INFECTION AND IMMUNITY, Jan. 1977, p. 156-164
Vol. 15, No. 1

Printed in U.S.A.

Binding of Bacterial Endotoxin to Murine Spleen Lymphocytes

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Received for publication 26 July 1976

The early events in lipopolysaccharide (LPS)-induced B-cell activation were investigated by studying the binding of ^{14}C-labeled LPS to murine lymphocytes in vitro. In these studies we utilized intrinsically labeled ^{14}C-labeled LPS from *Salmonella minnesota* or the ^{14}C-labeled glycolipid derived from the Re mutant of *S. minnesota* (R595). Bone marrow-derived (B) lymphocytes bound more LPS than did thymus-derived (T) lymphocytes. Binding of LPS to murine spleen lymphocytes from strain C3H/HeN was compared with the binding to spleen lymphocytes from strain C3H/HeJ, a strain resistant to certain biological activities of LPS including mitogenesis. Spleen cells from both strains bound LPS equally well, suggesting that unresponsiveness of C3H/HeJ mice to LPS is due to factors other than a defect in binding of LPS. LPS binding to cells appeared to be due to a nonspecific interaction between the lipid moiety of LPS and the lipid components of the cell membrane. Thus, the highly lipophilic, polysaccharide-deficient glycolipid from R595 bound at least 20 times better than did LPS. Furthermore, partial removal of cell surface proteins with trypsin or sialic acids with neuraminidase enhanced glycolipid binding, suggesting that binding is not through a protein- or sialic acid-containing receptor. The binding of glycolipid to lymphocytes was only partially specific since unlabeled glycolipid R595, lipid A, and LPS did not completely inhibit the uptake of ^{14}C-labeled glycolipid R595. In addition, binding could be inhibited by a nonmitogenic phospholipid (phosphatidyl ethanolamine), which also is consistent with a nonspecific lipid-lipid interaction. Experiments were performed to determine the relationship of LPS binding to lymphocyte activation in the lymphocytes. The process of activation of lymphocytes by LPS was a slow one, since LPS was required to be present in culture for at least 24 h in order to obtain significant lymphocyte activation, suggesting that the amounts of LPS bound earlier are either quantitatively or qualitatively insufficient to irreversibly activate the cell.

A variety of substances have been reported to cause lymphocyte mitogenesis. Some of these agents, such as phytohemagglutinin (PHA) and concanvalin A (Con A), are plant lectins that stimulate thymus-derived (T) lymphocytes (3, 6, 11, 12). It is believed that there are surface receptors on lymphocytes to which PHA and Con A bind, and that this process of binding is a prerequisite for lymphocyte activation (19, 29, 38). The molecular properties of these surface receptors for PHA and Con A have been partially characterized (17).

On the other hand, bacterial endotoxins or lipopolysaccharides (LPS) induce cell division in bone marrow-derived (B) murine lymphocytes, and the lipid moiety is responsible for this mitogenic activity (2, 13, 32). LPS insolubilized to Sepharose matrices have been reported to be mitogenic, suggesting that the initial step in the activation of lymphocytes by LPS occurs at the lymphocyte cell surface (27). In addition, LPS is known to bind to lymphocytes in vitro (26, 37). However, unlike the T-cell mitogens, the nature and specificity of the LPS binding event has not been well characterized. We therefore undertook a comprehensive study to determine the nature of the interaction between LPS and lymphocytes, utilizing intrinsically radiolabeled LPS. We found that LPS binds more to B than to T lymphocytes, suggesting a possible mechanism for its mitogenic specificity. Furthermore, our data suggest that LPS binds via a nonspecific interaction between

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its lipid moiety and some lipid component of the cell membrane.

MATERIALS AND METHODS

Animals. Normal 6- to 8-week-old, female C3H/HeN (Division of Research Services, National Institutes of Health) or C3H/HeJ (Jackson Laboratories, Bar Harbor, Me.) mice were used throughout the study. In addition, athymic Nu/Nu mice (Division of Research Services, National Institutes of Health) were used where indicated.

Media and reagents. All tissue cultures were grown in RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) and were bufferd with HEPES (N-2-hydroxyethyl-*N'*-2-ethanesulfonic acid) (15 mM). Fetal calf serum (FCS, lot 2424, Industrial Biological Laboratories, Rockville, Md.) was used because it produced low background counts and high mitogen responses. This serum is "nonpermissive" in Mishell-Dutton culture systems (T. Chused, personal communication). Plastic tissue culture plates (no. 3040, Microtest II) were obtained from Falcon Products, Oxnard, Calif. Trypsin (2× crystallized, bovine pancreas) and neuraminidase (*Clostridium perfringens*) were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Endotoxin preparations. For binding studies, we used radiolabeled LPS and glycolipid isolated from *Salmonella minnesota* 1114 and the rough mutant of *S. minnesota* R595, respectively. These compounds (supplied by A. Nowotny, Temple University, Philadelphia, Pa.) were labeled intrinsically by growing the bacteria in the presence of [^{14}C]glucose. The ^{14}C-labeled LPS was extracted from the bacteria by the phenol-water procedure of Westphal (40). The crude product was further purified to remove proteins and nucleic acids by procedures involving repeated ultracentrifugation at 105,000 × *g* as described by Westphal and Jann (39). The ^{14}C-labeled glycolipid was extracted from *S. minnesota* R595 with chloroform-methanol according to the procedure described by Chen et al. (9). Both the ^{14}C-labeled LPS and the ^{14}C-labeled glycolipid were free of nucleic acids since the purified preparations did not show any absorption maxima at 260 nm. The specific activities of ^{14}C-labeled LPS and ^{14}C-labeled glycolipid were 0.23 and 0.35 μCi/mg, respectively.

