the final reaction temperature obtained was the temperature at which the PB lytic reaction mixture was to be assayed. The reaction mixture was maintained at this temperature in a water bath. Hemolysis was then recorded continuously as described above.

Our previous data suggested that rates of lysis were dependent upon the concentration of LPS used to sensitize the erythrocytes. To examine in more detail the dependence of hemolysis upon LPS and PB concentrations, RaRBC were treated either with increasing concentrations of *S. minnesota* R595 LPS at 4°C and then with excess PB at 37°C or with an

FIG. 2. RaRBC (10%) at room temperature were added to 10-times-greater volumes of LPS at different concentrations (25, 50, and 100 $\mu\text{g/ml}$) at 4°C. After 15.0 min the RaRBC were washed and suspended in cold buffer. Samples of the LPS-treated and washed cells were then added to equal volumes of PB at different concentrations (50, 100, and 200 $\mu\text{g/ml}$) at 37°C. Hemolysis was monitored turbidimetrically at 37°C.

optimal concentration of LPS at 4°C and then with increasing concentrations of PB at 37°C. The results of these experiments (Fig. 2) basically confirmed that hemolysis rates are dependent upon both the concentration of LPS used to sensitize the RaRBC and the concentration of PB used to mediate lysis.

Since *S. minnesota* R595 LPS represents a unique LPS in that it is devoid of all of the carbohydrate component characteristic of the outer core polysaccharide and O-antigen regions of LPS, it was of interest to determine whether the hemolytic response demonstrated in Fig. 2 was restricted to the Re type of LPS. Therefore, a variety of LPS chemotypes derived from *S. minnesota* mutants were assayed at several LPS concentrations. The results of this experiment (Fig. 3) confirmed that all R mutant LPS chemotypes except the LPS from the Ra mutant are capable of rendering RaRBC sensitive to PB-mediated hemolysis. We also demonstrated that LPS from *Escherichia coli* 055:B5 (smooth strain) is inactive in sensitizing RaRBC for PB-mediated lysis (data not shown). It is noteworthy that the efficiency of sensitization did not correlate precisely with core polysaccharide chain length, suggesting that other variables may contribute to the activity of the LPS (see below).

Our previous observations showed that LPS must interact with the erythrocytes before addition of PB if lysis is to occur (1). These findings suggest that PB interacts directly with LPS on the membrane surface of the RaRBC. However, another possible interpretation is that LPS modifies the membrane in such a manner as to allow subsequent interaction of PB with the LPS-altered membrane, resulting in cell lysis. To test whether PB must interact directly with LPS on the membrane surface, we performed experiments in which we used two LPS preparations with different capacities to bind PB. *S. typhimurium* strain SH9178 is an Rb LPS chemotype mutant. A substrain, strain SH7426, which was

FIG. 3. RaRBC (10%) at room temperature were added to 10-times-greater volumes of LPS at 4°C. The LPS had been extracted from various *S. minnesota* mutants having defined alterations in their core polysaccharide structures (Ra, Rb, Rc, Rd, and Re mutants). After 15 min samples of LPS-sensitized cells were added to equal volumes of a solution containing 200 µg of PB per ml at 37°C, and hemolysis was monitored turbidimetrically at 37°C.

FIG. 4. RaRBC (10%) at room temperature were added to 10-times-greater volumes of LPS at 4°C. The LPS had been extracted from PB-resistant *S. typhimurium* strain SH7426, from PB-sensitive parental strain *S. typhimurium* SH9178, and from *S. minnesota* R595. After 15 min samples of LPS-sensitized cells were added to equal volumes of a solution containing 200 µg of PB per ml at 37°C, and hemolysis was monitored turbidimetrically at 37°C.

