THE LIPOPROTEIN OF THE OUTER MEMBRANE OF *ESCHERICHIA COLI:* A B-LYMPHOCYTE MITOGEN

By FRITZ MELCHERS, VOLKMAR BRAUN, AND CHRIS GALANOS

(From the Basel Institute for Immunology, Basel, Switzerland, The University of Tübingen, Mikrobiologie II, Tübingen, West Germany, and The Max-Planck-Institut für Immunobiologie, Freiburg, West Germany)

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Mitogens such as bacterial lipopolysaccharides (LPS)[1] (1), the purified protein derivative of tuberculin (PPD) (2), or fetal calf serum (FCS) (3) stimulate a large part of all small, resting B-lymphocytes to proliferation and to the development of IgM-secreting plasma cells. This mitogenic stimulation can be monitored by an increase in thymidine uptake and by the appearance of direct plaque-forming cells (PFC) assayed with densely coupled trinitrophenylated sheep red cells (TNP-SRBC) (4). The general activation of B-cells makes possible biochemical analyses of changes that take place after stimulation. IgM synthesis and secretion increase selectively over synthesis and secretion of other cellular proteins. IgM synthesized at the increased rate is secreted as a 19S pentamer (5, 6).

Mitogens may help to understand molecular processes which occur when B cells are activated and which regulate proliferation and differentiation of B cells into plasma cells. Knowledge of the structure of the mitogen may help to direct the search for the sites of interaction and for the structural requirements of these interactions on B cells. Only the structure of LPS, however, has been elucidated. Lipid A has been found to be the mitogenic part (4). The structures of the mitogenic principles of PPD or FCS are unknown. We report here experiments which show that the lipoprotein of the outer membrane of *Escherichia coli* is a B-cell mitogen.

The lipoprotein of the outer membrane of *E. coli* is a major protein of the cell wall (7). The sequence of the 57 amino acids has been elucidated (8, 9). One-third of the lipoprotein is covalently bound to the murein (peptidoglycan) of the cell wall (10, 11). Two fatty acids are bound to glycerylcysteine at the N-terminal end of the polypeptide chain (12). An additional fatty acid residue occupies the N-terminal α-amino group of the polypeptide (12). The fatty acids are distinct from those present in the LPS (13) in that no β-hydroxy myristic acid occurs in the lipoprotein (12). The conformation of the lipoprotein is mainly α-helical (14). The lipoprotein aggregates in aqueous solution due to the covalently linked lipid and due to the arrangement of all hydrophobic amino acids along one side of the α-helix. This lipoprotein occurs in several *Enterobacteriaceae* (7, 15, 16).

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; LPS, lipopolysaccharide from gram-negative bacteria; PFC, plaque-forming cells; PPD, purified protein derivative of tuberculin; TNP, trinitrophenyl.

Because of its well-known structure and because of the possibilities to modify this structure specifically, by the lipoprotein may be a powerful mitogen in probing B-lymphocytes for their molecular mechanisms of activation.

Materials and Methods

Animals. C3H/HeJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine. C3H/Tif/BOM and BALB/c nu/nu-mice were obtained from G1. Bomholtgard, Ry, Denmark. All animals were between 6 and 10 wk of age.

Cells, Media, and Reagents. Cell suspensions were prepared as described (5, 6) and cultured in serum-free RPMI 1640-medium (Microbiological Associates, Inc., Bethesda, Md.) supplemented with fresh glutamine (2 mM/ml), streptomycin and penicillin (100 μg/ml each), HEPES buffer (10 mmol), and mercaptoethanol (5×10^{-5} M) at a cell density of 5×10^6 cells/ml in Falcon plastic tubes (no. 2054; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in 0.5-ml aliquots per tube. Assays were done at least in triplicate. LPS from *Salmonella abortus-equi* was a phenol-water preparation. PPD was batch RT27 from Statens Serum Institut, Copenhagen, Denmark and contained 50,000 IU/mg.

