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In Vitro Transformation of Mouse Bone-Marrow-Derived (B) Lymphocytes Induced by the Lipid Component of Endotoxin

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An analysis of which component of lipopolysaccharide, the lipid or the polysaccharide, is mitogenic for mouse B-lymphocytes has been performed. A purified glycolipid derived from a rough mutant of *Salmonella minnesota* (R595) that does not contain any o-polysaccharide at all is more mitogenic than an intact lipopolysaccharide derived from a smooth strain of *S. minnesota*. Results using fractions produced by several different chemical modifications of whole lipopolysaccharide confirm this result. Acid hydrolysis separates lipopolysaccharide into two components. The lipid fraction is mitogenic, whereas the polysaccharide fraction is not. Those procedures which degrade or modify only the lipid moiety while preserving the antigenic integrity of the polysaccharide also destroy mitogenicity. These include alkaline hydrolysis and deacylation by a more specific treatment with potassium methylate. The lipid preparations are fully active on highly purified B-lymphocyte populations (prepared by anti-theta antiserum and complement), whereas they have no effect on highly purified T-lymphocyte populations (prepared by anti-immunoglobulin and complement). These data demonstrate that the lipid moiety of endotoxin is the B-lymphocyte mitogen, whereas the polysaccharide has no demonstrable mitogenic activity.

Recent work has demonstrated that the endotoxic lipopolysaccharide (LPS) derived from a variety of gram-negative bacteria are specific B-cell mitogens in the mouse (2, 6, 17). The LPS molecule is large and extremely heterogeneous (mol. wt. $300 \times 10^5 - 3 \times 10^6$). The biological toxicity of LPS has been shown to reside in the lipid moiety, whereas the antigenicity has been associated with the polysaccharide (19, 21). It was, therefore, of interest to determine which component of LPS was mitogenic.

In the present study we have examined the mitogenic potential of the lipid moiety of LPS by using two different techniques. First, we have compared the mitogenicity of LPS from a smooth strain of *Salmonella minnesota* with that of a purified glycolipid derived from a rough mutant of *S. minnesota* R595 (11). This latter compound consists of a glycolipid plus 2-keto-3-deoxyoctanoic acid (KDO), without any o-polysaccharide or heptose that is present in the smooth strain LPS, and possesses all the endotoxic ability of the smooth strain LPS molecule (9, 10). Secondly, we have used several standard chemical techniques to dissociate the lipid from the polysaccharide or to specifically inactivate the lipid component of whole LPS. Our data indicate that it is the lipid rather than the polysaccharide moiety of LPS that is mitogenic.

MATERIALS AND METHODS

Animals. Normal female C57/Bl mice (Division of Research Services, National Institutes of Health) were used at 8 to 10 weeks of age.

Cell collection and tissue culture. Spleens were removed aseptically and single cell suspensions were prepared by gently teasing with forceps. The spleen cells were washed twice in medium and suspended at a concentration of 2×10^6 cells per ml in RPMI 1640 (Grand Island Biological, Grand Island, N.Y.) with added L-glutamine (2 mM/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2% fetal calf serum (Industrial Biologic Laboratories, Rockville, Md.). Triplicate 1-ml samples of cells were cultured

along with the test materials in 1-dram glass vials for 72 h at 37 C in a humidified atmosphere of 5% CO_2 in air and then pulsed with 3.0 μCi of tritiated thymidine (^{3}H-Tdr, 6.7 Ci/mM, New England Nuclear Corp., Boston, Mass.) for the last 4 h of culture. Cultures were harvested by the Millipore filter technique of Robbins et al., (18) and the trichloroacetic acid-insoluble radioactivity was measured as counts per minute 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.

Preparation and characterization of the glycolipid. Lipopolysaccharide from a smooth strain of *S. minnesota* was prepared by a standard Westphal extraction procedure (20). The o-polysaccharide deficient, endotoxic glycolipid was prepared from the rough mutant of *S. minnesota*, R595, by chloroform-methanol extraction. This glycolipid has been chemically analyzed and found to be essentially free of protein, nucleic acid, and reducing sugars other than glucosamine and KDO (C. H. Chen et al., J. Infect. Dis., in press; 8). In addition, several biological activities of this glycolipid preparation have been studied and found to be similar or better in potency compared to the smooth strain lipopolysaccharide (C. H. Chen et al., J. Infect. Dis., in press; 12).