derived from strain SH9178, has been characterized as relatively resistant to the antibactericidal effects of PB. Of importance to the experiments described here, the LPS isolated from this strain has been shown to bind significantly less PB than the LPS isolated from the parental strain (29), most probably due to increased substitution of lipid A with 4-amino-arabinose (28). When these two Rb LPS preparations were compared with *S. minnesota* Re LPS for sensitization of RaRBC to PB lysis, the results shown in Fig. 4 were obtained. LPS extracted from parent strain SH9178 rendered RaRBC sensitive to PB-mediated lysis, whereas LPS obtained from *S. typhimurium* SH7426 did not render the cells sensitive to PB lysis under similar conditions. In additional experiments (data not shown) we obtained preliminary evidence suggesting that the inability of PB to lyse strain SH7426 LPS-treated RaRBC was not due to an inability of this LPS preparation to interact with the RaRBC. Thus, these data provide support for the hypothesis that erythrocyte-bound LPS is, in fact, the target molecule with which PB must interact to initiate lysis.

Previously published data from our laboratory (9) and another laboratory (15) have suggested that the interaction between LPS and PB involves both ionic and hydrophobic interactions. The ionic interactions are probably mediated by LPS phosphate and carboxyl groups which bind to cationic amino groups of the diaminobutyric acid residues of PB. The hydrophobic interactions involve the terminal PB octanoic acid and lipid A localized ester- and amide-linked fatty acids (9). Therefore, we assessed the relative contributions of these interactions to the initiation of *S. minnesota* Re595-dependent PB-induced lysis.

As a first approach, we asked whether a strictly ionic interaction between RaRBC-bound LPS and various polyvalent cations induced lysis. We took advantage of the fact that PB may be selectively modified by treatment with

FIG. 5. RaRBC were sensitized with *S. minnesota* R595 LPS (50 µg/ml) as described in the legend to Fig. 2. Samples of the sensitized and washed cells were then added to equal volumes of a solution containing 50 µg of PB per ml at 37°C along with different concentrations of Ca^{2+} , poly-L-lysine (PLL), and PBNP. The capacities of the cations (at specific concentrations) to inhibit PB-mediated hemolysis are shown.

ficin so that the terminal amino acid and octanoic acid are cleaved, generating a PBNP without the hydrophobic "tail" (Fig. 1). As shown in Fig. 5, neither calcium, poly-L-lysine, nor PBNP, even at molar concentrations that were an order of magnitude greater than the effective PB concentration, was able to initiate lysis. Interestingly, however, as shown in Fig. 5, high concentrations of these inactive cations blocked lysis initiation by PB. Thus, these observations support the hypothesis that an ionic interaction between RaRBC-bound LPS and PB may be necessary, but not sufficient, for the induction of erythrocyte hemolysis. Specifically, the presence of the cationic polypeptide PBNP alone appears not to be sufficient to initiate lysis, even though preparations containing PBNP still have the capacity to block lysis initiated by the intact PB. Importantly, these results also suggest that the terminal hydrophobic octanoic acid is critical for effective induction of hemolysis.

Our data are consistent with the hypothesis that hydrophobic interactions of the PB octanoic acid with either the RaRBC phospholipid bilayer or the erythrocyte-localized LPS contribute to hemolysis. To provide additional support for this hypothesis, we investigated the temperature dependence of the rate of lysis induced by PB. The results of this experiment (Fig. 6A) clearly showed a marked dependence of lysis upon temperature. When the data were analyzed by a Van't Hoff plot (Fig. 6B), a straight line was obtained, with an activation energy of about 83.7 to 167.4 KJ/mol.

DISCUSSION

In this study we examined the mechanism by which LPS-sensitized erythrocytes are lysed by PB. Our results are consistent with the hypothesis that PB-mediated lysis of erythrocytes sensitized with LPS depends upon several distinguishable biochemical interactions. Initially, PB binds to LPS on the erythrocyte membrane through a combination of ionic and hydrophobic interactions. This is followed by hydrophobic interaction of the PB fatty acid side chain with the lipid bilayer, resulting in membrane disruption and lysis.

