THE MITOGENIC EFFECT OF LIPOPOLYSACCHARIDE ON BONE MARROW-DERIVED MOUSE LYMPHOCYTES

LIPID A AS THE MITOGENIC PART OF THE MOLECULE*

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Lipopolysaccharides (LPS)[1] consist of three regions with different chemical and biological properties (for a review see reference 1). The O-specific polysaccharide (region I) carries the main serological specificity of bacteria. It is linked to a core polysaccharide (region II) that is common to groups of bacteria. This polysaccharide core is linked through a 2-keto-3-deoxyoctonate(KDO)-trisaccharide to the lipid component (region III), termed lipid A. Smooth (S) form bacteria contain the full structure of all three regions, while rough (R) form mutants of these bacteria synthesize lipopolysaccharides of different degrees of incompleteness. They lack the O-specific polysaccharides or, in addition, parts of the core polysaccharide. In the cell wall of gram-negative bacteria the lipopolysaccharides form complexes through lipid A with other lipids and proteins.

Lipopolysaccharides from S-form bacteria (LPS-S) are mitogenic for bone marrow-derived (B) mouse lymphocytes in in vitro cell cultures (2). In this report, S- and R-form lipopolysaccharides and degradation products are used to specify the mitogenically active part of the molecule: (*a*) *LPS-S*, complete with O-antigen polysaccharide, core polysaccharide, and lipid A; (*b*) *LPS-R345*, isolated from an Rb-form mutant containing lipid A and the larger part of the core polysaccharide; (*c*) *LPS-R595*, isolated from an Re-form mutant containing only lipid A and KDO; (*d*) *lipid A* obtained from LPS-R by mild acid hydrolysis (3–5) either complexed to bovine serum albumin or in a carrier-free water-soluble form; and (*e*) alkali-treated preparations of LPS-S (6). Mitogenic effects are measured as increases in DNA synthesis, protein as well as immunoglobulin M (IgM) synthesis and secretion, and in the number of direct plaque-forming cells to the 2,4,6-trinitrophenyl (TNP), the 4-hydroxy-3-iodo-5-nitrophenacetyl (NIP), and the 4-hydroxy-3,5-dinitrophenacetyl (NNP) haptenic determinants coupled to red blood cells.

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[1] *Abbreviations used in this paper:* B cell, bursa equivalent bone marrow-derived lymphocyte; BSA, bovine serum albumin; HRC, horse red blood cells; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; LPS-R, lipopolysaccharide from rough-form bacteria; LPS-S, lipopolysaccharide from smooth-form bacteria; NIP, 4-hydroxy-3-iodo-5-nitrophenacetyl; NNP, 4-hydroxy-3,5-dinitrophenacetyl; PFC, plaque-forming cells; SRC, sheep red blood cells; TNP, 2,4,6-trinitrophenyl.

Materials and Methods

Cells.—Spleen cells from mice with congenital thymic aplasia ("nude" mice) were used as a source of B cells. The mice were purchased from Bomholtgaard, Ltd., Ry, Denmark, and used at 4–8 wk of age. Single-cell suspensions were prepared for 1-ml cultures containing 1×10^6 or 5×10^6 living lymphocytes per ml of medium set up in tissue culture plastic Petri dishes or tubes (no. 3005 or no. 2058; Falcon Plastics, Los Angeles, Calif.) under conditions described earlier (7, 8).

Mitogens.—Lipopolysaccharide from the S-form bacteria *Escherichia coli* 055:B5 (LPS-S) was prepared by phenol-water extraction (9). Lipopolysaccharides from the R-form bacteria *Salmonella minnesota* 595 and 345 (LPS-R) were obtained by extraction with phenol-chloroform-petroleum ether (10). Lipid A was prepared from the R-form *S. minnesota* 595 by acetic acid hydrolysis (4). Two forms of soluble lipid A were employed. The first was prepared by complexation in a 1:1 ratio with bovine serum albumin (BSA) as described earlier (3). The second form of soluble lipid A was obtained by a newly developed procedure. Briefly, LPS that had been converted to the acid form by deionization in an electrodialysis apparatus was heated in 1% acetic acid at 100°C for 90 min. The lipid A thus obtained was converted into a water-soluble form by neutralizing with triethylamine and lyophilized. Because lipid A and the various R lipopolysaccharides are insoluble in physiological media, aqueous stock solutions of the preparation were prepared. This was achieved by suspending the respective preparations in distilled water (5 mg/ml), warming to about 50°C, and where necessary sonicating for a short time. From these stock solutions amounts were transferred to the incubation mixtures as required. To avoid precipitation of the lipid A it was added to cultures at 0°C; thereafter, the cultures were brought to incubation temperature.