The glycolipid from *S. minnesota* R595 is insoluble in aqueous solution. Therefore, for use in binding and mitogen studies, an ultrafine dispersion was prepared by sonication (33). The glycolipid was first suspended in pyrogen-free distilled water at a concentration of 1 mg/ml and sonicated for 1 min at 1.7 A (Branson Instruments, Co., Stamford, Conn.). It was then made isotonic with appropriate amounts of sterile, pyrogen-free 9% NaCl. This dispersion remains stable for weeks. Heated LPS samples as used in this investigation were obtained by heating LPS (both labeled and unlabeled) at a concentration of 1 mg/ml in phosphate-buffered saline at 100°C for 3 h.

For mitogen studies, we used LPS from *Escherichia coli* K235 (supplied by F. McIntire, Abbott Laboratories, Chicago, Ill.), isolated by the phenol-water procedure. Details of the extraction procedure have been described elsewhere (25).

Purification of lymphocytes. (i) Removal of macrophages. Macrophages were removed from mouse spleen cells by passage through a column containing sterilized Sephadex G-10 (25 by 2.5 cm) as described by Ly and Mishell (24). Two to four milliliters of cells (1.5 × 10^8/ml) was applied to the top of the column, allowed to penetrate the gel bed, and then washed through with 25 to 30 ml of medium containing 5% FCS. Approximately 50% of the cells were recovered in the effluent. A similar percentage of recovery has been reported by Ly and Mishell (24). The effluent cells were checked for possible macrophage contamination by examining stained smears (Wright) with a Zeiss microscope equipped with phase-contrast optics (Carl Zeiss, Inc., New York). Using this separation procedure, the proportion of surface immunoglobulin-bearing and Thy 1,2 (θ)-bearing cells in the starting and the effluent populations remain unaltered (T. Chused, personal communication).

(ii) Removal of erythrocytes. Erythrocytes were removed from spleen cells by lysis with tris(hydroxymethyl)aminomethane (Tris)-buffered ammonium chloride (pH 7.2) according to the procedure described by Boyle (8). After the treatment with Tris-buffered ammonium chloride, lymphocytes were washed several times with the medium to remove any erythrocyte stroma.

Separation of T and B lymphocytes using nylon wool columns. Mouse spleen cells were separated into T and B populations according to the procedure described by Handwerger and Schwartz (16). The purity of the nylon-adherent (B) and nylon-nonadherent (T) populations was checked by immunofluorescent techniques with fluorescein-conjugated rabbit anit-mouse immunoglobulin (14). Lymphocytes (5 × 10^6/ml) were incubated at 4°C in 0.1 ml of undiluted fluorescein-conjugated rabbit anti-mouse immunoglobulin (Meloy Laboratories, Springfield, Va.) in the presence of 10 mM sodium azide for 30 min. Cells were washed four times with medium, mounted on Vaseline cover slips, and counted using an ultraviolet microscope (E. Leitz, GMBH, Germany). The nylon-adherent cell populations (B) were found to be 85% immunoglobulin positive, whereas the nonadherent populations (T) contained very few immunoglobulin-positive cells (<3%).

LPS binding. All binding studies were performed using purified (macrophage- and erythrocyte-depleted) lymphocytes except where specified.

(i) Time dependence of binding of endotoxins to lymphocytes. Purified lymphocytes (5 × 10^6) were incubated in 0.5 ml of the medium with a known amount of ^{14}C-labeled LPS or ^{14}C-labeled glycolipid at 37°C. At different time intervals, cells were washed with the medium until no radioactivity was observed in the culture supernatant. Then the cell pellet was dissolved in scintillation fluid (Hydromix, Yorktown, N.J.), and bound radioactivity was determined in a liquid scintillation spectrometer (Packard Tri-Carb, Downers Grove, Ill.).

(ii) Specificity of glycolipid binding. Purified

lymphocytes (5×10^6/ml) were incubated for 1 h at 37°C with ^{14}C-labeled glycolipid (50 μg) along with increasing amounts of competing substances such as unlabeled glycolipid A, LPS, and phosphatidyl ethanolamine (10 to 200 μg), which were added at the same time as was the LPS. Cells were washed and the cell-bound radioactivity was measured as before.

