INFECTION AND IMMUNITY, June 1979, p. 685-696
0019-9567/79/06-0685/12$02.00/0

Vol. 24, No. 3

Characterization of the Chemical and Physical Properties of a Novel B-Lymphocyte Activator, Endotoxin Protein

GAIL W. GOODMAN† AND BARNET M. SULTZER*

Department of Microbiology and Immunology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

Received for publication 1 March 1979

Endotoxin protein, a novel mouse B-lymphocyte mitogen, is a hydrophobic, acidic compound composed of approximately 85% protein and 2.2% glucosamine, but no 2-keto-3-deoxyoctonate. Endotoxin protein also contains lipid, and analysis of the fatty acids in this material demonstrated the presence of β-hydroxymyristate, a marker for lipid A. In addition, analysis of endotoxin protein by polyacrylamide gel electrophoresis showed that it is heterogeneous, containing four or five major polypeptides, depending upon the bacterial species from which it was isolated. The mitogenicity of endotoxin protein was diminished by alkaline hydrolysis, but not by treatment with hydrochloric or acetic acid. Furthermore, its activity was resistant to digestion with trypsin, chymotrypsin, and pronase and was only partially degraded by papain.

A number of outer membrane proteins of gram-negative bacteria can be found associated with lipopolysaccharide endotoxin (LPS), when the LPS is extracted by a variety of techniques (3, 20, 26, 27, 36, 37). More than 30 years ago Morgan and Partridge (22, 23), Binkley and coworkers (2, 10), and Freeman and associates (8, 9) characterized the LPS from species of *Shigella* and *Salmonella* and found that all contained similar protein moieties (2, 8–10, 22, 23). These proteins were separated from LPS with alkali or phenol and, after separation, the polysaccharide and protein could recombine (23). Additionally, the protein moiety was readily soluble in dilute alkali or phenol (2, 8, 10, 22, 23) and was resistant to digestion with trypsin (2, 9, 10, 23). More recently, these observations have been confirmed and expanded. Homma (14) has shown that the protein associated with *Pseudomonas aeruginosa* endotoxin can be isolated from the bacterial outer membrane separately from the endotoxin and that this protein is acidic in nature, being soluble in dilute alkali. Wober and Alaupovic' have found that the protein moiety withstands both trypsin and pronase degradation (39, 40). Furthermore, analysis of the fatty acids in these preparations revealed the presence of β-hydroxymyristic acid, a characteristic marker of lipid A, leading Wober and Alaupovic', as well as Wu and Heath, to conclude that the protein is conjugated to LPS through the lipid A moiety (39–41). This linkage is phenol sensitive (35, 39) and, after treatment, some of the lipid A may remain associated with the protein, whereas the bulk of the lipid A remains attached to the polysaccharide component (35, 39).

Since the protein could be removed by both chemical and enzymatic means without affecting the basic biological properties of LPS, it was considered to be superfluous, functioning primarily as a carrier of the biologically active moieties (39). However, Kim and Watson suggested that a peptide present in LPS acted as a secondary toxophore by eliciting delayed-type hypersensitivity in the host (16, 17). Freedman et al. (7) observed that only LPS which contained protein could enhance the sensitivity of the host to a subsequent dose of LPS and suggested that their protein might be the peptide or "related antigen" described by Kim and Watson (16). In addition, Freedman et al. (7) speculated that the increased sensitivity which they observed might be related to the secondary toxicity described by Kim and Watson (16, 17).

Recently, it was shown that LPS which contained protein (Boivin-LPS) stimulated proliferation of lymphocytes from C3H/HeJ mice, whose B-cells are normally unresponsive to the mitogenic signal of protein-free LPS or lipid A (24, 33). Furthermore, the protein component, endotoxin protein (EP), was separated from Boivin-LPS with hot phenol and found to stimulate the B-cells but not the T-cells of C3H/HeJ mice. Subsequently, EP has been demonstrated

† Present address: Department of Microbiology and Immunology, Albany Medical College, Albany, NY 12208.

to be a potent mitogen and polyclonal activator of B-lymphocytes from mice and humans (G. W. Goodman and B. M. Sultzer, Fed. Proc. **35**:1388, 1978; G. W. Goodman and B. M. Sultzer, J. Exp. Med., in press), as well as of splenic lymphocytes from rats, rabbits, and guinea pigs (G. W. Goodman and B. M. Sultzer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, E29, p. 47; G. W. Goodman and B. M. Sultzer, J. Immunol., in press).

