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# Polymyxin B Suppresses the Endotoxin Inhibition of Concanavalin A-Mediated Erythrocyte Agglutination

JOHN R. WARREN

*Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611*

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The lectin agglutinability of human erythrocytes has been utilized to examine interactions of gram-negative endotoxin with mammalian cell plasma membranes. Erythrocytes treated in buffer with *Escherichia coli* 0127:B8 lipopolysaccharide (LPS) or *Salmonella minnesota* Re595 glycolipid for 1 h became resistant to agglutination by the lectin concanavalin A (ConA) in buffer free of LPS or glycolipid. Polymyxin B, a cationic cyclic lipopeptide which specifically binds to the lipid A toxophore, was tested for possible effects on the LPS and glycolipid inhibition of ConA erythrocyte agglutination. The presence of polymyxin B during the initial 1-h treatment with LPS or glycolipid blocked the ability of the endotoxins to render erythrocytes refractory to agglutination by ConA. Inhibition by polymyxin B was stoichiometric, and in repeated experiments, LPS was completely suppressed in the hemagglutination assay at a polymyxin B:LPS weight ratio of 1:4.1 (increasing polymyxin concentration, constant LPS concentration) and 1:5.1 (constant polymyxin concentration, increasing LPS concentration). These stoichiometry values are similar to values obtained for inhibition by polymyxin B of LPS lymphoid cell activation. It was concluded, therefore, that endotoxin inhibition of ConA erythrocyte agglutination reflects interactions of erythrocyte membranes with the lipid A region of endotoxin. In addition, the stoichiometry of polymyxin B inhibition suggests a similar extent of lipid A-dependent LPS interaction with erythrocytes and lymphoid cells.

The cell wall of gram-negative bacteria contains endotoxic lipopolysaccharide (LPS) as a major structural component. Recent evidence indicates that lymphoid cells, especially bone marrow-derived macrophages or macrophage-like cells, play a primary role in endotoxin-induced lethality (8, 19). The implication of lymphoid cells in endotoxicity mandates an understanding of the mechanisms by which LPS activates cells. Information obtained in studies of biologically active ligands such as hormones, mitogens, and antigens points to ligand-plasma membrane interactions as crucial initiating events in cell activation. However, the structural complexity of LPS permits the interaction of LPS with a variety of membrane phospholipids (2, 18, 20) and proteins (21), and consequently, quantitative measurements of LPS binding to cells have not provided meaningful information concerning specific LPS actions on cell surfaces (12). As an alternative approach to this problem, my laboratory has utilized the lectin agglutinability of human erythrocytes as an in vitro assay for the study of LPS actions on cell membranes. The lectin agglutinability of erythrocytes is a sensitive indicator of glycoprotein and glycolipid configurations on cell membranes (16). Binding of *Escherichia coli* LPS to human red cells has been found to inhibit agglutination of the cells by concanavalin A (ConA) (23).

For testing the potential biological importance of LPS-membrane interactions reflected in the ConA erythrocyte agglutination assay, the activity of polymyxin B in this assay was evaluated. Polymyxin B forms a stable molecular complex with the toxic lipid A region and thereby neutralizes the endotoxicity of LPS (15). In addition, the ability of *Salmonella minnesota* Re595 glycolipid to inhibit ConA erythrocyte agglutination was evaluated. Glycolipids from Re mutants are potent endotoxins which contain only lipid A and 2-keto-3-deoxyoctonate (7). I now report that *S. minnesota* Re595 glycolipid inhibits ConA erythrocyte agglutination and that polymyxin B blocks the inhibition by both *E. coli* LPS and the *S. minnesota* Re595 glycolipid of ConA-induced erythrocyte agglutination. Furthermore, the stoichiometry of polymyxin B inhibition of LPS actions on ConA agglutination was similar to that observed for polymyxin B inhibition of LPS activation of cultured splenic lymphoid cells. The LPS inhibition of ConA erythrocyte agglutination appears to reflect,

therefore, interactions with erythrocyte membranes of biologically active LPS configurations dependent upon the lipid A moiety.

