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Characterization of Lipoteichoic Acid Binding to Polymorphonuclear Leukocytes of Human Blood

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Human polymorphonuclear leukocytes (PMN) were shown to possess specific binding sites for lipoteichoic acid (LTA). LTA binding was reversible and time and temperature dependent. Scatchard plot analysis revealed an apparently single population of 6.6×10^6 LTA binding sites per PMN with a dissociation constant of 5.6 μM. Attachment of an avirulent, unencapsulated, M-negative strain of group A streptococci to PMN was inhibited by LTA, but not by other bacterial somatic antigens tested. Occupation of 30% of the LTA binding sites resulted in greater than 70% inhibition of streptococcal attachment to PMN. In contrast, LTA failed to block attachment of *Escherichia coli* or antibody-coated streptococci, indicating that binding sites for *E. coli* and the Fc portion of immunoglobulin G are distinct from those for LTA. Immunofluorescent studies demonstrated that LTA remained uniformly bound to PMN membranes for as long as 2 h at 37°C. Cross-linking of PMN-bound LTA with anti-LTA resulted in rapid capping of LTA receptor sites. The results suggest that LTA is a monovalent ligand interacting with mobile receptors in the plasma membrane of PMN.

Lipoteichoic acid (LTA) of gram-positive bacteria has been shown to bind to a variety of mammalian cells via its lipid moiety (3-5, 15, 19, 28, 29). An intact lipid moiety is necessary for LTA to induce its biological activities such as bone resorption (14), mitogenicity (4), nephritis (27), and arthritis (17). In addition, there is evidence that LTA may play an important role in modulating the defense mechanisms of the host. LTA has been shown to suppress the immune responses and to promote carbon clearance by the reticuloendothelial system in mice (16) and to stimulate the release of lysosomal enzymes by macrophages (20).

The binding of LTA to host cells appears to be an important prerequisite for biological activity (4). Most previous studies of the binding of LTA to host cells have employed nonphagocytic cells. Since professional phagocytes play a central role in defense against bacterial infections and because LTA seems to exert a modulating influence on such cells in vivo, the present study was undertaken to determine the characteristics of the binding of streptococcal LTA to human polymorphonuclear leucocytes. Our data suggest that polymorphonuclear leukocytes (PMN) possess a single population of LTA receptors, and that LTA bound to its receptors was not ingested by PMN, but caused receptor capping when cross-linked by anti-LTA.

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MATERIALS AND METHODS

Preparation of LTA. [^{3}H]LTA (specific activity, 20,000 to 30,000 cpm/μg) was obtained by growing streptococci in the presence of radiolabeled glycerol as previously described (19). Briefly, group A streptococci, strain 1RP41 (originally obtained from Rebecca Lancefield, The Rockefeller University, New York, N.Y.), were grown in 5-liter batches in van de Rijn-Kessler defined medium (26) containing 2.5 mCi of [2-^{3}H]glycerol (specific activity, 200 mCi/mmol; New England Nuclear Corp., Boston, Mass.) per liter. After 16 h of growth at 37°C, the bacteria were harvested by centrifugation, washed with distilled water, and extracted with phenol. The aqueous phase of the extract was dialyzed exhaustively against distilled water and lyophilized. Free lipids were removed from aqueous solutions of the lyophilized extract with equal volumes of chloroform-methanol (2:1). The aqueous phase of the chloroform-methanol extraction was further purified on an albumin-Sepharose 6B column (24). Unlabeled LTA was prepared in the same manner, except no [^{3}H]glycerol was added to growth medium.