Murein and Lipoprotein. Murein-lipoprotein complex was isolated as previously described (10, 17). In short *E. coli* cells were disrupted with glass beads and the cytoplasm was separated from the envelope by differential centrifugation. The cell envelope was solubilized in 4% boiling sodium dodecyl sulfate. The large murein-lipoprotein complex (mol wt, 4×10^9) was spun down and washed four times with water to remove the detergent and with it the last detectable traces of LPS and phospholipids. The LPS content of this murein-lipoprotein complex was certainly below 1% as judged by the content of hydroxymyristic acid of this preparation.

Muropeptide-containing lipoprotein was prepared by degradation of murein-lipoprotein complex with lysozyme (18). Lipoprotein was freed from the enzyme and the murein-degradation products by chromatography on Sephadex G75 (18). The resulting lipoprotein contains on the average three muropeptides covalently bound.

Murein-free lipoprotein was obtained by incubation of murein-lipoprotein complex with trypsin for 30 min at 37°C. Most of the released lipoprotein stayed with the murein due to its insolubility in buffer solution. Liprotein was solubilized in 4% sodium dodecyl sulfate and precipitated again by addition of an equal volume of acetone. The lipoprotein pellet was resuspended four times in 80% acetone in water and thus freed from the detergent.

Isolation of Protein from Salmonella. The preparation represents a mixture of proteins obtained from the *Salmonella minnesota* R595 mutant (19). Briefly R595 bacteria were treated with 0.05 M EDTA in 4% NaCl, at 25°C for 30 min. They were centrifuged, the supernate dialyzed against phosphate-buffered saline, and lyophilized. A Morgan-Elson reaction as described by Stominger et al. (20) on 0.5 mg of the preparation hydrolysed in 6 N HCl at 100°C for 6 h showed no detectable glucosamine. Also, in lethal toxicity tests in adrenalectomized mice (21), the mean lethal dose (LD_{50}) of the preparation was found to be higher than 100 μg. The homologous R595 LPS was found to contain 12.5% of glucosamine and its LD_{50} was of the order of 0.01 μg.[2] The absence of glucosamine and the low toxicity of the isolated proteins are interpreted as evidence for the absence of any significant amounts of LPS.[3]

Assays for Mitogenicity. Uptake of radioactive thymidine (22), the development of direct PFC assayed with densely coupled TNP-SRBC (4), incorporation of radioactive leucine in cell cultures, and the serological determination of leucine-labeled IgM and its characterization by size and polypeptide chain composition on polyacrylamide gels (5, 6) were done as described in detail previously. Data in Tables I, II, IV, and V include standard errors of the means of triplicate determinations calculated by Student's *t* test.

Results

Mitogenic Activity of Lipoprotein. Lipoprotein from the outer membrane of *E. coli* was tested with different lymphocyte populations for its capacity to

[2] Galanos, C., et al. Manuscript in preparation.

[3] Galanos, C., R. Geyser, O. Lüderitz, and O. Westphal. Manuscript in preparation.

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increase thymidine uptake, to induce the development of direct PFC assayed with densely coupled TNP-SRBC, and to increase synthesis and secretion of [^{3}H]leucine-labeled IgM selectively over synthesis and secretion of other cellular proteins. Two kinds of lipoprotein were tested for their capacity to stimulate lymphocytes. Both kinds of lipoprotein differ only in the carboxyl-terminal end of the polypeptide chain. The "muropeptides containing lipoprotein" were released from murein by lysozyme and consists of the complete lipid-polypeptide chain to which two to three muropeptides remain attached (18). The "murein-free lipoprotein" released by trypsin consists of the lipid-polypeptide chain lacking the carboxyl-terminal amino acid sequence tyrosyl-arginyl-lysine (9, 17). Activation of the different cell populations by two known B-cell mitogens, PPD and LPS, and by one known T-cell mitogen, concanavalin A (Con A) (23) was also tested. Tables I, II, and III summarize the results.