Determination of DNA Synthesis.—1-ml cell suspensions containing 1×10^6 lymphocytes were cultured for 72 h. [^{3}H]thymidine at a final concentration of 1 μCi/ml was added to the cultures 24 h before harvest. Determinations of acid-insoluble radioactivity incorporated by the cultures were performed as described in detail elsewhere (7). The data are expressed as counts per minute per 10^6 lymphocytes originally placed in culture.

Determination of Protein and Immunoglobulin Synthesis and Secretion.—Cell suspensions at 5×10^6 cells per ml of Eagle's medium minus leucine were incubated for 4 h with 60 μCi/ml radioactive leucine L-[4,5-^{3}H]leucine (The Radiochemical Centre, Amersham, England; batch no. 36, 54 Ci/mM) as previously described (11). Separation of the labeled cells from their supernatant medium, lysis of cells with Nonidet P-40 (Shell Chemical Co., Zürich, Switzerland) nonionic detergent, dialysis and centrifugation of cell lysates and supernatant medium, and determinations of radioactivities incorporated into protein and into immunoglobulin M, serologically precipitable by IgM-specific antisera, were done as described in detail previously (11). Protein and IgM synthesis denominates the amount of protein and IgM found inside the cells, while protein and IgM secretion comprises the amount of radioactive protein and IgM that has been synthesized and then secreted by the cells. All data are given for a constant time period of incorporation of radioactive leucine of 4 h. Identification of the immunoglobulin polypeptide chains by polyacrylamide gel electrophoresis was also done as described previously (8, 11). The reader is referred to these publications for a detailed description of the methods employed.

Determination of Antibody-Producing Cells.—The local hemolysis of gel assay (12), as modified by Bullock and Möller (13), was used to detect single antibody-producing cells against the TNP, NIP, and NNP haptenic determinants. TNP was coupled to horse red blood cells (HRC) by the method of Rittenberg and Pratt (14) using 40 mg of 2,4,6-trinitrobenzene sulfonic acid per ml of packed red cells. NIP and NNP were coupled to sheep red blood cells (SRC) by the method of Pasanen and Mäkelä (15) using 2 mg of NNP-azide or NIP-azide per ml of packed SRC. The red cells were subsequently washed once in phosphate-buffered saline containing 2.2 mg/ml glycylglycine and twice in balanced salt solution. Lym-

phocytes were harvested on day 3 and cells from three dishes were pooled and assayed as described earlier (2, 13). Only direct plaque-forming cells (PFC) were scored and the data expressed as anti-TNP, anti-NIP, or anti-NNP PFC per 10^6 cells after subtraction of the HRC or SRC PFC responses.

RESULTS

The B Cell Mitogenic Effects of Lipopolysaccharides with Different Chemical Structure.—The mitogenic actions of lipopolysaccharides with different chemical structures, isolated from S-form bacteria (LPS-S) and from two R-form mutants (LPS-R595 and LPS-R345), and of lipid A were investigated. Fig. 1 summarizes the effects of these substances on DNA synthesis, on protein synthesis and secretion (i.e. newly synthesized intracellular and excreted protein including IgM), on IgM synthesis (i.e. intracellular) and secretion (i.e. extracellular), and on the number of direct plaque-forming cells against the NNP-haptenic determinant coupled to red blood cells.

DNA Synthesis and Total Intracellular Protein Synthesis.—LPS-S, LPS-R595, LPS-R345, and free and BSA-complexed lipid A all stimulated B lymphocytes to increased DNA and protein synthesis (Fig. 1, *A* and *B*, *a–e*). LPS-S and LPS-R595 had the highest stimulating effect at the highest concentration employed in the experiments (100 μg/ml, Fig. 1, *A* and *B*, *a* and *b*). The same was found for the stimulation of DNA synthesis by LPS-R345 (Fig. 1, *A*, *c*), while this mutant lipopolysaccharide had an optimum for stimulation of intracellular protein synthesis at 10 μg/ml (Fig. 1, *B*, *c*). For free and BSA-complexed lipid A, optimal stimulation of DNA synthesis and intracellular protein synthesis occurred at 10 μg/ml (Fig. 1, *A* and *B*, *d* and *e*).