Chemical treatment of endotoxin. Intact lipopolysaccharide was modified by the following chemical procedures:

(i) Acid hydrolysis. A 10-mg amount of *S. minnesota* LPS in 1.0 N hydrochloric acid was heated at 100 C for 30 min. This treatment splits the LPS into an insoluble precipitate (lipid A) that retains some endotoxic activity and a soluble, partially degraded polysaccharide that retains its antigenicity (haptenic polysaccharide) (13, 14, 20).

(ii) Alkaline hydrolysis. A 1-mg amount of *S. minnesota* LPS in 1 ml of 0.1 N sodium hydroxide was heated at 100 C for 4 h. This procedure destroys the endotoxicity of the LPS by cleaving all ester linkages from the lipid moiety while preserving the antigenic polysaccharide (19, 21).

(iii) Detoxification by deacylation. A 100-mg amount of *Serratia marcescens* 08 LPS prepared as previously described (16) was refluxed with 0.3 M potassium methylate in anhydrous methanol for 60 min, and then filtered, washed, and dried. This procedure cleaves only certain ester-bound, long-chain fatty acids from the lipid moiety of the LPS. The final preparation, termed endotoxoid, has a greatly reduced biological toxicity, but has an intact polysaccharide component which is antigenic (7, 15).

Prior to use in tissue culture, all the chemically modified LPS preparations and their PBS-treated controls were dialyzed against four changes of RPMI 1640. The nonstimulatory preparations were also checked for a possible direct toxic effect on lymphocytes in vitro, by testing their effect on spleen cells cultured with either intact LPS or 1 μg of Concanavalin A (Con A) per ml (2 $\times$ crystallized, Nutritional Biochemical Corp., Cleveland, Ohio). Since the glycolipid and the lipid A were water-insoluble, these were sonically treated in pyrogen-free distilled water for 1 min at 1.7 A (Branson Instruments, Stanford, Conn.) to produce a microdispersion. Prior to use in vitro, the suspensions were mixed with an appropriate amount of sterile pyrogen-free 9% NaCl to make them isotonic. As a control, all other preparations also received similar treatment before being tested.

Preparation of bone marrow-derived (B) and thymus-derived (T) lymphocytes. B-lymphocytes were prepared by selectively killing the T-lymphocytes in the spleen cell suspension with anti-theta antiserum plus complement. Spleen cells (50×10^6) in 1 ml of medium were incubated with 1.5 ml of AKR anti-theta (C_3H) antiserum at 4 C for 30 min. They were then washed once, suspended in 3 ml of guinea pig serum (1:3 dilution) (Texas Biological Co., Fort Worth, Tex.), and reincubated at 37 C for 30 min.

T-lymphocytes were prepared by selectively killing the B-lymphocytes with a heterologous anti-immunoglobulin antiserum plus complement. Spleen cells (50×10^6) in 1 ml of medium plus 1 ml of undiluted guinea pig serum were incubated for 10 min at 22 C in the presence of 10 mM sodium azide. A 0.5-ml amount of goat anti-mouse immunoglobulin was then added and the mixture was incubated at 37 C for 30 min. The use of sodium azide in this procedure has been found to greatly enhance the specific cytotoxicity of anti-immunoglobulin antiserum presumably by preventing the modulation of membrane immunoglobulin from the surface of the B lymphocyte (T. Chused, manuscript in preparation). Sodium azide alone had no detectable effect on B-lymphocytes.

After the cytotoxic procedure, the cell suspensions were washed three times in medium and cell viability was determined by the exclusion of trypan blue dye. Treatment with anti-theta antiserum and complement killed 40% of mouse spleen cells whereas anti-immunoglobulin antiserum and complement killed 50% of mouse spleen cells. These numbers correspond to the approximate percentages of B and T cells in mouse spleen as determined by other methods. The treated cell suspensions were then reconstituted to a concentration of 10^6 live cells per ml along with specific stimulants.