To investigate the biochemical requirements of the erythrocyte-bound LPS, we used LPS isolated from a variety of bacterial strains. In particular, we isolated LPS from a

PB-resistant gram-negative mutant of *Salmonella* and its PB-sensitive parent. Both the PB-resistant bacteria and LPS extracted from these bacteria bound significantly less PB than the parental PB-sensitive strain or LPS derived from this strain (29). This may have been the result of increased levels of 4-amino-arabinose which substituted for one of the phosphate moieties of the lipid A region of the mutant LPS (28). The finding that LPS extracted from the PB-resistant strain is ineffective in sensitizing RaRBC to PB (Fig. 4), even when PB is used at a concentration that is 10 times greater than the normal effective concentration, constitutes strong evidence in support of the hypothesis that there is an ionic interaction in the binding of PB to membrane-associated LPS, most probably involving lipid A-bound phosphate residues. We confirmed that LPS from the PB-resistant bacteria, LPS from the PB-sensitive parent, and LPS from

FIG. 6. RaRBC (10%) at room temperature were added to a 10-times-greater volume of LPS (50 µg/ml) at room temperature. A sample of the sensitized RaRBC was added to an equal volume of PB (50 µg/ml) at a temperature so that the appropriate PB lytic reaction temperature was obtained (37, 33, 30, or 23.5°C). (A) Extents of PB-induced hemolysis at different lytic reaction temperatures after PB addition, monitored turbidimetrically. (B) Logarithm of the relative rate of hemolysis (A) plotted versus the reciprocal of the absolute temperature of the PB lytic reaction.

an Rb mutant of *S. minnesota* all manifest qualitatively similar binding to RaRBC. Using a 3-deoxy-D-mannooctulosonic acid assay to monitor the amount of LPS absorbed by RaRBC, we found that these cells absorbed 20, 35, and 25% of the PB-resistant, PB-sensitive, and *S. minnesota* Rb LPS, respectively. Thus, we concluded that the lack of lysis observed with the PB-resistant LPS was not the result of an inability of this LPS to bind to RaRBC.

The available evidence indicates that the interaction of PB with LPS is at least partially dependent on ionic interactions between cationic amino groups of the PB and LPS-associated phosphate and carboxyl groups (9, 15). The hypothesis that these ionic interactions are relevant for erythrocyte lysis is further supported by the finding that other cations, including PBNP, Ca^{2+} , and poly-L-lysine, can all block PB-mediated hemolysis under appropriate conditions. PBNP and poly-L-lysine were found to be more efficient inhibitors of PB-mediated lysis than Ca^{2+} . These results suggest that the greater charge or the molecular configuration of PB, PBNP, and poly-L-lysine may allow more efficient interaction of these molecules than of Ca^{2+} with membrane-associated LPS. Importantly, however, PBNP inhibited PB-mediated hemolysis only when it was used at concentrations significantly greater than the lytic concentration of PB. PBNP may interact less well than PB with membrane LPS due to the loss of one of the five positive charges or to the loss of the hydrophobic fatty acid moiety of PB. In support of this hypothesis, Teuber and Miller (22) have shown that native PB displaces radiolabeled PB which lacks one of the five positive charges of PB from negatively charged membrane surfaces (22).

It is possible that cations do not compete with PB for surface-associated LPS but stabilize the RaRBC membrane in some undefined manner. We believe that interpretation of the blocking data in conjunction with the results of other experiments (i.e., PB-resistant LPS experiments) favors an ionic association of PB with surface LPS susceptible to cationic inhibition. Also, our findings that poly-L-lysine at concentrations significantly higher than the concentrations that block PB-mediated lysis can lyse LPS-sensitized RaRBC and that pretreatment of RaRBC with calcium does not protect them from LPS and PB suggest that membrane stabilization by cations does not play a dominant role in preventing PB lysis. These ionic interactions of PB with surface-bound LPS may be influenced by the core polysaccharide structure of LPS (Fig. 3).