Murein-free and muropeptides-containing lipoprotein both stimulate splenic lymphocytes from C3H/Tif and from BALB/c nu/nu mice, but not thymus cells from C3H/Tif mice, to increased thymidine uptake Table I), to the development of PFC (Table II), and to a selectively increased rate of IgM synthesis and secretion (Table III). Free murein is not active in any of the assays. Con A stimulates only thymidine uptake but no development of PFC, and does so also in thymus cells, as expected from a T-cell mitogen. We conclude that the lipoprotein, either in free form or with bound muropeptides is a B-cell mitogen.

The stimulation of murine B-lymphocytes by lipoprotein is dose dependent. Free lipoprotein exhibits one rather narrow dose optimum between 1 and 10 μg/ml, while the muropeptides-containing form shows two dose optima around 10 μg/ml and between 50 and 100 μg/ml (Fig. 1). The cause for the two dose optima for the muropeptides-containing form of lipoprotein is at present unknown.

Experiments performed with splenic lymphocytes separated according to size by velocity sedimentation (5) (data not shown) indicate that, as with PPD and LPS, the main lipoprotein-sensitive cells are small, resting B cells. Small spleen cells from BALB/c nu/nu mice which lack functional T cells are equally well activated by lipoprotein than unseparated spleen cells from C3H/Tif mice which possess functional T cells. This indicates that the activation of B cells by lipoprotein is T-cell independent and does not need serum factors or adherent cells.

Mitogenic Activity of Lipid-Depleted Lipoprotein. Ester-linked fatty acids located at the NH_2 terminal end of the lipoprotein can be removed by alkaline hydrolysis (12). This leaves a lipoprotein in which only the amide-linked fatty acid remains bound. Results in Table IV show that such a lipid-depleted lipoprotein preparation has lost its mitogenic activity. It suggests that ester-linked fatty acids in the lipoprotein are involved in the mitogenic action on B cells. They may be involved in the proper anchoring of the lipoprotein in the lipid bilayer of the B-lymphocyte plasma membrane.

Different Sites of Action of LPS and of Lipoprotein on Murine B Lymphocytes. B lymphocytes from C3H/HeJ mice are low responders to mitogenic stimulation by LPS, but high responders to stimulation by PPD (24, 25), while B lymphocytes from C3H/Tif mice are high responders to the stimulation by both LPS and PPD. Two distinct sites of mitogenic action have

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TABLE I

Stimulation of Thymidine Uptake by Lipoprotein*

Mitogen	Cell source‡	Radioactivity ($10^{-3} \times$ ^{3}H-labeled cpm/2.5×10^6 cultured cells)
None	1	0.2 ± 0.1
	2	0.2 ± 0.1
	3	0.2 ± 0.1
	4	0.1 ± 0.1
LPS (50 μg/ml)	1	14.0 ± 0.7
	2	19.6 ± 1.0
	3	1.5 ± 0.5
	4	0.2 ± 0.1
PPD (50 μg/ml)	1	6.2 ± 0.4
	2	8.4 ± 0.7
	3	6.6 ± 0.8
	4	0.3 ± 0.1
Con A (5 μg/ml)	1	0.4 ± 0.1
	2	10.7 ± 0.7
	3	9.3 ± 0.8
	4	15.6 ± 0.9
Murein-free lipoprotein (4 μg/ml)	1	24.4 ± 1.5
	2	20.4 ± 2.0
	3	24.8 ± 2.5
	4	0.5 ± 0.1
Muropeptides-containing lipoprotein (10 μg/ml)	1	22.4 ± 2.5
	2	26.5 ± 3.5
	3	23.4 ± 2.0
	4	0.1 ± 0.1
Murein§ (25 μg/ml)	1	0.5 ± 0.1
	2	0.4 ± 0.1
	3	0.4 ± 0.1
	4	0.1 ± 0.1

* Cells cultured for 48 h were incubated for 1 h with 10 μCi [*methyl*-^{3}H]thymidine (5 Ci/mmol, The Radiochemical Centre, Amersham, England) and uptake into DNA measured as described in reference 18.