RESULTS

Comparison of mitogenic activity of the lipopolysaccharide and the glycolipid. The proliferative response of mouse spleen lymphocytes induced by a broad concentration range of the smooth strain *S. minnesota* lipopolysaccharide or the rough mutant glycolipid is shown in Fig. 1. Lipopolysaccharide produced barely significant stimulation (>2.0) at concentrations of 10^{-4} and 10^{-3} μg/ml. From 10^{-3} to 1 μg/ml the response increased linearly (from 2.9 to 20.0) and then remained constant up to concentrations of 100 μg/ml. In contrast, the glycolipid produced significant stimulation

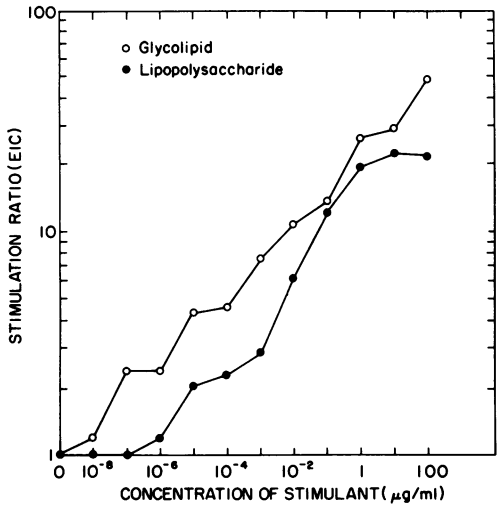

FIG. 1. *In vitro proliferation of mouse spleen lymphocytes induced by a purified glycolipid in comparison to whole lipopolysaccharide. Glycolipid was derived from the O-polysaccharide deficient, rough mutant of S. minnesota R595. Lipopolysaccharide was derived from a smooth strain of S. minnesota. Results are the mean of four experiments plotted on a log scale as the stimulation ratio (E/C). Baseline proliferative response, 1,165 counts per min per 10^6 cells.*

at a concentration of 10^{-7} μg/ml and the response continued to increase up to a concentration of 100 μg/ml. Thus, it is clear that bacterial glycolipid, free of any polysaccharide, was fully mitogenic.

Effect of various chemical modifications on the mitogenic ability of lipopolysaccharide.

(i) Alkaline hydrolysis. *S. minnesota* LPS was heated at 100 C for 4 h in either PBS or 0.1 N NaOH and then tested for mitogenic activity. Heating in PBS alone had no effect on the stimulatory ability of LPS, illustrating the remarkable stability of this mitogen (Fig. 2). In contrast, alkaline hydrolysis completely destroyed the mitogenicity of LPS even when tested at a concentration as high as 100 μg/ml. These results indicate that an intact lipid moiety is required for mitogenicity.

(ii) Acid hydrolysis. *S. minnesota* LPS was heated at 100 C for 30 min in 1.0 N HCl or in 0.1 N NaOH or PBS as controls, and then tested for mitogenic activity by using a broad dose range of stimulant (0.01 μg/ml–300 μg/ml). The maximum stimulation obtained is presented in Table 1. When LPS is heated in HCl, it is fractionated into an insoluble lipid and a soluble polysaccharide. The lipid (lipid A) still retained mitogenic activity although this activity was decreased. In contrast, the "haptenic" polysaccharide had no mitogenic activity at all. Even under these conditions, treatment with 0.1 N sodium hydroxide, which degrades the lipid, still completely destroyed the mitogenic activity, whereas treatment with PBS alone again had no effect.

(iii) Deacylation. Both acid and alkaline hydrolysis result in degradation of the LPS molecule and produce molecular fragments of marked heterogeneity. In contrast, it is possible to detoxify endotoxin by using much milder chemical procedures. One such procedure, deacylation, involves treatment with potassium methylate, which only cleaves certain long-chain carboxylic acids from the lipid moiety and produces a molecule that is devoid of toxicity but is intact and antigenic. This compound is referred to as an endotoxoid (7, 15).