Preparation of PMN. The erythrocytes in heparinized (100 U/ml) venous blood from healthy donors were allowed to sediment for 1 h after adding 1 part of 6% dextran (Sigma Chemical Co., St. Louis, Mo.) to 5 parts of whole blood. The leukocyte-rich supernatant was layered over Ficoll-Paque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) and centrifuged for 25 min at $800 \times g$ (8). The pellet, containing PMN and residual erythrocytes, was resuspended in an erythrocyte-lysing solution (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.001 M ethylenediaminetetraacetate, pH 7.3) and incubated for 5 min at 4°C. The PMN were then washed two times with cold 0.02 M phosphate-0.15 M NaCl,

pH 7.4 (PBS), and finally suspended in PBS to the desired concentration. Viability of PMN always exceeded 95% based on trypan blue exclusion. Purity of the PMN preparations was 91 ± 4.6% as ascertained by morphological and staining characteristics. PMN monolayers were prepared by incubating 50 μl of PMN suspension on glass cover slips placed in a petri dish (no. 3001; Falcon Plastics, Oxnard, Calif.) for 5 min at ambient temperature. Nonadherent cells were removed by washing the cover slips three times with cold PBS and then covered with 2 ml of cold PBS and kept at 4°C until utilized.

Binding experiments. All binding assays were performed in duplicate on various days with different donors as a source of PMN. The time course of binding was determined by mixing PMN (27×10^6/ml) with [^{3}H]LTA (12.5 μg/ml) at 25°C. Duplicate samples of 100 μl containing 2.7×10^6 PMN were taken at the times indicated, filtered under reduced pressure, and washed with 10 ml of PBS. The polycarbonate membrane filters (pore size, 1 μm; Nuclepore Corp., Pleasanton, Calif.) were mixed with 10 ml of Scintiverse (Fisher Scientific Co., Fairlawn, N.J.) and counted in a liquid scintillation counter (Hewlitt Packard, Palo Alto, Calif.). For Scatchard plot analysis 8.3×10^6 PMN were incubated in 0.25 ml of PBS containing various amounts of [^{3}H]LTA (0.195 to 100 μg) for 60 min at 25°C. The assay was terminated by filtration under reduced pressure, washed, and counted as above. The temperature-dependent binding of LTA was determined by using 2.7×10^6 PMN incubated with various concentrations of [^{3}H]LTA (0.625 to 10 μg) in 0.25 ml of PBS for 30 min at 4, 25, and 37°C. The mixtures were filtered, washed, and counted as above. The effect of PMN concentration on [^{3}H]LTA binding was determined by mixing 1.25 μg of [^{3}H]LTA with increasing numbers of PMN in a total volume of 0.25 ml of PBS for 60 min at 25°C. Duplicate samples were then assayed for binding of [^{3}H]LTA.

Deacylation of LTA. LTA was deacylated by hydrolysis with ammonium hydroxide as previously described (19). LTA was resuspended in distilled water to a concentration of 5 mg/ml and mixed with an equal volume of 30% ammonium hydroxide. The mixture was incubated at room temperature for 8 h and then evaporated to dryness under nitrogen. The residue was redissolved in distilled water and extracted three times with chloroform-methanol (2:1), and the aqueous phase was lyophilized.

Preparation of anti-LTA. LTA was precipitated with methylated bovine serum albumin at low pH, emulsified with Freund incomplete adjuvant, and injected into New Zealand White rabbits as previously described (6). Immune sera were collected and stored at −70°C until used.

Immunofluorescent assay for PMN-bound LTA. Localization of LTA on the cell membrane of PMN was followed in two sets of experiments. In one set, PMN monolayers were incubated with 100 μl of PBS containing 1 to 1,000 μg of LTA for 15 min at 37°C, followed by washing in cold PBS and suspension in 1 ml of PBS. After further incubation for various time periods at 37°C, the monolayers were fixed with 1% glutaraldehyde, washed in PBS, and exposed to 1 ml of anti-LTA diluted 1:50 in PBS for 15 min at 37°C. The monolayers were then washed three times with PBS and further incubated with 1 ml of fluorescein-conjugated goat anti-rabbit immunoglobulin (Kallestad Laboratory, Minneapolis, Minn.) diluted 1:20 in PBS for 15 min at 37°C. The PMN monolayers were washed free of fluorescein label, mounted on slides with one drop of 30% glycerol in PBS, and screened for fluorescence. Controls consisted of monolayers treated in the same manner, except incubated with PBS instead of LTA. In the second set of experiments, the PMN monolayers were treated as above, except that anti-LTA was added to monolayers and incubated at 37°C for various time periods before fixation with glutaraldehyde.