Secretion of Protein.—Under the stimulatory effects of LPS-S and LPS-R595, secretion of protein from B lymphocytes was optimal at 10 μg/ml (Fig. 1, *B*, *a* and *b*). LPS-S affected secretion of protein to a higher degree than LPS-R595. While the stimulation of total protein synthesis inside B cells declined from 10 to 100 μg/ml with LPS-R345 and free or BSA-complexed lipid A, secretion of protein continued to increase.

Synthesis and Secretion of IgM.—Stimulation of intracellular IgM synthesis by LPS-S, LPS-R595, LPS-R345, and free and BSA-complexed lipid A occurred parallel to the stimulation of synthesis of total intracellular protein (Fig. 1, *C*, *a–f*). Secretion however was optimal at 10 μg/ml for all preparations tested. Furthermore, while the amount of total radioactive protein synthesized inside B lymphocytes within 4 h was higher than the amount secreted in that time, synthesis of radioactive IgM inversely was lower than secretion within 4 h. This indicates that the mitogenic effect on synthesis and secretion of IgM is different from that on the synthesis and secretion of other proteins in B cells, apparently favoring selective secretion of IgM.

A differential effect on synthesis and on secretion of IgM was observed between 10 and 100 μg/ml of LPS-S and LPS-R595. By comparing Fig. 1 *B* with *C* (*c*, *d*, and *e*) it appears that while secretion of proteins increases by