‡ 1, BALB/c nu/nu spleen; 2, C3H/Tif spleen; 3, C3H/HeJ spleen; and 4, C3H/Tif thymus.

§ Lower doses down to 0.0025 μg/ml also did not show any mitogenic effects.

therefore been postulated for B lymphocytes (22). Results in Tables I, II, and III and in Fig. 1 shown that C3H/HeJ and C3H/Tif B lymphocytes are high responders to the lipoprotein. This rules out a LPS contamination in the lipoprotein preparations as cause for the observed mitogenic activity. It indicates that LPS and lipoprotein from the outer membrane of gram-negative bacteria are two mitogens which act at two distinct sites on B lymphocytes.

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TABLE II

Stimulation of B Cells to the Development of PFC Against TNP-SRBC† by Lipoprotein

Mitogen	Cell source‡	PFC/2.5 × 10^6 cultured cells	
		No pretreatment	After pretreatment with anti-Ig antibodies§
None	1	25 ± 10	10 ± 5
	2	25 ± 10	15 ± 5
	3	35 ± 10	15 ± 5
	4	5 ± 5	ND‖
LPS (50 μg/ml)	1	850 ± 100	40 ± 15
	2	690 ± 80	30 ± 15
	3	30 ± 15	15 ± 5
	4	20 ± 10	ND
PPD (50 μg/ml)	1	1,170 ± 250	55 ± 25
	2	1,700 ± 300	25 ± 10
	3	1,500 ± 150	25 ± 15
	4	20 ± 10	ND
Con A (5 μg/ml)	1	20 ± 10	ND
	2	20 ± 10	ND
	3	30 ± 10	ND
	4	10 ± 10	ND
Murein-free lipoprotein (4 μg/ml)	1	1,150 ± 100	25 ± 10
	2	1,870 ± 180	45 ± 20
	3	1,555 ± 205	30 ± 15
	4	20 ± 10	ND
Muropeptides-containing lipoprotein (10 μg/ml)	1	2,650 ± 300	50 ± 20
	2	3,450 ± 350	55 ± 15
	3	2,800 ± 300	25 ± 10
	4	25 ± 10	ND
Murein ¶ (25 μg/ml)	1	45 ± 10	ND
	2	55 ± 10	ND
	3	ND	ND
	4	ND	ND

* 1 ml packed SRBC were coupled with 30 mg trinitrobenzene-sulphonic acid (Sigma Chemical Co., St. Louis, Mo.) and used in a direct plaque assay as described in reference 4.

‡ 1, BALB/c nu/nu spleen; 2, C3H/Tif spleen; 3, C3H/HeJ spleen; and 4, C3H/Tif thymus.

§ Cells were exposed to rabbit antimouse IgM antibodies 2 h before mitogenic stimulation with antibodies and under conditions described in detail in reference 23.

‖ ND, not determined.

¶ Lower doses down to 0.025 μg/ml also did not show any mitogenic effects.

Inhibition of Lipoprotein-Mediated B-Lymphocyte Stimulation by Anti-Ig Antibodies. Anti-Ig antibodies inhibit the LPS- and PPD-mediated mitogenic stimulation of B lymphocytes (26). Results in Tables II and III show that anti-Ig antibodies added to B cells before mitogenic stimulation also inhibit the

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TABLE III

Induction of Selectively Increased Rates of Synthesis and Secretion of Leucine-Labeled IgM over Rates of Synthesis and Secretion of all Cellular Proteins by Lipoprotein*

Mitogen	Cell source‡,§		Rate of IgM	
			Synthesis	Secretion
			% of total cellular protein	
None		1	2	3
		2	3	2
		3	2	4
	After pretreatment with anti-Ig antibodies‖	1	1	2
		2	2	2
		3	1	2
LPS (50 μg/ml)		1	15	60
		2	12	55
		3	3	3
	After pretreatment with anti-Ig antibodies	1	2	2
		2	2	2
		3	2	2
PPD (50 μg/ml)		1	12	45
		2	12	55
		3	15	60
	After pretreatment with anti-Ig antibodies	1	2	2
		2	2	3
		3	3	3
Murein-free lipoprotein (4 μg/ml)		1	10	45
		2	9	55
		3	8	50
	After pretreatment with anti-Ig antibodies	1	3	2
		2	2	2
		3	3	3
Muropeptides-containing lipoprotein (10 μg/ml)		1	12	50
		2	10	40
		3	8	60
	After pretreatment with anti-Ig antibodies	1	2	2
		2	2	2
		3	2	3