Binding of ^{14}C-labeled glycolipid by tryspin- and neuraminidase-treated lymphocytes. Purified lymphocytes (10^7/ml) were treated with trypsin (0.1% in phosphate-buffered saline at 37°C for 15 min). Cells were then washed three times with buffer and incubated at 37°C with ^{14}C-labeled glycolipid (100 μg/ml) in medium. After 1 h, the cells were washed and the cell-bound radioactivity was counted as before. Cells were treated with neuraminidase (0.1 U, 1 h at 37°C) in a similar way.

Lymphocyte proliferation. To compare the dose-response kinetics of LPS, heated LPS, and glycolipid, spleen cells (5×10^5) were cultured in microtiter plates with varying doses of these mitogens for a total period of 72 h at 37°C in a humidified atmosphere containing 5% CO_2. No serum was added to the culture medium. For the last 4 h of the culture, cells were pulsed with tritiated thymidine (0.5 μCi) and were harvested using an automatic cell harvester (Mash II, Microbiological Associates, Bethesda, Md.) with phosphate-buffered saline (pH 7.2), and then the filters were air-dried. With this harvesting technique, no trichloroacetic acid is required. The filter disks were placed in 3 ml of Hydromix (Yorktown Research, N.J.) and counted in a Packard Tri-Carb scintillation counter. The arithmetic mean of triplicate samples was determined, and the results were expressed as the stimulation ratio (E/C), where E/C is defined as the ratio of mean counts per minute in the stimulated cultures divided by mean counts per minute in the unstimulated cultures.

Kinetics of lymphocyte activation. To determine the amount of time LPS had to be in contact with lymphocytes to produce irreversible activation, 2×10^6 C3H/HeN spleen cells in 1 ml of medium containing 2% FCS were cultured in 3-ml glass vials along with 0.1 μg of LPS per ml. At various time intervals (5 min, 4 h, 24 h, 48 h), cells were washed free of unbound LPS and then incubated with fresh medium containing 2% FCS for the remainder of the 72-h culture period. Cells were pulsed with tritiated thymidine (5 μCi) for the last 4 h of the culture and harvested and counted as described previously.

RESULTS

Binding of LPS to murine spleen lymphocytes. We first sought to determine the characteristics of the binding of LPS to lymphocytes using ^{14}C-labeled LPS and its derivatives. Since both erythrocytes and macrophages bind LPS (7, 35), the binding studies were performed using lymphocytes depleted of these cells.

Spleen lymphocytes (5×10^6 cells) were incubated with ^{14}C-labeled LPS (100 μg) for various time periods at 37°C. The amount of ^{14}C-labeled LPS bound to spleen cell populations of non-macrophage-depleted, macrophage-depleted, and athymic Nu/Nu mice is shown in Fig. 1. It was found that macrophage-containing spleen cell populations bound more LPS than those depleted of macrophages. Lymphocytes depleted of macrophages bound only up to 0.5% of the total ^{14}C-labeled LPS added in the first few minutes. The uptake doubled in the first 4 h, and afterwards it increased slowly with time. After 48 h, 2% of the added LPS was bound to

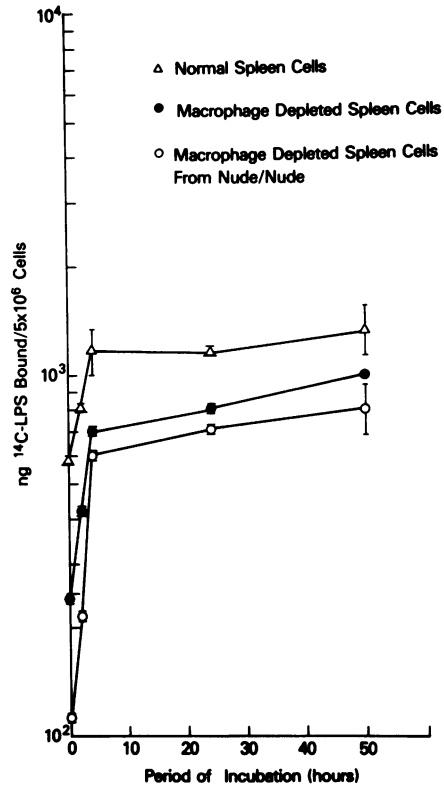

FIG. 1. *Binding of ^{14}C-labeled LPS by splenic lymphocytes from C3H/HeN or Nu/Nu mice as a function of time. Lymphocytes (5×10^6) were incubated at 37°C with ^{14}C-labeled LPS (100 μg) for various time periods. The zero-time point represents cells to which were pulsed with LPS and then immediately washed. Each point in the figure represents the arithmetic means ± 1 standard error of the mean of duplicate determinations. These lymphocytes were depleted of erythrocytes and macrophages as described in the text. Symbols: Δ, normal (nonmacrophage depleted) spleen cells from C3H/HeN mice; ○, macrophage-depleted spleen cells from C3H/HeN mice; ●, macrophage-depleted spleen cells from Nu/Nu. Because of the nonsynchronous growth of the lymphocytes in the culture medium, the plots after 20 h do not take into account cell surface area changes by blast formation, cell death, or division.*

lymphocytes. The uptake of ^{14}C-labeled LPS by macrophage-depleted spleen cells of nude mice which contained mostly B lymphocytes was essentially the same as that of macrophage-depleted spleen cells from normal mice (Fig. 1).