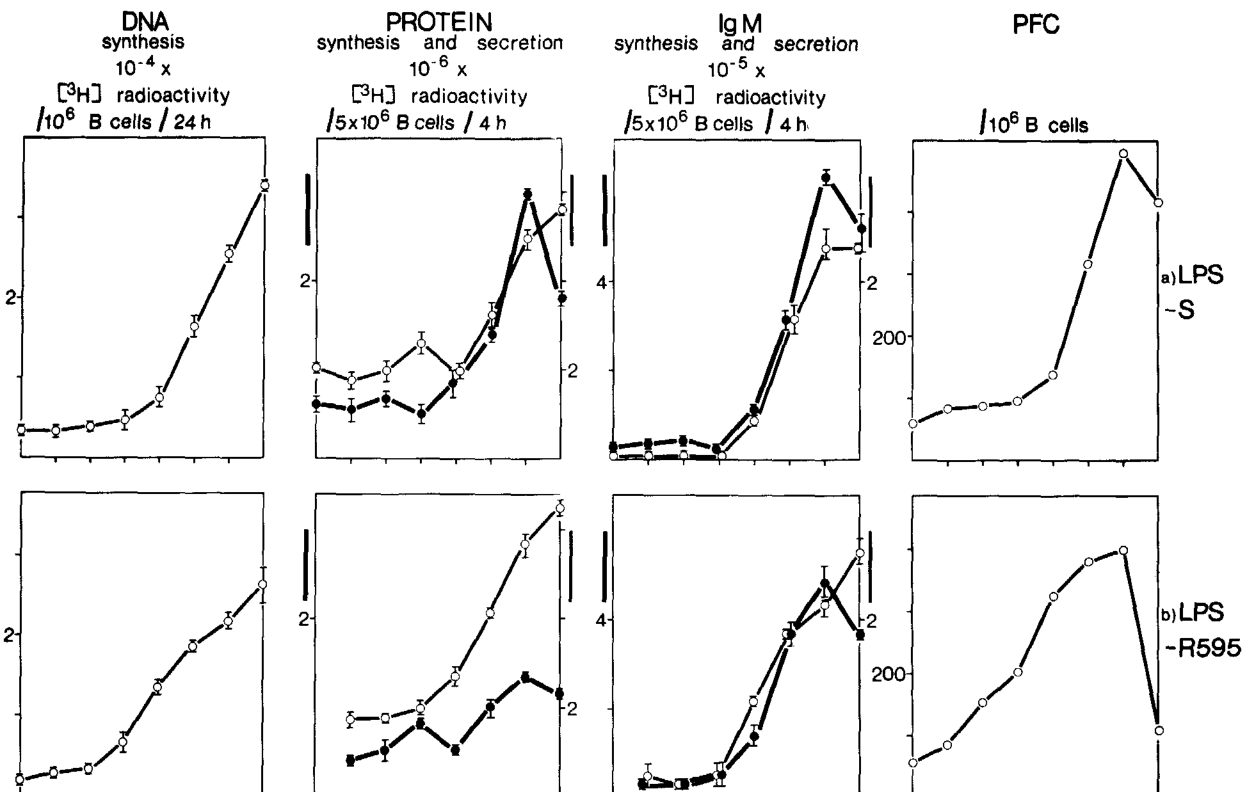
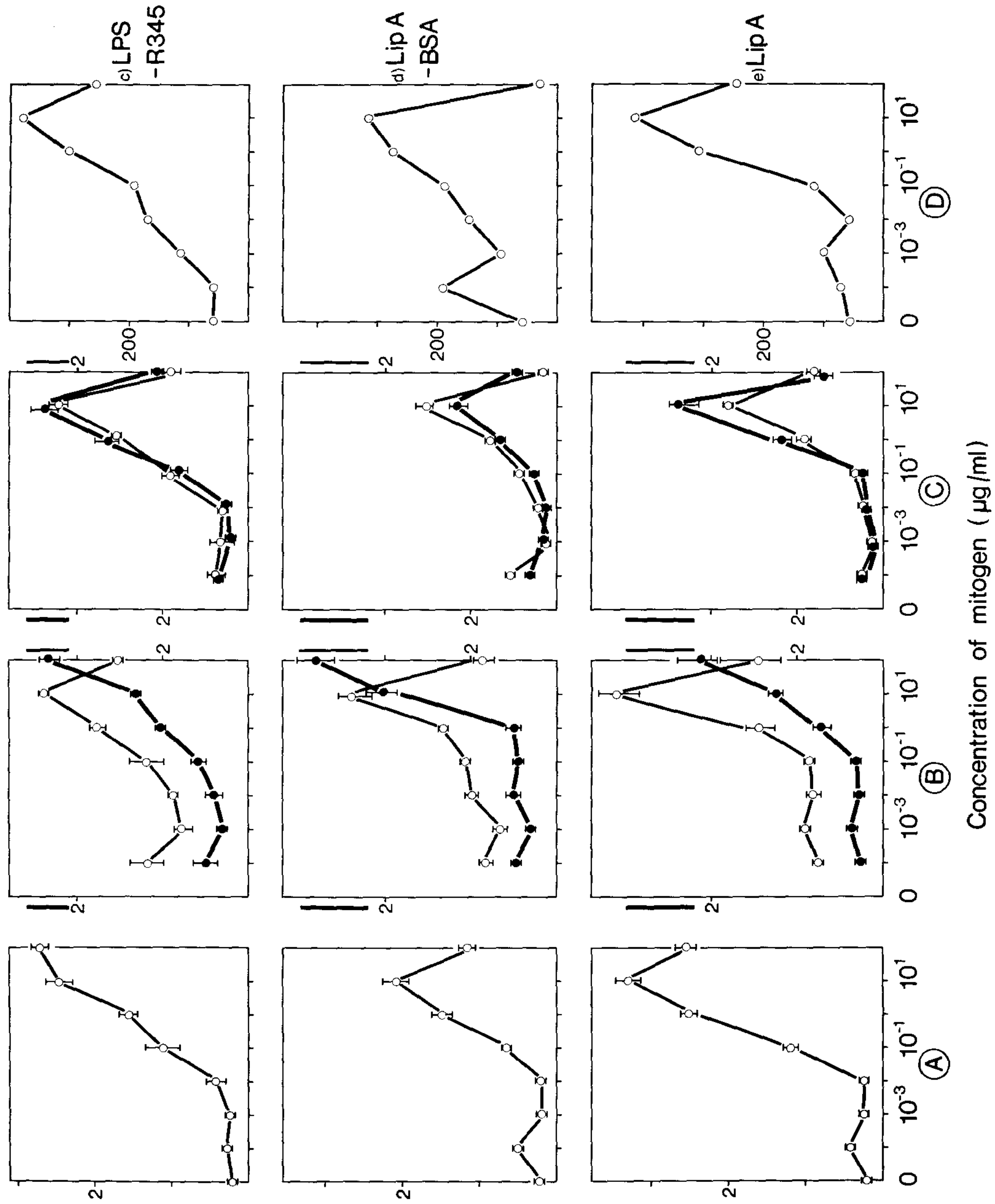