To better understand these new biological characteristics of EP, we examined its composition by polyacrylamide gel electrophoresis (PAGE), as well as by fatty acid and protein analyses. The susceptibility of EP to acid and alkali hydrolyses and to protease digestion was analyzed both by mitogenicity for murine lymphocytes and by PAGE. Preliminary separation of the polypeptides was accomplished by micropreparative PAGE, and the activity of the separated components was determined by assaying the proliferation and polyclonal activation of mouse splenic lymphocytes.

MATERIALS AND METHODS

Animals. CBA/J, C3H/HeJ, and C57Bl/5J mice were originally obtained from Jackson Laboratories, Bar Harbor, Maine, and were subsequently bred in our facilities. Mice of both sexes were used at 2 to 4 months of age and were maintained on water and Purina mouse chow ad lib.

Lymphocyte cultures. Single cell suspensions were prepared from spleens, and 10^6 cells per ml were cultured in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 100 U of penicillin per ml and 100 μg of streptomycin per ml as described previously (34). Deoxyribonucleic acid synthesis was measured by incorporation of [^{3}H]thymidine into acid-precipitable counts and measured as reported elsewhere (34). All cultures were done in triplicate, and the replicate values did not differ by more than 10%. The stimulation index is a ratio of the mean counts per minute of the experimental cultures to the mean counts per minute of the control cultures.

Polyclonal activation. A total of 10^7 cells per ml were cultured as described previously (25) and plaqued in a modified hemolytic plaque assay (25) against trinitrophenylated sheep erythrocytes (30).

Mitogens. EP was prepared from *Salmonella typhosa* O-901, *Salmonella typhimurium* W118-2, and *Escherichia coli* O127:B8 by hot phenol-water extraction of Boivin-LPS as described previously (33). Since the material is sparingly soluble in water and acid, stock solutions of EP were solubilized by suspending 2 mg in 0.95 ml of nonpyrogenic water and adding 0.05 ml of 0.1 N NaOH. The solution was immediately diluted in RPMI 1640 at pH 7.4 and stored at −20°C until used. Protein-free LPS (less than 1% protein) was prepared by the hot phenol-water method of Westphal et al. (38).

Chemical analysis. Protein was analyzed by the method of Lowry et al. (19), using bovine serum albumin as the standard; glucosamine was analyzed by the method of Rondle and Morgan (31); and 2-keto-3-deoxyoctonate (KDO) was analyzed by the method of Osborn (28). Before the determination of glucosamine, the protein was hydrolyzed in 6 N HCl for 18 h at 100°C. Fatty acids were analyzed by hydrolyzing the protein (5 mg/ml) in 0.1 N HCl at 90°C for 35 min (39). The fatty acids were chloroform extracted and then methylated by treatment with methanolic-HCl (15). The fatty acid methyl esters were washed with chloroform and dried. The esters were analyzed on a Hewlett Packard 900 gas chromatograph equipped with a SILAR-10C on a Gas-Chrom Q 100/120 column (outer diameter, 6 ft by 0.125 in. [1.83 m by 0.32 cm]; Applied Science Laboratories, State College, Pa.). The column was programmed as follows: 150°C for 6 min, 6°C/min to 190°C, and held at 190°C for 25 min or until all of the methylated fatty acids were eluted. The carrier gas was nitrogen (40 ml/min), and the areas under the peaks were determined by planimetry. All reference standards were obtained from Applied Science Laboratories.

Bacterial cell cultures. The bacteria were grown in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) in 500 ml of medium in a 2-liter flask for 18 h at 37°C. The flasks were shaken at a rate of 120 rpm. In addition, *E. coli* O127:B8 was grown in a 90-liter fermentation tank at the New England Enzyme Center (Boston, Mass.), and *S. typhosa* O-901 was grown in a similar manner by Kent Miller, University of Miami (Miami, Fla.). Both of these cultures were grown in Trypticase soy broth for 24 h at 37°C.