Bacterial attachment of PMN monolayers. An M protein-poor strain (type 1 av) of group A streptococci and a clinical isolate of *Escherichia coli* strain M-10 were grown for 16 h at 37°C in Todd-Hewitt broth and brain heart infusion broth (Difco Laboratories, Detroit, Mich.), respectively. The bacteria were harvested, washed three times in PBS, and resuspended in PBS to an optical density of 0.4 at 530 nm. Assays for the attachment of bacteria to PMN monolayers were performed as previously described (18), with slight modifications. Briefly, 1 ml of the bacterial suspension containing the desired concentration of test substance was added to each monolayer. Ice-cold buffers were used throughout, and the monolayers were kept at 4°C to prevent ingestion (12). After shaking of the monolayers and the bacterial mixture for 30 min at 4°C, the monolayers were washed three times with PBS, fixed, stained, and examined by light microscopy. The number of streptococci attached to 100 PMN was scored. In other experiments, the monolayers were pretreated with desired concentrations of test substances for 30 min at 4°C, washed three times with PBS, and then incubated with 1 ml of bacterial suspension for 30 min at 4°C. In some experiments the streptococci were preincubated with anti-T serum (Centers for Disease Control, Atlanta, Ga.) diluted 1:10 in PBS for 30 min at 4°C, washed three times in PBS, and resuspended to original concentration in PBS before adding to the monolayers. Lipopolysaccharide (LPS) from *E. coli* was obtained from Difco, and fatty acid-free bovine serum albumin was obtained from Fisher Scientific Co.

RESULTS

Binding of [^{3}H]LTA to PMN. Binding of [^{3}H]LTA to human PMN was linear with increasing cell concentration (Fig. 1) and was time (Fig. 2) and temperature dependent (Fig. 3). Maximum binding was approached in 30 min, similar to that reported for lymphocytes (4). Up to 80% of PMN-bound [^{3}H]LTA was displaced by a 50-fold excess of unlabeled LTA. In contrast, a 50-fold excess of deacylated LTA displaced only 2% of bound [^{3}H]LTA (Table 1). The radioactivity that was undisplaceable by unlabeled LTA was considered to represent nonspecific binding. In all experiments, therefore, duplicate incubations were performed with and without the addition of excess unlabeled LTA.

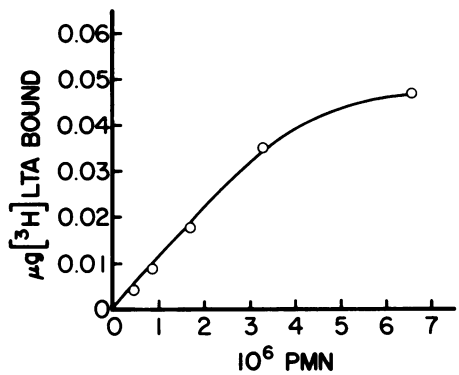

FIG. 1. *Binding of [^{3}H]LTA to PMN as a function of increasing cell number. [^{3}H]LTA (1.25 μg) was mixed with the indicated number of PMN in 0.25 ml of PBS for 60 min at 25°C. The reaction was stopped by filtration under reduced pressure and washing with 10 ml of cold PBS. The washed filters were then assayed for radioactivity (see text).*

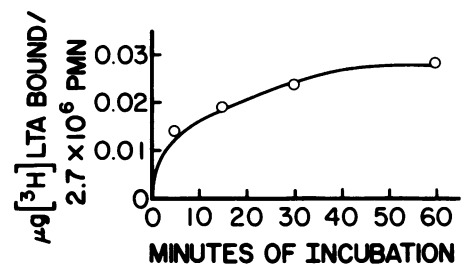

FIG. 2. *Binding of [^{3}H]LTA to PMN as a function of time. PMN (27 × 10^6/ml) were mixed with [^{3}H]LTA (12.5 μg/ml) at 25°C. Duplicate samples of 0.1 ml containing 2.7 × 10^6 PMN were taken at the indicated time intervals and assayed for radioactivity.*