Fig. 1. Stimulation of (*A*) DNA-synthesis, (*B*) protein synthesis (i.e. intracellular) (○——○) and secretion (i.e. extracellular) (●——●), (*C*) IgM synthesis (i.e. intracellular) (○——○) and secretion (i.e. extracellular) (●——●), and (*D*) of the number of direct plaque-forming cells against the NNP (13) haptenic determinant coupled to red cells by (*a*) LPS-S, (*b*) LPS-R595, (*c*) LPS-R345, (*d*) lipid A, complexed with BSA, and (*e*) free lipid A. Please notice the different scales for expression of synthesis (right) and secretion (left). For details of the experiments see the Materials and Methods section.


c) LPS
-R345
d) Lip A
-BSA
e) Lip A
A
B
C
D
Concentration of mitogen (μg/ml)

increasing amounts (up to 100 μg/ml) of lipopolysaccharides, the secretion of IgM gives a maximum at 10 μg/ml. This may indicate that lipopolysaccharides of S- and R-form stimulate secretion of IgM at concentrations between 0.1 and 10 μg/ml and inhibit secretion of IgM at concentrations above 10 μg/ml.

Specific Direct Plaque-Forming Cells.—The dose response curves for the induction of an increased number of direct plaque-forming cells against the TNP-, NNP- and NIP-haptenic determinants coupled to red cells followed, as may be expected, those for the secretion of IgM. For the sake of clarity, only the anti-NNP PFC response is depicted (Fig. 1, *D*, *a–f*). From the simultaneous increase in the number of direct plaque-forming cells against the three different antigenic determinants (Table I), it is concluded that all preparations tested had stimulated a large part of the B lymphocytes in an antigen-unspecific way. The use of hapten-coupled erythrocytes in this assay system results in much greater numbers of PFC compared with nonhaptenated red cells. This is probably due to the high epitope density of hapten on the red cell surface, which permits binding and therefore lysis by low avidity antihapten immunoglobulins (15). Since this greatly increases the sensitivity of the assay system by permitting detection of a broader spectrum of Ig-secreting cells, we chose to use haptenated red cells throughout this investigation.

As shown in Table I, very few, if any, antigenic determinants on the LPS cross-react with TNP or NNP as revealed by the inability of 1 mg/ml of LPS-S to inhibit the antihapten PFC response. Soluble hapten-protein conjugates showed the expected inhibitions of the appropriate antihapten PFC responses. Thus, stimulation of B lymphocytes by LPS results in poly-

TABLE I

Hapten Specificity of the Direct Plaque-Forming Cells Stimulated by Lipopolysaccharides

Addition to the agar	PFC/10^6 cultured cells	
	Anti-TNP	Anti-NNP
—	215	529
LPS-S 1 mg/ml	194	477
NNP-HGG 1 mg/ml	225	3
TNP-HGG 1 mg/ml	20	425

5×10^6 nude spleen lymphocytes were cultured for 70 h in the presence of 20 μg/ml LPS-S. The cells were assayed as described in Materials and Methods with the addition of either the mitogen used for induction or human gamma globulin (HGG) conjugated with the same haptenic determinants used for detection of PFC in the hemolytic assay. NNP-HGG was prepared as described by Bullock and Möller (13) and contained 45 mol NNP/mol HGG. TNP-HGG was prepared according to Rittenberg and Amkraut (29) and contained 20 mol TNP/mol HGG. Unstimulated cultures gave 60 anti-NNP PFC and 50 anti-TNP PFC, respectively.

clonal proliferation and product formation in a major part of the lymphocyte population (19).