## MATERIALS AND METHODS

**Erythrocytes and splenic lymphoid cells.** Human erythrocytes were obtained from recently outdated units of packed erythrocytes provided by the United Blood Services Center, Chicago. The erythrocytes were washed three times with pH 7.2 phosphate-buffered saline (PBS) (140 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4$, 1 mM $NaH_2PO_4$) and stored at 4°C at a packed cell volume of 4% (vol/vol) in PBS supplemented with 5% (wt/vol) dextrose. Splenic lymphoid cells were isolated from 3-month-old C57BL/6J female mice (The Jackson Laboratory, Bar Harbor, Maine).

**Preparation of LPS and Re595 glycolipid.** Lyophilized preparations of *E. coli* 0127:B8 LPS (Westphal) (control 674687 and 650011) were purchased from Difco Laboratories, Detroit, Mich., and lyophilized preparations of *S. minnesota* Re595 glycolipid (lot 7) were purchased from List Biological Laboratories, Campbell, Calif. The LPS and Re595 glycolipid were activated for erythrocyte binding by mild alkaline hydrolysis of 10 mg of LPS or glycolipid per ml in a 0.25 N NaOH solution at 56°C for 1 h. Alkaline hydrolysis was terminated by neutralization with 0.25 N HCl, and the solution was incubated overnight at 4°C. The resulting precipitate was removed by centrifugation at 300 × *g*, 1 volume of the clear supernatant solution was extracted three times with 4 volumes of chloroform, and the aqueous phase of the chloroform extraction was precipitated overnight at 4°C with a 10-fold volume excess of ethanol. The precipitate which formed in ethanol solution was collected by centrifugation at 500 × *g*, washed once with cold (4°C) ethanol, suspended in distilled water, and dialyzed overnight at 4°C against large volumes of distilled $H_2O$. The LPS or Re595 glycolipid preparation was finally lyophilized and stored at 4°C as a dry powder until used.

**Chemicals and reagents.** ConA purified by chromatography on Sephadex was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Polymyxin B sulfate was from Sigma Chemical Co., St. Louis, Mo. The RPMI 1640 medium, fetal calf serum, penicillin, and streptomycin were purchased from Kansas City Biologicals, Lenexa, Kans.

**ConA erythrocyte agglutination.** Erythrocytes (group A or AB, $Rh^+$) were treated by incubation in PBS containing LPS, Re595 glycolipid or polymyxin B at a temperature of 22 to 24°C and a packed cell volume of 2% (vol/vol). Control erythrocytes were incubated in PBS not containing LPS, glycolipid, or polymyxin B. The erythrocytes were then washed three times with PBS, suspended to 1% (vol/vol) in PBS, and added in 125-μl aliquots to glass tubes containing 125 μl of PBS with various concentrations of ConA. The erythrocyte suspensions were gently mixed, and a 200-μl portion was transferred from each glass tube into a microtiter plate well (V-shaped bottoms; Dynatech Laboratories, Inc., Alexandria, Va.). The microtiter plates were faced by transparent plastic tape, and the effacement of wells was evaluated after 3 h at room temperature (22 to 24°C). Complete effacement of a microtiter plate well was recorded as positive (++) for erythrocyte agglutination, and the presence of a dense button of red cells at the bottom of the well surrounded by a clear zone of buffer was recorded as absence (0) of erythrocyte agglutination. Partial effacement (50 to 90%) was recorded as +.