Each value of binding was corrected by subtracting the radioactivity bound to PMN incubated with excess unlabeled LTA. The radioactivity in the presence of the excess unlabeled LTA was never greater than 18% of a duplicate incubation performed in the absence of unlabeled LTA (Table 1). The specificity of binding inhibition was demonstrated by the abilities of LTA from *Streptococcus pyogenes* (1RP41) and *Streptococcus salivarius*, but not of M protein, C-carbohydrate, or lipopolysaccharide of *E. coli* to block binding. Bovine serum albumin, which is known to possess fatty acid binding sites (24), also was able to inhibit binding of [^{3}H]LTA (Table 1).

The number and affinity of binding sites for LTA was determined by adding increasing amounts of [^{3}H]LTA to PMN and analyzing the data by Scatchard plots (21) assuming a molecular weight of 8,500 for LTA (24). The data (Fig. 4) indicated an apparently single population of 6.6 × 10^6 binding sites per PMN with a K_d of 5.6 μM.

Immunofluorescent assay for PMN-bound LTA. LTA remained bound to PMN membranes for as long as 2 h, as visualized by

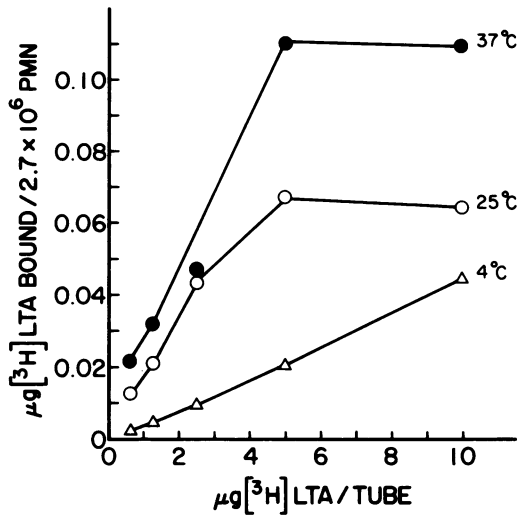

FIG. 3. *Binding of [^{3}H]LTA to PMN as a function of temperature. Various amounts of [^{3}H]LTA (0.625 to 10 μg) were incubated with 2.7 × 10^6 PMN in 0.25 ml for 30 min at the temperatures indicated. Assays were terminated by filtration and washing under reduced pressure as described in the text.*

TABLE 1. *Inhibition of LTA binding to and elution of bound LTA from human PMN*

Test substance	% Inhibition of [^{3}H]-binding[a]	% of bound [^{3}H]LTA eluted[b]
S. pyogenes, LTA	85	79
S. salivarius LTA	68	ND[c]
Deacylated *S. pyogenes* LTA	0	2
Bovine serum albumin, fatty acid free	84	48
M protein (M6)[d]	2	4
Group A C-polysaccharide[e]	23	9
Lipopolysaccharide of *E. coli*	17	14

[a] A constant amount (20 μg) of [^{3}H]LTA was incubated with 1.2 × 10^6 PMN for 60 min at 25°C in a total volume of 0.25 ml either simultaneously with a 50-fold excess by weight of test substance or in PBS. Percent inhibition = [1 − (micrograms of [^{3}H]LTA bound with test substance/micrograms of [^{3}H]LTA bound in PBS control)] × 100.

[b] A 50-fold (wt/wt) excess of test substance was used to elute bound [^{3}H]LTA from PMN.

[c] ND, Not done.

[d] Purified preparation of M protein prepared from a limited pepsin extract of type 6 streptococci as previously described (7).

[e] C-polysaccharide was extracted from type 6 streptococci by the method of Fuller (11).