Binding of LPS by T or B lymphocytes. Since LPS is a specific mitogen for murine B lymphocytes, binding studies were performed on isolated T and B populations to investigate whether the mitogenic specificity for LPS was due to a difference in binding properties of T and B cells.

Macrophage-depleted spleen lymphocytes were fractionated into T and B populations on a nylon wool column. It was observed that ^{14}C-labeled LPS bound to both B and T cells (Fig. 2). B cells bound about twice as much LPS as did T cells, although the kinetics of LPS uptake was similar for both cell types.

To determine if B cells bound more LPS because they were larger, the size distribution of T and B cells in our splenic populations was determined after fractionation but before culture, using a Coulter counter with an automatic cell-sizing attachment (Fig. 3). T cells possessed a mean volume of 175 μm^3, whereas that of B cells was 135 μm^3, indicating that the splenic T cells were slightly bigger than B cells. Since the two cell types did not differ significantly in size, the increased binding of LPS by

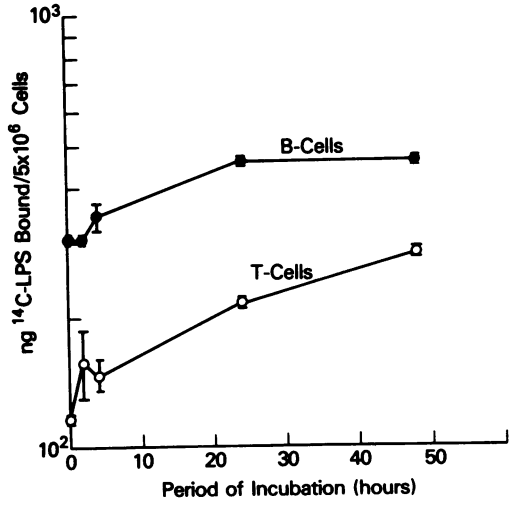

FIG. 2. *Binding of ^{14}C-labeled LPS by B and T lymphocytes prepared from C3H/HeN mice. Cells were fractionated into B and T populations using a nylon wool column. Lymphocytes (5 × 10^6) were incubated at 37°C with ^{14}C-labeled LPS (50 μg) for various time periods. Each point in the figure represents the arithmetic mean ± 1 standard error of the mean of duplicate determinations.*

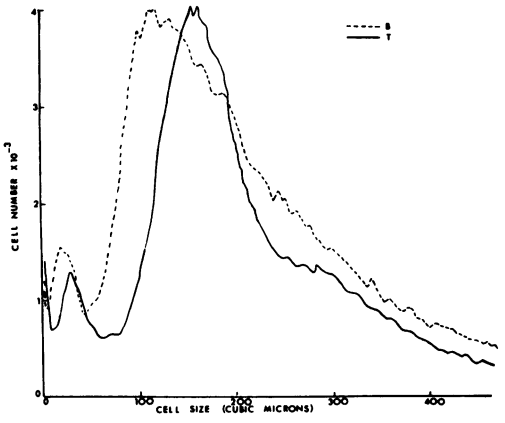

FIG. 3. *Size distribution of T and B cells using a Coulter counter with an automatic cell-sizing attachment. Cells were sized immediately after their isolation from the nylon wool column.*

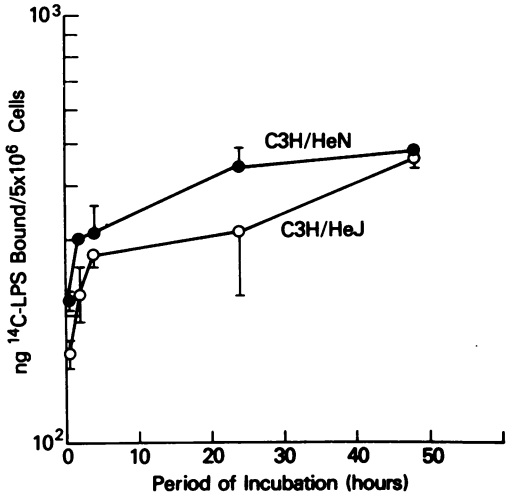

FIG. 4. *Binding of ^{14}C-labeled LPS by erythrocyte- and macrophage-depleted spleen cells (5 × 10^6) from C3H/HeN or C3H/HeJ mice. Each point represents the arithmetic mean of duplicate determinations. No bars are indicated for second and fifth points for C3H/HeN spleen lymphocytes as no significant difference was observed between the duplicate determinations. Lymphocytes were incubated with 50 μg of ^{14}C-labeled LPS.*

B cells may reflect an increased LPS-receptor density on the surface of these cells.