Gel electrophoresis. Analytical PAGE was performed with the discontinuous sodium dodecyl sulfate buffer system of Laemmli (18). The separating gel was prepared from a stock solution of 30 g of acrylamide (J. T. Baker Chemical Co., Phillipsburg, N.J.) and 0.8 g of *N,N'*-methylenebisacrylamide (J. T. Baker Chemical Co.) in 100 ml of deionized water and was a continuous 5 to 15% acrylamide gradient. The stacking gel was 3% acrylamide from the same stock. The catalysts were ammonium persulfate (Bio-Rad Laboratories, Richmond, Calif.) and *N,N,N',N'*-tetramethylethylenediamine (Bio-Rad Laboratories). The samples (40 μg) were applied to the gel in a sodium dodecyl sulfate-glycerine buffer. Slab gels were run overnight on an upright apparatus (Fred A. Schneider, Dumont, N.J.) against a constant voltage (30 V) or during the day at 60 V for the first hour and 120 V thereafter. Gels were stained with 0.1% Coomassie brilliant blue R-250 (Bio-Rad Laboratories) in 40% methanol and 7.5% acetic acid for 1 h at 37°C and destained with 7% acetic acid. Densitometer tracings were done with a Quick Scan Jr. (Helena Laboratories Corp., Beaumont, Tex.). Preparative gels were run using the same buffer system. The separating gel was 10% acrylamide, and the stacking gel was 3% acrylamide. The gels were run in a micropreparative PAGE chamber TP-13 (Tara Scientific Ltd., E. Rockaway, N.Y.) at 120 V. The buffer was eluted at a rate of 6 to 10 ml/h. The protein in the eluate was monitored at 280 nm with a UA-5 absorbance monitor (Instrument Specialties Co., Lincoln, Nebr.), and the fractions were collected in a

model 328 fraction collector (Instrument Specialties Co.).

Alkaline hydrolysis. Alkaline hydrolysis of EP was performed by dissolving 50 mg of EP in 3 ml of 0.25 N NaOH and heating the solution at 56°C for various times as described below. The hydrolyzed material was neutralized with an equal volume at 0.25 N HCl and diluted to 1 mg/ml with RPMI 1640. For assay of mitogenesis, further dilutions were made from this stock.

Acid hydrolysis. *E. coli* EP was hydrolyzed at 5 mg/ml in 0.1 N HCl at 90°C for 35 min (39) or in 10% acetic acid at 100°C for 3 h (37). The EP was initially insoluble in the acid. It solubilized with heating, and further treatment resulted in a floccular precipitate. Both the aqueous layer and the precipitate were extracted three times with chloroform and dialyzed against nonpyrogenic water. After chloroform extraction, the aqueous layer and the precipitate were combined, lyophilized, and assayed for mitogenic activity. The chloroform layer was washed three times with nonpyrogenic water and dried under vacuum. The residue was dissolved in a small amount of triethylamine and diluted in RPMI 1640 for assay of lymphocyte activation.

Protease treatment. *S. typhosa* and *E. coli* EP (4 mg/ml) were treated with an equal amount of trypsin (2× recrystallized, Sigma Chemical Co., St. Louis, Mo.), pronase (grade B, Calbiochem, La Jolla Calif.), chymotrypsin (3× recrystallized, Worthington Biochemicals Corp., Freehold, N.J.), or papain (2× recrystallized, Worthington Biochemicals Corp.). All proteases were run at their optimal pH, and papain was activated with 0.0005 M cysteine and 0.002 M ethylenediaminetetraacetic acid (Aldrich Chemical Co., Milwaukee, Wis.). The reactions were run for 24 h at 37°C to achieve maximum digestion. The trypsin digestive activity was blocked by the addition of soybean trypsin inhibitor (Aldrich Chemical Co.), and the digestive activity of all the other enzymes was blocked by treatment at 90°C for 45 min. This procedure does not affect the mitogenic activity of EP. For assaying biological activity, the reaction mixture was diluted so that the concentration of EP was 1 mg/ml. From this stock, further dilutions were used in the mitogen assay.