**LPS stimulation of spleen cell DNA synthesis.** Splenic lymphoid cells were aseptically obtained from C57BL/6J mice and dispersed into complete culture medium at a density of $2.5 \times 10^6$/ml. Complete culture medium consisted of RPMI 1640 medium containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 5% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The spleen cell suspension was distributed in 1.0-ml volumes into plastic culture tubes (12 by 75 ml; Falcon Plastics, Oxnard, Calif.). The cells were treated by a 1-h incubation with LPS, polymyxin B, or both at a temperature of 37°C. Control cells were incubated in complete culture medium not containing LPS or polymyxin B. The spleen cells were then washed twice with complete medium, and the cells in each tube were suspended in 1.0 ml of complete culture medium and incubated at 37°C for an additional 45 h. One microcurie of tritium-labeled thymidine (2.0 Ci/mmol; Research Products International, Elk Grove Village, Ill.) was then added to each culture. The incubation was terminated at 50 h by a wash with cold (4°C) PBS, the radioactive spleen cell DNA was precipitated with 10% trichloroacetic acid overnight, and acid-insoluble precipitates were trapped on glass-fiber filters (no. 25; Schleicher & Schuell Co., Keene, N.H.). The filters were washed with cold (4°C) PBS and transferred to scintillation vials. Acid-insoluble precipitate on the filters was digested with NCS solubilizer (Amersham/Searle, Arlington Heights, Ill.) at room temperature for 16 h, Ready-Solv EP liquid scintillation solution (Beckman Instruments, Inc., Fullerton, Calif.) was added to the vials, and the radioactivity was measured as disintegrations per minute in a Beckman LS 9000 liquid scintillation counter.

## RESULTS

**Effect of polymyxin B on *E. coli* LPS inhibition of ConA erythrocyte agglutination.** Erythrocytes incubated for 1 h with *E. coli* LPS at concentrations of 50 μg/ml or higher and then thrice washed in LPS-free buffer were completely refractory to agglutination by 20 μg of ConA per ml in repeated experiments ($n = 15$). In each of these experiments, ++ erythrocyte agglutination was noted in the presence of 20 μg of ConA per ml for cells not treated with LPS. When erythrocytes were treated for 1 h with 50 μg of LPS per ml in the presence of increasing polymyxin B concentrations, washed with PBS, and then tested for agglutination by 20 μg of ConA per ml, polymyxin B at concentrations of 10 μg/ml or above completely suppressed the ability of LPS to inhibit ConA agglutination (Table 1, experiment 1). Polymyxin B alone had no effect on ConA erythrocyte agglutination. The polymyxin B:LPS stoichiometry (wt/wt) observed for the inhibitory action of polymyxin B in experiment 1 was 1:5. In seven separate experi-

TABLE 1. Effect of polymyxin B on the LPS inhibition of ConA erythrocyte agglutination

| Polymyxin B concn (μg/ml) for indicated expt | LPS concn (μg/ml) | Erythrocyte agglutination[a] |
|---|---|---|
| Expt 1 | | |
| 0 | 0 | ++ |
| 25 | 0 | ++ |
| 0 | 50 | 0 |
| 0.5 | 50 | 0 |
| 1 | 50 | 0 |
| 2 | 50 | 0 |
| 5 | 50 | 0 |
| 10 | 50 | ++ |
| 25 | 50 | ++ |
| Expt 2 | | |
| 0 | 0 | ++ |
| 0 | 10 | ++ |
| 0 | 50 | 0 |
| 0 | 100 | 0 |
| 0 | 200 | 0 |
| 0 | 300 | 0 |
| 0 | 400 | 0 |
| 25 | 0 | ++ |
| 25 | 10 | ++ |
| 25 | 50 | ++ |
| 25 | 100 | ++ |
| 25 | 200 | + |
| 25 | 300 | 0 |
| 25 | 400 | 0 |

[a] ++, Complete effacement of microtiter well by 20 μg of ConA per ml; +, partial effacement; 0, no effacement.

ments in which the protocol of experiment 1 was used, polymyxin B at a concentration of 10 or 25 μg/ml blocked 50 μg of LPS per ml, yielding stoichiometry values for polymyxin B inhibition ranging from 1:2 to 1:5 and a mean value of 1:4.1. In an additional group of experiments, polymyxin B concentration was kept constant while the LPS concentration was varied during the 1-h treatment. Erythrocytes treated with LPS in the presence of 25 μg of polymyxin B per ml became refractory to ConA agglutination only at LPS concentrations exceeding 200 μg/ml (Table 1, experiment 2). In 11 separate experiments in which the protocol of experiment 2 was used, LPS escaped the blocking activity of 10 or 25 μg of polymyxin B per ml only at LPS concentrations of 50 μg/ml or greater. Calculation from combined results of the 11 experiments indicated a mean stoichiometry value of 1:5.1, with a range of 1:1 to 1:10 for complete inhibition by polymyxin B.