Binding of LPS to spleen cells of LPS-responsive and -nonresponsive mice. The C3H/HeJ mouse strain responds poorly to LPS in vivo (27) and in vitro (37). Investigations were carried out to determine whether this phenomenon of unresponsiveness was due to differences in LPS binding by splenic lymphocytes of C3H/

HeJ mice. The kinetics of ^{14}C-labeled LPS uptake by macrophage-depleted spleen cell populations of C3H/HeJ mice was similar to that of C3H/HeN mice, which responded well to LPS (Fig. 4). This finding suggests that unresponsiveness to LPS in C3H/HeJ mice is due to some defect other than absent or diminished binding of LPS.

Binding of various preparations of LPS to spleen lymphocytes. (i) Heated LPS. According to Lüderitz et al. (22), a preparation of heated LPS binds much more radily to erythrocytes than does unheated LPS. Experiments were performed to determine whether the binding of heated LPS to purified lymphocytes was also enhanced. LPS was heated for 3 h at 100°C.

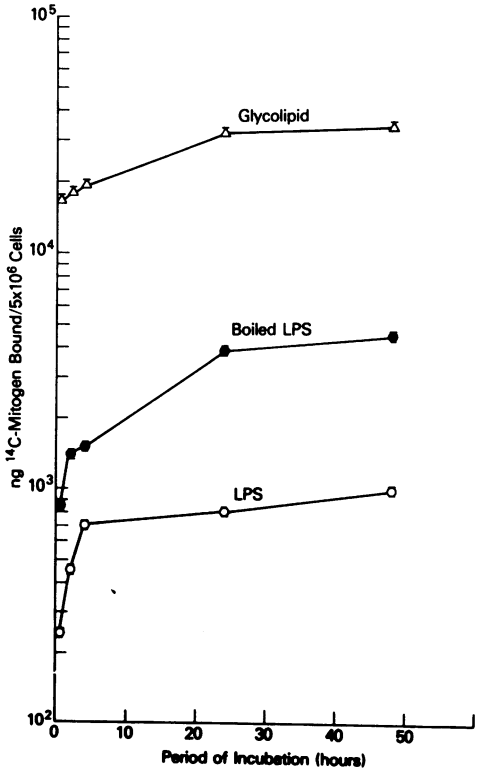

FIG. 5. *Binding of ^{14}C-labeled LPS, heated LPS, and glycolipid by spleen lymphocytes from C3H/HeN mice as a function of time. ^{14}C-labeled LPS was heated in phosphate-buffered saline buffer (pH 7.1) for 3 h at 100°C. The ^{14}C-labeled glycolipid was obtained biosynthetically from the heptoseless rough mutant of S. minnesota R595. Cells (5 × 10^6/ml) were incubated with 100 μg of the radiolabeled mitogen for various time periods and washed, and cell-bound radioactivity was counted as described in the text. Each point represents the arithmetic mean ± standard error of the mean of duplicate determinations.*

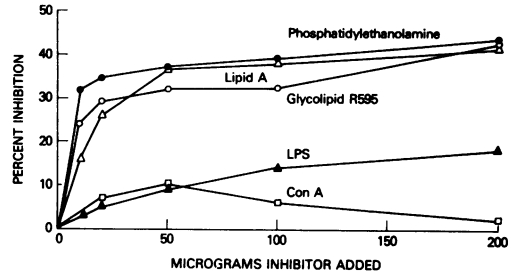

FIG. 6. *Percent inhibition of the binding of ^{14}C-labeled glycolipid R595 from S. minnesota by increasing amounts (10 to 200 μg) of unlabeled lipid A, glycolipid R595, phosphatidyl ethanolamine, LPS, and Con A. Lymphocytes (5 × 10^6/ml) were incubated for 1 h at 37°C with ^{14}C-labeled glycolipid (50 μg) and increasing amounts of unlabeled competing substances. Cells were washed and the cell-bound radioactivity was measured as described in the text.*

Within the first few minutes, twice as much heated LPS bound to lymphocytes as compared with unheated LPS, and the binding was increased at least fourfold after 48 h (Fig. 5).

(ii) Glycolipid from *S. minnesota* R595. To evaluate the role of the lipid moiety of LPS in this binding phenomenon, studies were carried out with glycolipid from rough mutant of *S. minnesota* R595. This glycolipid possesses neither the antigenic O-specific nor the core polysaccharide (21), but is still a potent mitogen for murine spleen lymphocytes (32). The kinetics of ^{14}C-labeled glycolipid uptake is also shown in Fig. 5. Glycolipid bound much more avidly (18 μg/5 × 10^6 cells) to the cell surface than did LPS (250 ng/5 × 10^6 cells). Fifteen percent of the total glycolipid added was bound in the first few minutes, and this binding increased to 40% after 48 h.