Effect of Alkaline Hydrolysis on the B Cell Mitogenic Properties of Lipid A.— In order to determine whether parts of the lipid A or the polysaccharide part are responsible for the observed effects, LPS-S was hydrolyzed with 1 N NaOH at 56°C for various lengths of time (21). This treatment removes ester-linked fatty acids from the lipid A component but leaves the polysaccharide portion intact (3, 16). As shown in Fig. 2, treatment of LPS-S with alkali results in a

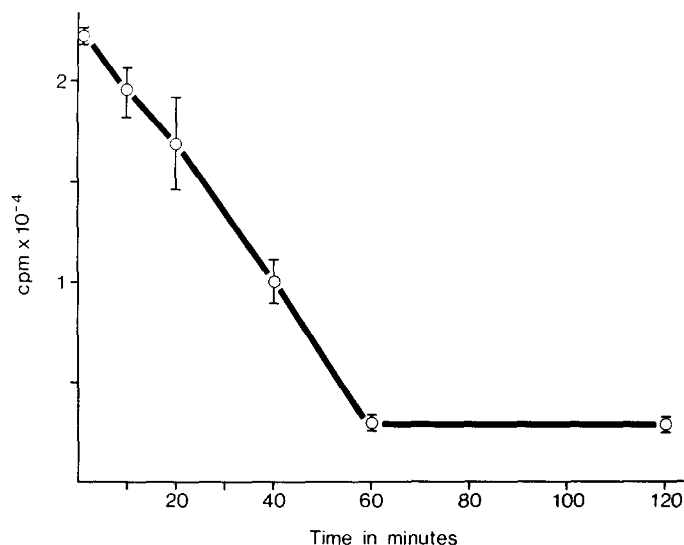


FIG. 2. Response of normal B lymphocytes to 10 μg/ml of LPS-S hydrolyzed by 1 N NaOH at 56°C for various lengths of time. The response was determined by [^{3}H]thymidine incorporation into DNA as described in Materials and Methods.

gradual decrease in B cell mitogenic activity with increasing time. Thus, treatment with alkali for 40 min resulted in a 50% reduction of mitogenic activity and after hydrolysis for 60 min, mitogenicity for B cells as measured by [^{3}H]-thymidine incorporation into DNA was completely abolished. Analogous findings were obtained for antibody synthesis and secretion. The capacity of LPS-S to induce an anti-TNP and anti-NNP PFC response was abolished by alkali treatment (60 min) over the entire dose range tested ($10^{-4} - 10^2$ μg/ml) (Fig. 3).

These results indicate that lipid A is the active part of lipopolysaccharide in mitogenic stimulation of B lymphocytes. Within the lipid A part, ester-linked fatty acids play an important role in this stimulation, while the polysaccharide portion is inactive.

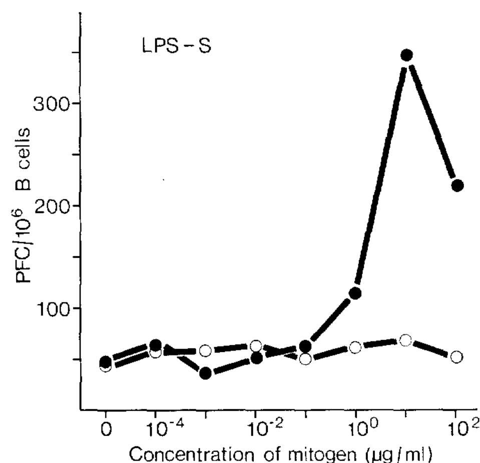


FIG. 3. Response of nude spleen cells to various concentrations of LPS-S untreated (●) or hydrolyzed by 1 N NaOH for 60 min (○). The response was determined as anti-TNP PFC/10^6 cells after 70 h of culture.

DISCUSSION

In the present investigation the nonspecific stimulation of B lymphocytes from nude mice and their polyclonal immunoglobulin production after treatment with lipopolysaccharide were studied in order to define the chemical structures in the lipopolysaccharide molecule responsible for these activities. DNA, protein and IgM synthesis, and protein and IgM secretion, as well as direct plaque-forming cells against TNP and NNP, were determined after stimulation with complete S-form lipopolysaccharide, incomplete R-form lipopolysaccharide, and BSA-complexed and free lipid A. The results show that the stimulatory effect of these preparations was almost identical, indicating that the polysaccharide portion of lipopolysaccharide is not essential and that lipid A is the active component. Cleaving of ester-linked fatty acids from lipid A abolished its mitogenic activity indicating that long chain fatty acid substituents represent an essential part for mitogenicity of the lipid A molecule. Analogous results had been obtained previously (17) when these preparations were tested for nonspecific endotoxic reactions such as pyrogenicity, toxicity, and complement inactivation.