The importance of a fatty acid-dependent hydrophobic interaction of PB with membrane-associated LPS is suggested by the lack of a lytic effect of PBNP on LPS-sensitized RaRBC. PBNP possesses neither the fatty acid moiety nor the terminal positively charged diaminobutyric acid amino acid of PB. We showed that PBNP blocks the effects of PB. These data suggest that although PBNP retains the capacity to bind to LPS on the erythrocyte surface, the presence of the terminal PB-associated fatty acid is critical for erythrocyte lysis. Morrison and Jacobs (9) have previously suggested that association of PB and fluid phase LPS is dependent on hydrophobic interactions, as well as ionic interactions.

Teuber et al. (21) showed that association of negatively charged phospholipids with PB renders aqueous PB soluble in an organic solvent (that is, in a hydrophobic medium), and they proposed that association of PB with membrane-bound LPS may allow PB penetration into the membrane lipid bilayer. The results of studies in which scanning electron microscopy (8), differential scanning calorimetry, electron

spin resonance, nuclear magnetic resonance (13), and fluorescence polarization (20) were used support the hypothesis that PB can insert itself into lipid bilayer membranes.

Imai et al. (7) have suggested that PB fatty acid insertion into bilayers depends on model membrane fluidity, whereas other workers have presented evidence showing that penetration of bacterial envelopes by fluorescently labeled PB is temperature dependent (16). The finding that the temperature dependence of PB bacterial killing strictly correlates with the fluidity of the bacterial envelope and shows a transition at the phase temperature constitutes very interesting suggestive evidence that the PB bactericidal effect depends on membrane fluidity-dependent insertion of PB into the bacterial membrane (6). The temperature dependence of PB-mediated lysis of LPS-sensitized RaRBC (Fig. 6) is consistent with a role for a membrane fluidity-dependent insertion of the PB fatty acid moiety; greater rates of PB-induced lysis at higher temperatures may be attributable to enhanced PB fatty acid insertion into more fluid membranes.

Characterization of the PB lytic reaction mechanism may facilitate an understanding of the nature of the time and temperature decrease in the PB response of LPS-sensitized RaRBC, as well as an understanding of the bactericidal mechanism of PB. The premise that PB-mediated hemolysis of LPS-sensitized RaRBC is due to membrane fluidity-dependent insertion of the PB fatty acid moiety into the membrane suggests a mechanism for the time- and temperature-dependent decrease in the PB response other than that which we have previously proposed (1). LPS has been shown to decrease the fluidity and permeability of various model membranes (12, 14), and an LPS-induced decrease in RaRBC membrane fluidity could inhibit PB fatty acid insertion and abrogate PB-mediated hemolysis of LPS-sensitized cells. Studies in which the levels of PB bound to LPS-sensitized RaRBC are investigated coincident with the decay in PB-mediated lysis and experiments in which LPS-induced alterations in RaRBC membrane fluidity are monitored should result in more extensive information on the nature of the decay in the PB response. The bactericidal effect of PB, which may represent loss of the cytoplasmic membrane permeability barrier (20), may be analogous to PB-mediated lysis of LPS-sensitized RaRBC since both effects depend on the presence of the PB fatty acid (26, 27) (Fig. 5) and on temperature (16) (Fig. 6). Therefore, our findings are consistent with those of Hodate and support the hypothesis that PB fatty acid insertion into the bacterial cytoplasmic membrane after outer membrane disruption of ionic interactions (28) may mediate the bactericidal effect of PB.

In conclusion, the results of the experiments described in this paper are consistent with the hypothesis that erythrocyte membrane-associated LPS can serve as a receptor for PB and that PB mediates membrane damage through insertion of its fatty acid moiety. Elucidation of the nature of PB-mediated damage of LPS-sensitized membranes will contribute to our understanding of potential mechanisms for the time- and temperature-dependent decrease in PB susceptibility of LPS-sensitized RaRBC (1), as well as possible mechanisms for the bactericidal effect of PB.

ACKNOWLEDGMENTS

We gratefully acknowledge the generous gifts of the PBNP and *S. typhimurium* strains SH9178 and SH7426, which were provided by Marti Vaara, Helsinki, Finland. We also acknowledge the excellent secretarial assistance of Grace Vrell.

This research was supported by Public Health Service research grants AI17115 and AI17304 from the National Institutes of Health.

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