Specificity of glycolipid binding. To determine the specificity of glycolipid binding, competitive binding studies were performed with unlabeled glycolipid R595, LPS, lipid A, phosphatidyl ethanolamine, and Con A. Lipid A, obtained by the acid hydrolysis of LPS, is a structural analogue of glycolipid R595. Phosphatidyl ethanolamine is a common phospholipid constituent of the cell membrane and is known to inhibit the attachment of LPS to erythrocyte membranes (35). The results of these competitive binding studies are shown in Fig. 6. Unlabeled glycolipid, lipid A, and phosphatidyl ethanolamine all partially inhibited (45%) the binding of glycolipid to lymphocytes and were approximately twice as effective as unlabeled LPS. Con A, a structurally unrelated compound, was ineffective in blocking the binding of ^{14}C-labeled LPS, suggesting that Con A

acts at sites different from those of endotoxin. These results suggest that although a part of the glycolipid binding is specific, a significant amount of binding is also nonspecific and irreversible.

Effect of trypsin and neuraminidase treatment on the binding of glycolipid. To determine the role of cell surface proteins or sialic acid in this binding, purified lymphocytes were treated with trypsin or neuraminidase, and binding of ^{14}C-labeled glycolipid to the enzyme-treated cells was detemriend. Treatment of lymphocytes with neuraminidase led to an increased binding (40%) of glycolipid over untreated lymphocytes (Table 1). A similar increased binding of glycolipid was observed when lymphocytes were treated with trypsin. These findings suggest that cell surface trypsin-sensitive proteins or sialic acids are not required for LPS binding.

Comparison of mitogenic activity of LPS, heated LPS, and glycolipid. We next investigated the relationship between binding affinity of LPS and its derivatives to their mitogenic potential. The proliferative response of mouse spleen cells induced by LPS, heated LPS, and glycolipid is shown in Fig. 7. Although all of these compounds were mtiogenic, LPS was the most mitogenic, heated LPS was less mitogenic, and the glycolipid used in this investigation was the least mitogenic. Thus, there appears to be an inverse correlation of binding to mitogenicity. An analogous decrease in in vivo immunogenicity of heated LPS has been reported by Neter et al. (28).

Relationship between lymphocyte binding of mitogen and irreversible cell activation. Since we found that LPS was able to bind to cell surfaces after a relatively short incubation time, we next investigated how long an exposure of lymphocytes to LPS was required for the induction of an irreverisble state of activation in the lymphocytes. Spleen cells were cultured with LPS, and at varying time intervals, cells were washed to remove LPS and placed back in culture with fresh medium. All cultures were harvested at the end of 72 h.

TABLE 1. *Effect of trypsin and neuraminidase treatment on the binding of glycolipid R595 by mouse spleen lymphocytes*

Cells	Glycolipid bound/10^7 cells (μg)	
	Exp 1	Exp 2
Normal	30.25 ± 0.12	33.12 ± 2.2
Neuraminidase-treated	39.50 ± 0.13	46.63 ± 0.27
Trypsinized	ND[a]	46.25 ± 0.328

[a] ND, Not determined.

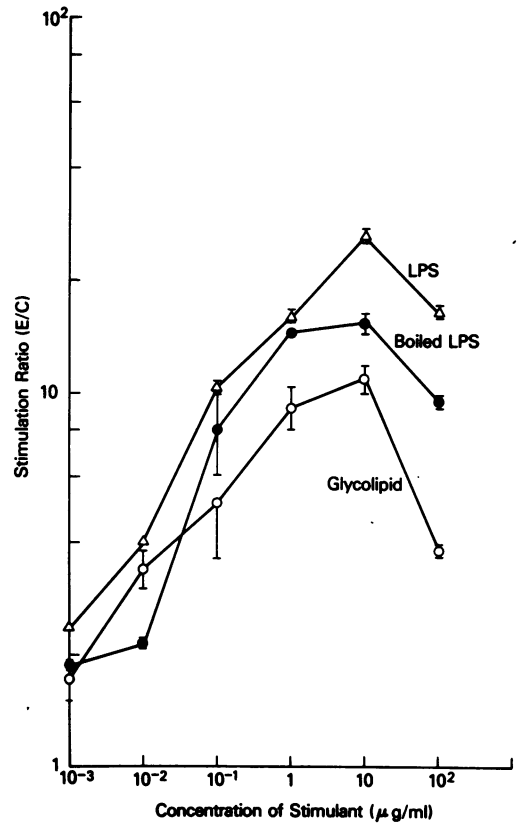

FIG. 7. *In vitro proliferative response of spleen lymphocytes from C3H/HeN mice induced by LPS, heated LPS, and glycolipid. LPS, obtained from E. coli K235, was heated in phosphate-buffered saline at 100°C for 3 h. The glycolipid R595 was sonicated before use. The results are expressed as the arithmetic mean ± standard error of the mean of triplicate cultures and plotted as the stimulation ratio (E/C). Cells were harvested after 72 h of culture. The baseline proliferative response was 1,700 cpm per 5 × 10^5 cells.*