To determine whether the inhibitory action of polymyxin B was due to interactions with LPS or with erythrocytes, we preincubated erythrocytes for 1 h with polymyxin B, washed the cells, and then treated them for 1 h with LPS. We found that preincubation of erythrocytes with polymyxin B did not block the ability of LPS to render the cells refractory to ConA agglutination (Table 2). In the experiment shown in Table 2, polymyxin B was strongly inhibitory when added in the presence of LPS. The results reported in Table 2 are representative of three separate experiments. These results indicate that inhibition by polymyxin B in this system depended upon direct interactions of polymyxin with LPS.

**Effect of polymyxin B on *E. coli* LPS spleen cell stimulation.** Lymphocyte mitogenicity correlates in a positive fashion with both the lethal toxicity (13) and pyrogenicity (5) of native and modified LPS. To assess the biological activity of the hydrolyzed LPS used in the present study, and also because of the demonstrated role of lymphoid cells in endotoxicity (8, 19), the mitogenic activity toward lymphocytes of the hydrolyzed LPS and the blocking of mitogenic activity by polymyxin B were evaluated. In these experiments, C57BL/6J spleen cells were treated in complete culture medium containing *E. coli* LPS or LPS and polymyxin B for 1 h, washed, and then incubated for an additional 49 h without LPS or polymyxin B. Increasing the concentration of polymyxin B progressively suppressed spleen cell stimulation by 50 μg of LPS per ml (Fig. 1), with complete suppression obtained at 10 μg of polymyxin B per ml. This is equivalent to a polymyxin B:LPS stoichiometry (wt/wt) of 1:5 for complete inhibition by polymyxin B of

TABLE 2. Effect of preincubation with polymyxin B on the LPS inhibition of ConA erythrocyte agglutination

| Preincubation condition[a] | Treatment condition[b] | Erythrocyte agglutination[c] |
|---|---|---|
| PBS | PBS | ++ |
| PBS + polymyxin B | PBS | ++ |
| PBS + polymyxin B | PBS+ 25 μg of LPS/ml | 0 |
| PBS + polymyxin B | PBS + 50 μg of LPS/ml | 0 |
| PBS | PBS + 25 μg of LPS/ml + polymyxin B | ++ |
| PBS | PBS + 50 μg of LPS/ml + polymyxin B | ++ |

[a] Erythrocytes were preincubated for 1 h in PBS without or with 10 μg of polymyxin B per ml.

[b] After preincubation and washing, cells were treated for 1 h in PBS without or with LPS or LPS plus 10 μg of polymyxin B per ml.

[c] ++, Complete effacement of microtiter well by 20 μg of ConA per ml; 0, no effacement.

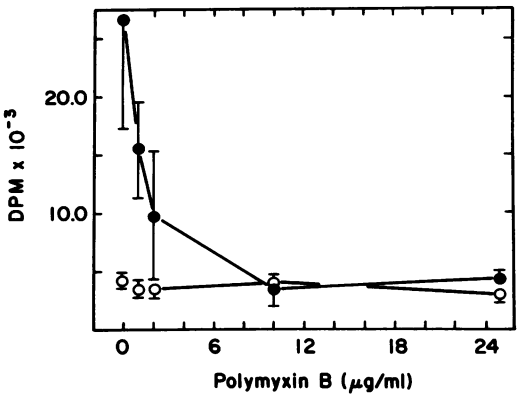


FIG. 1. Effect of polymyxin B on stimulation of splenic lymphoid cells by LPS (endotoxin). Cells were treated with increasing polymyxin B concentrations in the absence (○) or presence (●) of 50 μg of LPS per ml. Symbols represent the mean values ± standard errors of three replicate cultures.