* Incorporation of [4,5-^{3}H]leucine (55 Ci/mmol, 66 μCi/ml, the Radiochemical Centre, Amersham, England) into cells cultured for 72 h was done for 4 h under conditions described in references 5 and 6. Serological determinations of radioactive protein were carried out as also described there. Secreted Ig from LPS-, PPD-, and lipoprotein-activated cell cultures was found to over 90% as 19S IgM consisting of $H\mu^-$ and L chains.

‡ In thymus cells no significant synthesis or secretion of leucine-labeled IgM could be detected.

§ 1, BALB/c nu/nu spleen; 2, C3H/Tif spleen; and 3, C3H/HeJ spleen.

‖ Pretreatment of cells with rabbit antimouse IgM antibodies was as described in the legend to Table II.

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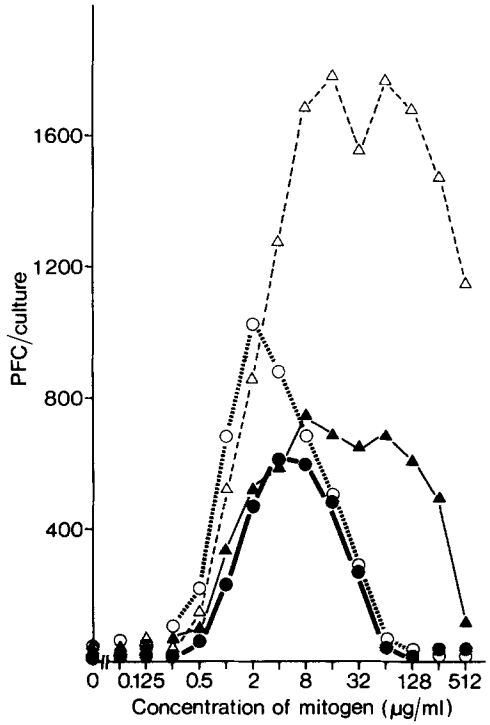

FIG. 1. Mitogenic PFC response of spleen cells of C3H/Tif (○, △) and of C3H/HeJ (●, ▲) mice with different doses of murein-free (●, ○) and of muropeptides-containing (▲, △) lipoprotein. 2.5×10^6 cells were cultured in 0.5 ml medium and assayed at day 3 of stimulation with densely coupled TNP-SRBC for direct PFC. For details see the Materials and Methods.

TABLE IV

Mitogenic Activity of Alkali-Treated, Lipid-Depleted Lipoprotein

Mitogen	PFC/2.5×10^6 cultured cells*
None	15 ± 10
LPS	
50 μg/ml	1,650 ± 150
Murein-free lipoprotein‡	
1 μg/ml	1,260 ± 70
10 μg/ml	2,220 ± 180
Alkali-treated murein-free lipoprotein‡	
1 μg/ml	80 ± 20
10 μg/ml	280 ± 20

* Assayed against TNP-SRBC as described in the legend to Table II with BALB/c nu/nu spleen cells.

‡ Murein-free lipoprotein was dissolved in 0.33 N NaOH at 250 μg/ml. One part was immediately neutralized with HCl and incubated for 1 h at 56°C (control), while the other part (alkali treated) was neutralized only after the 1 h incubation at 37°C. No loss of mitogenic activity was observed between the immediately neutralized solution and a lipoprotein solution, which was never exposed to either NaOH, HCl, or heat.