It has been the experience of most workers that the dose-response curve for LPS-induced mitogenesis is flat in a range from 0.1 to 100 μg/ml (32). Thus, if cells were incubated with 100 μg of LPS per ml, it would be necessary to remove 99.9% of the LPS before any significant reduction in the mitogenic action of LPS could be observed. For this reason, we pulsed our cells with 0.1 μg of LPS per ml, which is also a stimulatory concentration but is on the linear part of the dose-response curve.

In contrast to what is observed with lectin mitogens such as PHA (18), LPS seemed to require a prolonged incubation with lympho-

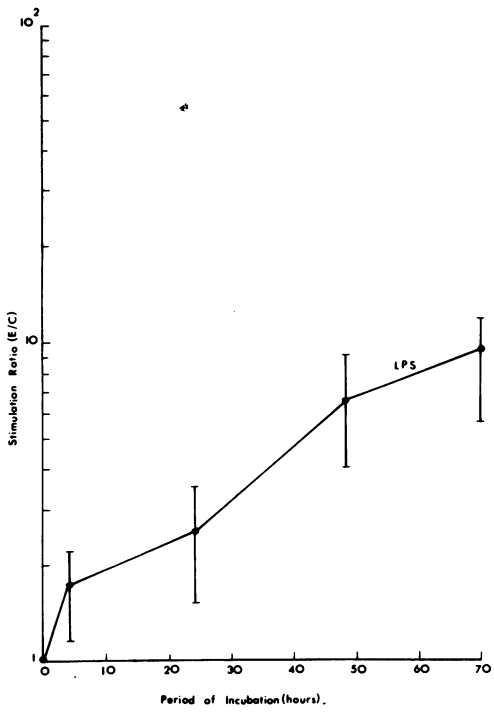

FIG. 8. *In vitro proliferative response of spleen lymphocytes from C3H/HeN mice incubated with LPS from E. coli K235 for varying time periods. Lymphocytes (2 × 10⁶/ml, 1 ml) were incubated with LPS (0.1 μg/ml) for different time periods, washed, and resuspended in the fresh medium containing 2% FCS. All lymphocytes were cultured for a total period of 72 h. The results are expressed as the arithmetic mean ± standard error of the mean of four experiments and plotted as the stimulation ratio (E/C). Base-line proliferative response, 3,500 cpm per 2 × 10⁶ cells.*

cytes in order to produce irreversible cell activation. Thus, after a 4-h period of incubation with the cells, LPS was unable to produce any lymphocyte activation at all (Fig. 8). After 24 h of incubation with the cells, the stimulation induced was only one-fourth maximal, and maximum lymphocyte activation only occurred after LPS and cells were incubated together for a full 72 h.

DISCUSSION

In this investigation we have studied the nature of interaction between bacterial endotoxins and lymphocytes. We have found that LPS binds to both T and B lymphocytes. B lymphocytes bound twice as much as LPS as did T lymphocytes. An analysis of cell size indicated that T cells were larger than B cells, suggesting that B cells actually bind more LPS per unit area of cell membrane than do T cells. The reason as to why B lymphocytes bind more LPS than do T lymphocytes is not well understood. Since LPS interacts with membrane phospholipids (34), it is possible that there might be a difference in the phospholipid composition of B- and T-cell plasma membranes which might account for the difference in binding of LPS by these cells.

Möller et al. (26) studied the binding of LPS to mouse thymocytes and whole spleen cells and did not observe any difference in LPS binding by cells from these two organs. We have found that in murine spleens, B lymphocytes bound twice as much LPS as compared with T cells. A comparison of our findings with those of Möller et al. indicates that mouse thymocytes bind more LPS than do mouse splenic T cells. Mouse thymocytes possess less cell surface sialic acid than do splenic T cells (10). Since our findings suggest that removal of cell surface sialic acid increases LPS binding, it is possible that the increased binding of LPS by thymocytes may be related to their decreased content of cell surface sialic acid.