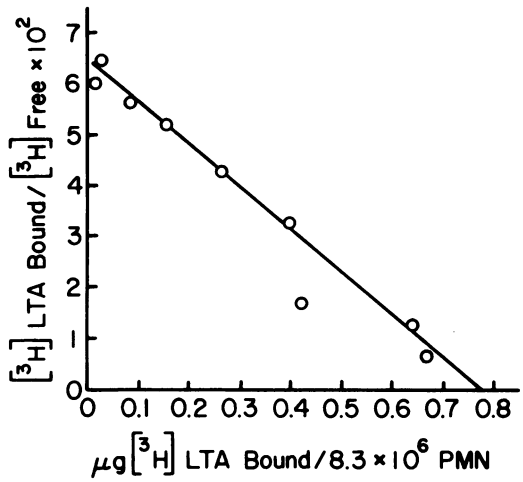

FIG. 4. *Scatchard plot of [^{3}H]LTA binding to PMN. Increasing amounts of free [^{3}H]LTA (0.195 to 100 μg) were added to 8.3 × 10^6 PMN in a total volume of 0.25 ml and incubated for 60 min at 25°C. The intercept with the abscissa corresponds to an approximation of the number of LTA binding sites at saturation.*

accessibility of the polyglycerol phosphate moiety of LTA to immunofluorescence labeling (Fig. 5A). The ring pattern of immunofluorescence observed on PMN remained constant during the 2 h at concentrations of LTA as low as 10 μg/ml. In contrast, the immunofluorescent label migrated to one pole of the PMN within 5 min after adding anti-LTA (Fig. 5B and C) to unfixed cells. This effect was not seen in PMN monolayers incubated with LTA followed by anti-LTA at 4°C or when the monolayers were fixed with glutaraldehyde just before adding anti-LTA (data not shown). These results suggest that the receptors for LTA in the PMN membranes are mobile.

Inhibition of the binding of streptococci to PMN by LTA. LTA inhibited the binding of group A streptococci to PMN monolayers in a dose-related fashion (Fig. 6). Furthermore, pretreatment of the monolayers with 1 mg of LTA per ml followed by washing in PBS blocked the binding of streptococci subsequently exposed to the monolayers, suggesting that LTA prevents streptococcal attachment by binding to LTA receptors in the PMN membranes. The inhibitory effect of LTA was specific for streptococci; attachment of *E. coli* was not affected by LTA (Fig. 6), but could be blocked by mannose (1). Moreover, the binding of streptococci preincubated for 30 min at 4°C with anti-T serum also was unaffected by LTA. These results indicated that the mannose-specific binding sites for *E. coli* as well as the Fc binding sites for immunoglobulin G were distinct from those for LTA.

The relationship between LTA binding and inhibition of streptococcal atrachment to PMN was further investigated by exposing PMN to radiolabeled LTA and subsequently exposing the same cells to streptococci. As the number of binding sites occupied by LTA increased, the attachment of streptococci correspondingly decreased (Fig. 7). Occupation of as few as 30% of the total number of LTA binding sites blocked the binding of streptococci by 70%.

DISCUSSION

Although LTA has long been known to bind spontaneously to a wide variety of animal cells (28), only recently have the characteristics of the binding of LTA to a number of mammalian cells been investigated. Lymphocytes (4), erythrocytes (5), platelets (3), and oral epithelial cells (unpublished observation) each were found to possess a single population of binding sites. PMN were similarly found to possess a single population of binding sites and, in agreement with previous studies (2–5, 19), binding of LTA was dependent upon its lipid moiety as demonstrated by the ability of unlabeled, but not deacylated, LTA to block the binding of radiolabeled LTA to PMN.

The nature of the receptor for LTA remains unknown at present. It may be argued that LTA binds to membranes by intercalating its lipid moiety within the phospholipid bilayer. That this is one possible mechanism for binding of LTA to membranes was shown by Silvestri et al., who used synthetic phosphatidylcholine vesicles to purify LTA (22). The binding to such synthetic vesicles, however, may represent only the irreversible, nonspecific type of binding. Moreover, our observation in the present study that LTA binding sites on the PMN membrane were mobile and able to form caps, and the observation made previously that LTA binds 10 times better to right-side-out than to inside-out erythrocyte vesicles (9) support the idea of more than simple insertion of LTA via its lipid end into the membrane phospholipid bilayer.