Intact LPS derived from *S. marcescens* was subjected to deacylation and then tested for mitogenicity. *S. marcescens* LPS alone at a concentration of 10 μg/ml produced a maximum stimulation of 12.8 (Table 1). The endotoxoid, on the other hand, was almost devoid of mitogenic ability even when tested at concentrations up to 300 μg/ml. These results indicate that specific ester-bound long-chain fatty acids must

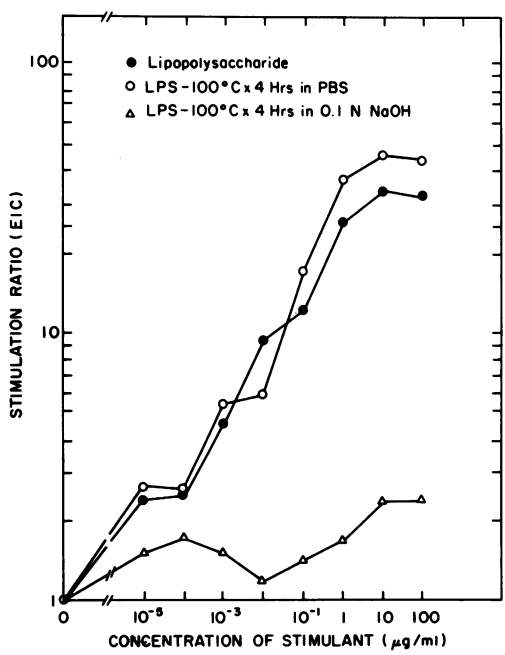

FIG. 2. *Effect of alkaline hydrolysis on the mitogenicity of S. minnesota lipopolysaccharide. LPS was heated at 100 C for 4 h in either PBS or 0.1 N NaOH. Results are the mean of three experiments plotted on a log scale as the stimulation ratio (E/C). Baseline proliferative response, 1,120 counts per min per 10^6 cells.*

TABLE 1. *Effect of various chemical treatments on the mitogenic activity of bacterial lipopolysaccharide*

Starting material	Treatment	Final product	Maximum lymphocyte proliferative response (E/C)[a]		
			Treated endotoxin alone	Treated endotoxin plus	
				Con A[b]	LPS[c]
1. *S. minnesota* LPS	None	LPS	26.2 ± 3.0		
S. minnesota LPS	Boiling	LPS	33.6 ± 7.7		
S. minnesota LPS	Acid hydrolysis				
S. minnesota LPS	Insoluble precipitate	Lipid A	10.8 ± 1.3		
S. minnesota LPS	Soluble phase	Haptenic polysaccharide	1.5 ± 0.3	89.2 ± 9.3	22.5 ± 2.3
S. minnesota LPS	Alkaline hydrolysis	Polysaccharide plus degraded lipid	2.4 ± 0.8	77.5 ± 6.3	24.1 ± 2.6
2. *S. marcescens* LPS	None	LPS	12.8 ± 0.3		
S. marcescens LPS	Deacylation	Endotoxoid	2.2 ± 0.4	90 ± 10.1	12.8 ± 1.3

[a] Baseline proliferative response = 970 counts per min per 10^6 cells.
[b] Concanavalin A at 1 μg/ml produced stimulation of 79.5 when added by itself to spleen cells.
[c] *S. minnesota* LPS (E/C = 26.2) was added back to treated preparations made from *S. minnesota* LPS and *S. marcescens* LPS (E/C = 12.8) was added back to the endotoxoid preparation.

be present in order for the lipid moiety of LPS to exert its mitogenic effect.

To be certain that the chemically altered endotoxins were not toxic to cells in vitro, the chemically-treated preparations were added to cultures that were stimulated with either concanavalin A (Con A) or intact endotoxin (Table 1). None of the treated LPS preparations had any significant effect on simultaneous Con A stimulation so that these preparations were neither inhibitory nor toxic to cells in culture (Table 1). In addition, they did not block the mitogenic effect of intact LPS so that their absence of stimulatory ability was not due to molecular fragments that were interfering with LPS-induced lymphocyte stimulation.

Stimulation of isolated B and T lymphocyte populations by several lipid preparations. To demonstrate that the lipid was a specific B cell mitogen, all the active preparations were tested for their effect on isolated B- or T-lymphocyte populations. B cells were prepared by treatment of mouse spleen cells with anti-theta serum and complement and T cells were prepared by treatment with a heterologous anti-immunoglobulin antiserum and complement. The purity of each cell population is demonstrated by the response to the T cell mitogen, Con A. Thus, the B-cell population did not respond at all to Con A, whereas the response of the T-cell population was equivalent to that of the whole spleen cell population (Table 2). Each of the active lipid preparations, LPS, glycolipid, and lipid A were as stimulatory for the pure B-cell population as for the unfractionated spleen cells. In contrast, none of the lipid preparations had any effect on the T-cell population.