The nature of the LPS-cell interaction has also been investigated. All our findings are consistent with the concept that LPS binding is due to a nonspecific interaction between the lipid moiety of LPS and a lipophilic component of the cell membrane. Thus, the binding of glycolipid is equally inhibited by a mitogenic lipid (lipid A) and a nonmitogenic one (phosphatidyl ethanolamine). Furthermore, the less lipophilic but still mitogenic LPS inhibits glycolipid binding less well than the more lipophilic compounds. Consistent with the concept that binding results from a lipid-lipid interaction is our finding that treatment of lymphocytes with trypsin and neuraminidase caused an increased binding of ^{14}C-labeled glycolipid by these cells. A similar increased binding of the ^{14}C-labeled glycolipid to trypsinized and neuraminidase-treated rat embryo fibroblasts has been reported by Bara et al. (4). Our interpretation of these results is that by reducing the protein and sialic acid content of the cell membrane, we have reduced the steric factors that hinder the incorporation of the glycolipid into the membranes, thus allowing freer access to the membrane lipids. Other mechanisms such as a change in surface charge may also be involved. Nevertheless, these results certainly argue against a trypsin-sensitive protein or sialic acid containing LPS receptor. Earlier, Adler et al. (1) studied the interactions of LPS with neuraminidase-treated lymphocytes by fluorescence microscopy and observed that treatment with neuraminidase increased the

degree of membrane binding. However, such treatment prevented the internalization of LPS. A possible explanation for this apparent disparity is that Adler et al. studied binding of LPS, whereas in our neuraminidase studies and in those of Bara et al. (4) the binding of a glycolipid was studied. Perhaps cell surface sialic acids bind the polysaccharide moiety of LPS but inhibit or delay access of the lipid moiety to the cell membrane lipids. This concept of a lipid-lipid interaction between LPS and a cell is certainly not new and has been amply documented in other systems (5, 34).

We have also attempted to investigate the correlation between LPS binding and the process of lymphocyte activation. It is clear that lymphocyte binding of LPS alone does not automatically result in cell activation. Thus, although T cells do bind LPS, they are not activated. This phenomenon is similar to what is observed with T-cell mitogens, where B cells bind PHA and Con A but are not activated (15). Similarly, we found that spleen cells of C3H/HeJ mice, which are resistant to the mitogenic effects of LPS, still bind this compound. These findings are essentially identical to those of Watson and Riblet (37), even though they used a nonintrinsically labeled LPS preparation and did not examine erythrocyte- and macrophage-depleted cells. Therefore, unresponsiveness or lack of activation of spleen cells by LPS in C3H/HeJ mice is not due to the lack of LPS binding. Finally, we have also found that the glycolipid from *S. minnesota* R595 and the heated LPS, which both exhibit an increased binding to spleen lymphocytes, were less mitogenic than LPS. Therefore, we would conclude from these findings that although the binding of LPS to lymphocytes may be a prerequisite for activation, this binding is not by itself sufficient to cause activation. In fact, we suspect that there must be an additional cell alteration induced by the bound LPS that actually determines whether activation takes place.

Our findings suggest that this second phase of LPS-cell interaction may be delayed in time since we have found that a prolonged presence of LPS is necessary for maximum cell stimulation. The mitogenic activity of LPS is associated with the lipid A moiety of the molecule (2). Because a prolonged presence of LPS in the medium is necessary before appreciable deoxyribonucleic acid synthesis takes place, the initial step in the process of activation of lymphocytes might involve an insertion of the lipid A moiety of LPS into the lipid bilayer of the B-cell membrane, which may require a longer time than simple binding to the surface. An alternate explanation for this requirement for a prolonged presence of LPS in culture is that bound LPS may be rapidly turned over, either shed or internalized. Cell activation, on the other hand, may require the continuous presence of LPS in or on the membrane. If LPS turnover is rapid, then this requirement for a continual presence could be furnished only if LPS was present in culture, where it could constantly rebind to the lymphocyte surface.

This finding that LPS is required almost continually in culture is somewhat controversial since Adler et al. (1) reported that a brief exposure (1 to 4 h) of lymphocytes to LPS was sufficient to induce deoxyribonucleic acid synthesis. However, despite this apparent discrepancy, these results are not in total disagreement for several reasons. First, inspection of their data reveals that after 4 h of incubation with LPS, activation of the cells was only a small fraction (26%) of that obtained after a full 72-h incubation. Secondly, these authors incubated their cells with a higher concentration of LPS (3.3 μg/ml) than we did (0.1 μg/ml). In preliminary studies, we found that when cells were pulsed with higher concentrations of LPS, washing was incomplete and enough LPS remained in the culture to produce activation. This is undoubtedly due to the dose-response curve of LPS where stimulation is often observed at concentrations below 0.01 μg/ml (32). Thus, when starting with a concentration of 3.3 μg/ml, a washing procedure that removes as much as 90% of the unbound LPS would still leave behind sufficient LPS (0.03 μg/ml) to produce cell activation. Furthermore, we have also found in preliminary studies that other B-cell mitogens such as polymerase I will activate cells after a short incubation period. The LPS used in these studies was nucleotide and protein free. It is possible that different LPS preparations might contain other mitogenic contaminants that might produce the effects observed by Adler et al. (1). Finally, the data presented in this report represents the results of four individual experiments performed in this fashion, whereas the data of Adler et al. (1) represent only one. In addition, we have repeated these studies many times using different LPS preparations and different LPS concentrations. The ability to reproduce these findings many times makes us confident that LPS must remain in prolonged contact with cells before irreversible activation can take place.

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