Several studies have suggested a specific protein or glycoprotein for the binding of amphipathic molecules to cell membranes. A lipoglycoprotein of erythrocyte membranes was found to serve as a receptor for the lipid A portion of LPS (13, 25). The hydrophobicity of niflumic acid was found necessary for its binding to band 3 protein of erythrocytes (10). A protein or glycoprotein has been suggested by Simpson et al. (23, 24) as a membrane receptor for LTA based on the finding that albumin which contains fatty acid binding sites competitively inhibited bind-

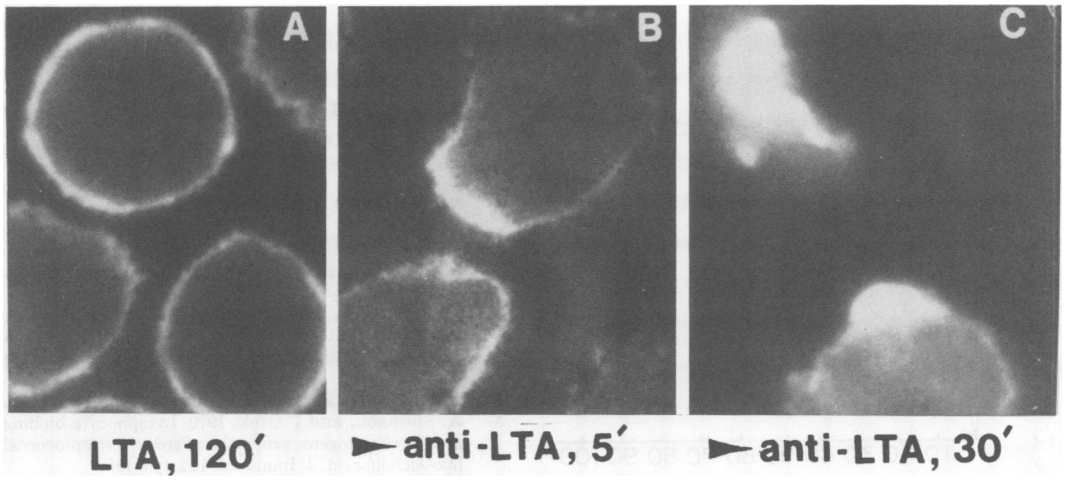

FIG. 5. *Immunofluorescent labeling of PMN-bound LTA. (A) PMN monolayers were incubated with 100 μl of LTA (10 μg to 10 mg/ml) for 15 min at 37°C, washed three times, suspended in PBS, and incubated at 37°C for various time periods of up to 120 min. The monolayers were then fixed with glutaraldehyde, treated with anti-LTA followed by fluorescein-labeled goat anti rabbit immunoglobulin as described in the text. The figure illustrates the fluorescence after 120 min of incubation at 10 mg/ml. No differences in pattern and only subtle differences in intensity of staining could be detected at any concentration or time studied. (B) PMN monolayers were incubated with LTA as above, washed with PBS, and then incubated with anti-LTA for 5 min before fixation with glutaraldehyde and staining with fluorescent antibody. (C) PMN monolayers were treated the same as in B, except the monolayers were incubated with anti-LTA for 30 min.*

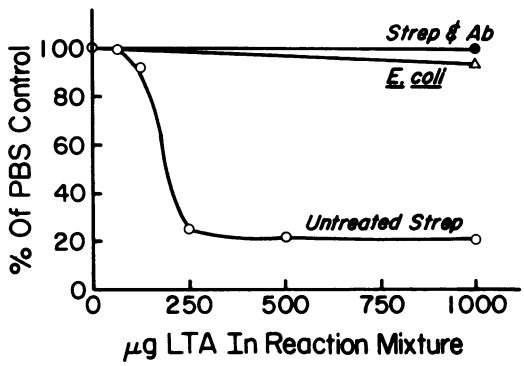

FIG. 6. *The inhibition of streptococcal attachment to PMN by LTA. LTA was added to a 1-ml suspension of untreated streptococci (○), antibody-coated streptococci (●), or E. coli (△). Each of the above mixtures was added to PMN monolayers and incubated for 30 min at 4°C. After washing, the number of bacteria attached to the monolayers was estimated by microscopy. The attachment of untreated streptococci in control incubations with PBS was considered as 100%.*

ing of LTA to erythrocytes. In the present study, fatty acid-free albumin was found similarly to inhibit the binding of LTA to PMN. The presence of a protein with fatty acid binding sites in cell membranes, therefore, may serve as the receptor for LTA. Proof that such a receptor exists in host cell membranes awaits the isolation of a membrane protein with LTA binding activity.