Lipopolysaccharides are in many respects constructed of repeating units, and may react as polyvalent macromolecules. The O-specific chains consist of repeating oligosaccharide units (1). Lipid A consists of several substituted diglucosamine repeating units that are interlinked by pyrophosphate bridges (6). These covalently-linked units aggregate in water solution. Thus the number of lipid A units lying side by side is increased. As a consequence, the core oligosaccharides of R-lipopolysaccharide that are attached to the lipid A units

occur repeatedly along the aggregate. Therefore, lipopolysaccharide S, lipopolysaccharide, R, and lipid A can be looked at as multivalent macromolecules containing well-ordered repeating units.

When lipopolysaccharide of S forms are treated with mild alkali, the stimulatory activity is continuously lost and after 60 min (1 N NaOH, 56°C) totally abolished. It is well known that alkali treatment of lipopolysaccharides results in an enhancement of their capacity for complex formation with lipids and proteins and for their fixation to cell membranes (18). This demonstrates that mere reaction with the lymphocyte membrane does not result in its stimulation. With alkali, ester-linked fatty acids are removed from the lipid A part of lipopolysaccharides, which results in the loss of lipidic character and, consequently, better water solubility and desaggregation (16). Furthermore, most probably pyrophosphate bridges are cleaved whereby the lipid A units are separated. The destruction by alkali of the repeating units of lipid A, i.e. its polyvalency, may be the reason for the loss of mitogenicity.

Binding of the antigenic determinants to the antigen-binding regions of the receptor immunoglobulin molecules in the outer (plasma) membrane of the B lymphocytes is prerequisite for their activation (19, 20). Selection of cells, which are to be triggered, is therefore achieved by the specific antigenic determinant, while triggering of such cells may be affected by a suitable spatial arrangement of repeating antigenic determinants on a carrier molecule (T cell-independent antigens) (21), or by the cooperation of other cells (T cell, macrophages) that present the antigenic determinants in a suitable spatial arrangement of repeating determinants (22, 23) and/or produce antigen-unspecific factor (T cell-dependent antigens) (24). All these mechanisms would lead to an increase in the affinity of the determinants to the receptor immunoglobulin molecules and lower the threshold for triggering (21).

By this definition B cell mitogens do not select, but trigger directly and without the help of either T cells or macrophages (25). Mitogens must thus have affinities for "mitogen receptors" present on the plasma membrane of (almost) all B cells. Since lipid A is recognized as the active part of mitogenic lipopolysaccharide, it may be anticipated that it confers to the lipopolysaccharide molecule the capacity to insert into the lipid bilayer of the plasma membrane of B lymphocytes. The property of lipopolysaccharide to integrate into membrane structures is well documented (26). So far no direct experimental proof has been obtained with B lymphocytes that would support this hypothesis. If it does insert into the plasma membrane, lipid A may act by perturbing the conformation of the lipid bilayer directly, which could result in a subsequent change in conformation of other (protein) parts of the membrane, or lipid A may show affinities for protein components, i.e. receptor IgM molecules and, upon combining with them, change their conformations within the membrane. An aggregation of lipopolysaccharide-binding protein components of the membrane may be the result, analogous to the aggregation ("cap-

ping") observed when surface IgM molecules are complexed with anti-Ig antibodies (27, 28).

SUMMARY

Lipopolysaccharides with different structure, isolated from different mutant strains of *Escherichia coli* and *Salmonella* bacteria, and chemical degradation products of these lipopolysaccharides have been employed to investigate which part of the lipopolysaccharide molecule exerts mitogenic effects on bone marrow-derived mouse lymphocytes. Within the structure of lipopolysaccharide consisting of lipid A, a core polysaccharide, and the O-polysaccharide antigen, lipid A was found to be the mitogenic part. The mitogenic effect of lipid A, consisting of phosphorylated glucosamine disaccharide units with ester- and amide-linked fatty acids, was lost after alkali treatment, which removes ester-linked fatty acids. Insertion of the lipid A portion of lipopolysaccharides into the lipid bilayer of the plasma membranes of bone marrow-derived lymphocytes is discussed as the initial mitogenic action.

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