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lipoprotein-mediated stimulation to the development of PFC and to a selectively increased IgM synthesis and secretion. It indicates that, as it was discussed previously (23), surface membrane-bound Ig receptor molecules are part of a functional complex of structures containing the LPS-, the PPD-, and the lipoprotein-specific receptor sites which are involved in the regulation of B-lymphocyte stimulation.

Mitogenic Activity in LPS-Free Protein Preparation from Salmonella. Salmonella contains a lipoprotein in the outer membrane which cannot be distinguished from that of *E. coli* by amino acid analysis (15). From this close similarity in structure one may expect that the lipoprotein from Salmonella acts as a B-cell mitogen.

Data in Table V show that an LPS-free protein preparation from Salmonella exhibited mitogenic activity with splenic lymphocytes from C3H/Tif and from C3H/HeJ mice. After alkaline treatment the protein preparation lost its mitogenic activity. Although the protein preparation contains many different bacterial proteins these results may be taken as a first indication that this mitogenic activity of Salmonella may reside in its lipoprotein of the outer membrane.

Discussion

The lipid A portion of LPS and the lipoprotein both are highly conserved structures in gram-negative bacteria. It is predictable that lipoprotein from other gram-negative bacteria will prove to be mitogenic for B cells. It is tempting to speculate that the active component(s) of PPD and of FCS are lipoprotein-like molecules. Preliminary experiments in our laboratory indicate that this may be so: alkaline hydrolysis performed on PPD and FCS under conditions described in the Results (Table IV) abolishes the mitogenic activities of PPD and FCS.

Gram-negative bacteria are abundant in the bacterial flora of the gut and the tonsils. A long symbiosis of bacteria and mammals has occurred during evolution. This may explain why recognition structures exist on mammalian cells such as lymphocytes for bacterial membrane structures which are different from

TABLE V

Mitogenic Activity of a Protein Preparation from Salmonella

Mitogens	PFC/2.5 × 10^6 cultured cells*	
	C3H/Tif splenocytes	C3H/HeJ splenocytes
None	20 ± 10	15 ± 10
LPS (50 μg/ml)	1,500 ± 200	40 ± 20
PPD (50 μg/ml)	1,250 ± 100	1,080 ± 150
Salmonella protein‡ (50 μg/ml)	1,750 ± 350	1,250 ± 250
Salmonella protein after alkaline hydrolysis§ (50 μg/ml)	200 ± 50	120 ± 30

* Cultured in serum-free medium and assayed against TNP-SRBC as described in the legend to Table II.

‡ Prepared as described in the Materials and Methods.

§ Alkaline hydrolysis was performed as described in Table IV.

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antigen-binding sites of antibody molecules. It is, however, puzzling that these bacterial membrane components, LPS and lipoprotein in *Enterobacteriaceae*, induce the polyclonal proliferation and differentiation of resting B lymphocytes into PFC. It remains unclear at present what biological function such a polyclonal stimulation of B cells may have in ontogeny, differentiation, and regulation of function of antigen-reactive lymphocytes.

Summary

The lipoprotein of the outer membrane of *Escherichia coli* is a B-cell mitogen in mice. Polyclonal activation of B lymphocytes was measured by an increase in thymidine uptake, by the development of plaque-forming cells against densely coupled trinitrophenylated sheep red cells, and by selectively increased rates of synthesis and secretion of leucine-labeled IgM. Murein-free and muropeptides-containing lipoprotein are effective in B-cell activation, while free murein is inactive. Removal of ester-linked fatty acids from the amino-terminal end of the lipoprotein by alkaline hydrolysis abolishes the mitogenicity of the lipoprotein. B lymphocytes from high responder (C3H/Tif and BALB/c nu/nu) or from low responder (C3H/HeJ) mice to the mitogen lipopolysaccharide (LPS) both respond well to the lipoprotein. Anti-immunoglobulin antibodies inhibit the mitogenic stimulation of B cells by lipoprotein. A complex of structures including the Ig-receptor molecules, the LPS receptor, and the lipoprotein receptor appear involved in the regulation of mitogenic stimulation of B cells to proliferation and differentiation to IgM-secreting cells.

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