Whatever their nature, LTA receptors appear to be centrally involved in the attachment of group A streptococci to host cells. LTA receptors on the surface of oral epithelial cells were shown to mediate adherence of streptococci; blocking of these receptors with LTA markedly inhibited streptococcal adherence (29). Similarly, coating of PMN with LTA prevented attachment of streptococci. In contrast, LTA did not affect the binding of *E. coli* or antibody-coated streptococci, suggesting that LTA receptors are distinct from Fc receptors or mannose-containing receptors which mediate the attachment of certain *E. coli* strains (1) to PMN.

The relationship between occupation of LTA binding sites on PMN and inhibition of streptococcal attachment was shown by performing an LTA-binding assay concomitant with a streptococcal attachment assay, utilizing the same PMN suspension. The data showed that occupation of fewer than one-third of the total LTA binding sites resulted in greater than two-thirds inhibition of streptococcal attachment to PMN.

LTA of group A streptococci consists of polyglycerol phosphate covalently joined to a glycolipid at one end (29). On the average, each molecule contains about one fatty acid residue (24) and, therefore, must behave as a monovalent ligand in its receptor-binding capacity. That

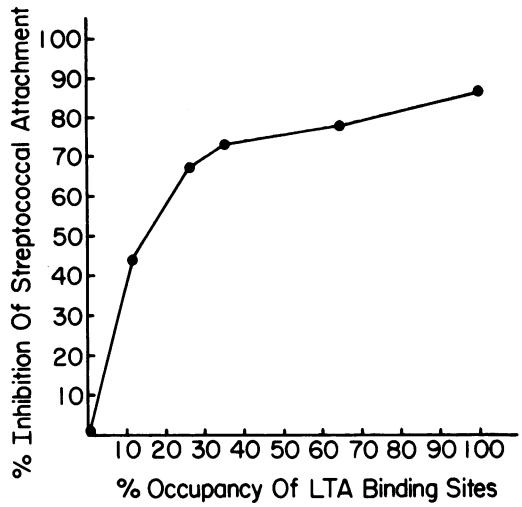

FIG. 7. *Relationship between the occupation of LTA binding sites and inhibition of streptococcal attachment to PMN. Tubes containing 4.5 × 10⁶ PMN were incubated with various concentrations of [³H]LTA for 60 min at 4°C in a total volume of 0.2 ml. After LTA binding, 0.8 ml of streptococci or PBS was added, and the mixtures were incubated an additional 60 min at 4°C. PMN were then assayed for bound LTA and for adherent streptococci. The binding data were plotted according to the method of Scatchard as in Fig. 4, and the intercept with the abscissa was taken as 100% occupancy. The percent occupancy for each point was then calculated by the following formula: percent occupancy = [(micrograms of [³H]LTA bound at each concentration of added LTA)/(micrograms of [³H]LTA bound at saturation)] × 100.*

this is the case is supported by our immunofluorescence studies. The ring pattern of immunofluorescence on PMN demonstrated that LTA remained bound uniformly over the entire surface of PMN for as long as 2 h. Capping of LTA receptors was induced only when the polyglycerol phosphate determinants of the PMN-bound LTA were cross-linked by anti-LTA. These results strongly suggest that LTA monovalently binds only to a single site on the cell membranes.

In summary, our studies suggest that human PMN contain a single population of LTA receptors and that these receptors mediate the attachment of streptococci in vitro. Further studies are needed to determine the role such receptors may have in modulating the diseases produced by group A streptococci.

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