LPS spleen cell activation. In three separate experiments performed in identical fashion, the mean value of the stoichiometry of inhibition by polymyxin B was 1:6.3, with a range of 1:4 to 1:10. Treatment of spleen cells with polymyxin B alone up to a concentration of 25 μg/ml neither stimulated nor suppressed spleen cell DNA synthesis (Fig. 1). In an additional set of experiments, the concentration of LPS was increased in the presence of a constant concentration (10 μg/ml) of polymyxin B (Fig. 2). A representative LPS dose-response curve is shown in Fig. 2, which shows that maximal lymphocyte stimulation was obtained at 10 μg of LPS per ml followed by a plateau up to 100 μg of LPS per ml. The presence of 10 μg of polymyxin B per ml during the 1-h treatment of spleen cells inhibited activation by LPS added to a concentration as high as 25 μg/ml (Fig. 2), equivalent to a polymyxin B:LPS stoichiometry (wt/wt) of 1:2.5. A ratio of 1:2.5 was obtained in three separate experiments performed in identical fashion.

**Inhibition of ConA erythrocyte agglutination by *S. minnesota* Re595 glycolipid.** Erythrocytes treated with Re595 glycolipid were not agglutinated by 20 μg of ConA per ml (Table 3). However, the minimum amount of Re595 glycolipid (500 μg/ml) required to render erythrocytes unresponsive to ConA was 10-fold greater than the minimum amount observed for LPS (50 μg/ml). The reason for this quantitative difference between the glycolipid and LPS (extent of aggregation in solution, dynamics of erythrocyte binding, or some other factor) is presently undetermined. Polymyxin B blocked the glycolipid inhibition of ConA agglutination when added at a polymyxin B: glycolipid stoichiometry of 1:10 (Table 3). In the same experiment, polymyxin B blocked LPS when added at a polymyxin B:LPS stoichiometry of 1:4.

## DISCUSSION

Localization of endotoxic LPS to the erythrocyte plasma membrane has been demonstrated by thin-section electron microscopy, using peroxidase-conjugated LPS (17). A putative lipoglycoprotein receptor for LPS has been isolated from human erythrocyte membranes (21). Endotoxic LPS can also penetrate monomolecular films of erythrocyte lipid as well as monolayers of phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine and can also decrease the stability of lecithin-cholesterol bilayers (2). Microelectrophoretic measurement of surface charge density of phosphatidylcholine liposomes indicates that LPS interacts with phosphatidylcholine both hydrophobically and electrostatically (18). It appears, therefore, that LPS can bind to a variety of proteins and lipids found in the erythrocyte membrane. Ciznar and Shands have observed that LPS binding to the erythrocyte membrane causes membrane disorganization with cell lysis and thus represents more than a passive surface adsorption of LPS (4). At LPS concentrations below those which induce erythrocyte lysis, we have observed inhibition of ConA agglutination (23, 24). However, treatment of erythrocytes by LPS was not found to result in inhibition of lectin-mediated agglutination of the treated cells by soybean agglutinin or *Phaseolus vulgaris* phytohemagglutinin. It was proposed, therefore, that at least a fraction of the membrane-bound LPS is localized to the ConA receptor regions and thereby disrupts

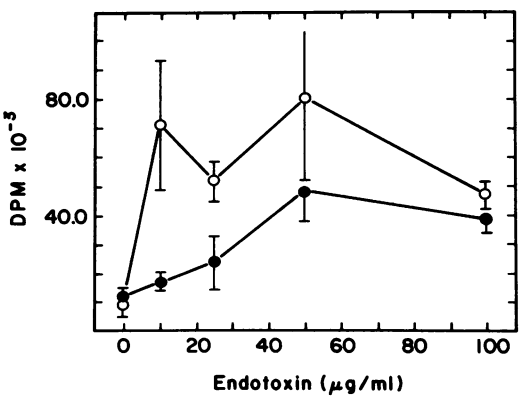


FIG. 2. Effect of polymyxin B on stimulation of splenic lymphoid cells by LPS (endotoxin). Cells were treated with increasing LPS concentrations in the absence (○) or presence (●) of 10 μg of polymyxin B per ml. Symbols represent the mean values ± standard errors of three replicate cultures.