DISCUSSION

In the present investigation we have determined which component of bacterial lipopolysaccharide, the lipid or the polysaccharide, was responsible for the mitogenic activity of endotoxin in vitro. Our data indicate that the lipid moiety is the mitogen. First, a purified glycolipid derived from a mutant strain of *S. minnesota* (R595) that does not produce o-polysaccharide, is more mitogenic than intact LPS. Secondly, when whole lipopolysaccharide is fractionated by acid hydrolysis into its lipid and polysaccharide components, the lipid is mitogenic, whereas the polysaccharide is not.

TABLE 2. *In vitro lymphocyte stimulation of isolated bone-marrow-derived (B) and thymus-derived (T) lymphocytes induced by several lipid preparations*

Stimulant	Maximum lymphocyte proliferative response (E/C)[a]		
	Spleen cells	B-Cells	T-Cells
Con A	79.5 ± 9.0	0.7 ± 0.1	87.6 ± 7.0
LPS	108.0 ± 1.5	106.3 ± 12	0.9 ± 0.3
Glycolipid	100.0 ± 9.1	126.6 ± 15	0.85 ± 0.1
Lipid A	28.5 ± 3.2	21.3 ± 3.5	0.66 ± 0.3

[a] Stimulants were tested at several different concentrations, and the maximum response was reported. Baseline proliferative response: spleen cells, 522 counts per min per 10^6 cells; B-cells, 242 counts per min per 10^6 cells; T-cells, 200 counts per min per 10^6 cells.

Also, those procedures that specifically inactivate only lipid with lesser effect on the integrity of the polysaccharide (alkaline hydrolysis and deacylation) completely destroy mitogenicity. Each of the active lipid preparations is fully mitogenic for isolated B- but not T-lymphocyte populations. Thus, we conclude that the lipid moiety alone is mitogenic for B-lymphocytes, whereas the polysaccharide appears to be completely devoid of mitogenic activity.

The mechanism by which LPS stimulates B-lymphocytes is not known. One possibility is that LPS binds directly to the lymphocyte surface membrane and this binding initiates transformation of the lymphocyte. Our data, that demonstrate the essential requirement of intact fatty acids in the lipid moiety, suggest that this binding may take place in the lipid layer of the surface membrane, since these fatty acids are the lipophilic component of the molecule. However, we presume that to be mitogenic the fatty acids must be bound to the glucosamine core since isolated fatty acids as are present in abundance in both the endotoxoid and the alkaline hydrolysate appear to be inactive.

On the other hand, recent work has suggested that B-lymphocytes may be stimulated in vitro by compounds like endotoxin or cobra venom factor through the activation of the third component of complement C3 (4). Since only B- and not T-lymphocytes possess a C3 receptor, this theory would explain the B-cell specificity of these compounds (3). In this regard, it is noteworthy that those preparations that we have found to be mitogenic, LPS, glycolipid, and lipid A, have been shown to activate C3, whereas the nonmitogenic compounds do not (7, 12).

Finally, it is possible to construct a hypothetical model that reconciles both alternative mechanisms. LPS may bind to the lymphocyte surface membrane where it may generate "activated" C3 which would then stimulate the B-lymphocyte. Bound LPS might facilitate stimulation by either producing large amounts of activated C3 in direct contact with the lymphocyte surface, or might possibly produce an "irreversible" and therefore "stimulatory" complex between the B-lymphocyte and activated C3.

These findings are important in several regards. First, the glycolipid is a relatively homogeneous, easily purified compound (C. H. Chen et al., J. Infect. Dis., in press), whereas lipopolysaccharide is very large and extremely heterogenous. Therefore, the use of this glycolipid could be an important tool in the investigation of the mechanism of B-cell activation in vitro. Secondly, our findings suggest a potentially interesting link between the in vivo biological activity of these compounds and their mitogenicity. It is possible that some of the in vivo biological activities of endotoxin may be mediated through the in vivo activation of B-lymphocytes rather than through a direct action of endotoxin. Several likely possibilities in this regard are the adjuvant effect, the tumor hemorrhage effect, and the production of interferon in vivo.

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ADDENDUM

Since this paper was written, two other groups using athymic mice as a source of B-lymphocytes have also reported that the lipid component of endotoxin is the B cell mitogen (1, 5).

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