TABLE 3. Effect of polymyxin B on the Re595 glycolipid and LPS inhibition of ConA erythrocyte agglutination

| Polymyxin B concn (μg/ml) | Glycolipid concn (μg/ml) | LPS concn (μg/ml) | Erythrocyte agglutination[a] |
|---|---|---|---|
| 0 | 0 | 0 | ++ |
| 400 | 0 | 0 | ++ |
| 0 | 500 | 0 | 0 |
| 10 | 500 | 0 | 0 |
| 50 | 500 | 0 | ++ |
| 400 | 500 | 0 | ++ |
| 0 | 0 | 100 | 0 |
| 10 | 0 | 100 | 0 |
| 25 | 0 | 100 | ++ |
| 50 | 0 | 100 | ++ |

[a] ++, Complete effacement of microtiter well by 20 μg of ConA per ml; 0, no effacement.

agglutination of erythrocytes by ConA (23). The present data indicate that *S. minnesota* Re595 glycolipid shares with *E. coli* 0127:B8 LPS the property of rendering human erythrocytes refractory to ConA agglutination and that polymyxin B completely blocks this property of the glycolipid and LPS. The Re595 glycolipid is a mutant endotoxin containing lipid A–2-keto-3-deoxyoctonate but completely deficient in core and O-antigenic oligosaccharide (7). Polymyxin B binds to the lipid A portion of LPS (15). The activities of Re595 glycolipid and polymyxin observed in the present study indicate that LPS inhibition of ConA erythrocyte agglutination is due to actions of lipid A-dependent LPS structures on the erythrocyte membrane.

Several groups of investigators have observed that polymyxin B suppresses the activity of LPS as a polyclonal lymphocyte mitogen (1, 3, 14). A careful study by Jacobs and Morrison revealed that polymyxin B suppression of *E. coli* 055:B5 stimulation of CBA/HWehi mouse spleen cell DNA synthesis was stoichiometric and that complete inhibition occurred when polymyxin B was added at a polymyxin B:LPS stoichiometry of 1:5 (wt/wt) (11). This reported value is very similar to values determined in the present study for polymyxin B inhibition of *E. coli* 0127:B8 LPS stimulation of C57BL/6J spleen cells (1:6.3 when increasing concentrations of polymyxin B were added to spleen cell cultures containing a constant LPS concentration; 1:2.5 when increasing concentrations of LPS were added to cultures with a constant polymyxin B concentration). Furthermore, values for the polymyxin B:LPS stoichiometry of polymyxin inhibition of LPS lymphoid cell stimulation are very close to those calculated for the polymyxin suppression of LPS actions on ConA erythrocyte agglutination (1:4.1 with increasing polymyxin B and constant LPS concentrations; 1:5.1 with increasing LPS and constant polymyxin B concentrations). This closeness of stoichiometry values can be interpreted as reflecting similar extents of lipid A-dependent interactions of LPS with erythrocyte and lymphoid cell membranes.

It has been reported that the specific receptors for ConA on the erythrocyte membrane are removed by proteolysis (9). ConA receptors are therefore presumably associated with glycoprotein (9). Evidence has also been obtained that the specific erythrocyte membrane receptor for ConA is a band 3 protein(s) (6). Band 3 proteins serve as sites for passive $Na^+$ and $K^+$ diffusion through erythrocyte membranes (10). Evidence has recently been reported from our laboratory that a primary action of LPS on erythrocytes is to increase plasma membrane permeability toward $Na^+$ and $K^+$ (22, 24). Investigations are thus now in progress to ascertain whether LPS affects the passive $Na^+$-$K^+$ permeability of lymphoid cells and whether LPS actions on erythrocyte and lymphoid cell Na+-K+ permeability show an apparent lipid A dependence similar to that reported here for LPS inhibition of erythrocyte agglutination. In addition, a colleague and I have recently discovered by scanning electron microscopy that human erythrocytes treated with increasing LPS concentrations undergo a biconcave disk-to-echinocyte-to-spherocyte transition (J. R. Warren and C. H. Wallas, unpublished data). This LPS-induced transition in erythrocyte shape occurred in the absence of ATP depletion of the erythrocytes. The erythrocyte is clearly an important model for defining actions of LPS on mammalian cell membranes and the relationship of such actions to cell activation and regulation of cell shape.

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