RESULTS

Chemical characteristics. As separated from endotoxin, EP was soluble in phenol and dilute alkali but not in water or dilute acid. Chemical analysis of the material from different organisms and from different lots revealed that EP was 84.6 ± 1.2% protein, 2.26 ± 0.4% glucosamine, 0.0% KDO (Table 1), and approximately 4.5% fatty acids by weight, as estimated from the gas-liquid chromatography data. The major fatty acids found in *E. coli* EP were stearate (13.8%), β-hydroxymyristate (43.4%), and an unidentified fatty acid (15%). Small quantities of other fatty acids were present, including myristate and palmitate (Table 2).

TABLE 1. *Chemical analysis of EP*

EP prepn	wt (%)		
	Protein	Glucosamine	KDO
S. typhosa			
Lot 1276	83.0	3.00	<1.0
Lot 9578	85.0	1.57	<1.0
E. coli			
Lot 1176	88.0	2.2	<1.0
S. typhimurium			
Lot 9778	82.5	ND[a]	ND

[a] ND, Not done.

TABLE 2. *Fatty acids present in E. coli EP*

Fatty acid	Total fatty acids detected (%)
Un[a]	2.8
C_{11}	1.8
C_{12}	—[b]
C_{14}	0.49
Un	1.3
C_{15}	2.7
Un	2.6
C_{16}	3.8
C_{17}	3.8
Un	0.9
C_{18}	13.8
C_{19}	3.2
Un	2.3
β-OH C_{14}	43.4
Un	15.0

[a] Un, Unknown.
[b] —, No fatty acid methyl ester detected.

The composition of EP was analyzed by PAGE, and the results are shown in Fig. 1. There was no difference in the polypeptide composition of EP in the presence or absence of 2-mercaptoethanol, indicating that the polypeptides were not linked by disulfide bridges. *E. coli* EP had 12 detectable peaks with molecular weights of 10,000 to 80,000. However, the polypeptides with molecular weights greater than 35,000 comprised less than 4% of the total protein and were visualized only when more than 40 μg of the protein was applied to the gel. The five major protein bands (molecular weights, 35,000, 17,000, 14,000, 12,000, and 10,000 to 11,000) were 86.4% of the total protein. As with *E. coli* EP, *S. typhosa* EP had many minor polypeptides, but only four major ones (molecular weights, 35,000, 32,000, 13,000 to 13,500, and 10,000 to 11,000). These four proteins comprised 83.5% of the total protein. Although there were some similar proteins in both EP preparations (for example, those at

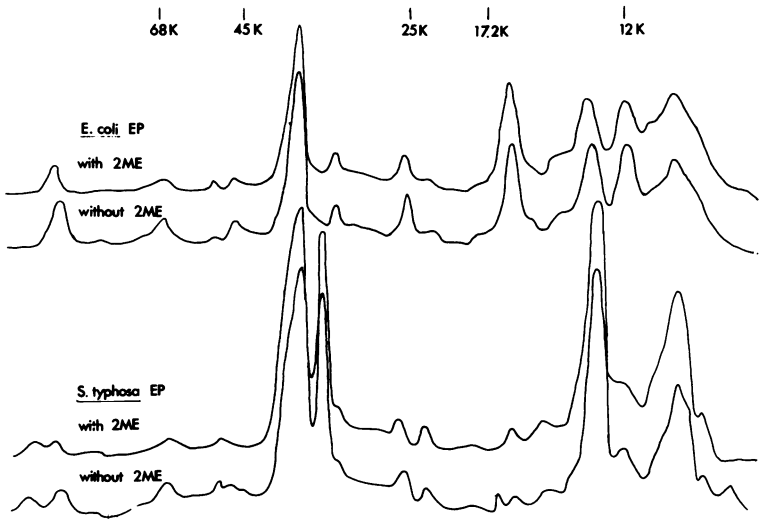

FIG. 1. *Densitometer tracings of E. coli EP and S. typhosa EP with and without 2-mercaptoethanol (2ME). The molecular weight markers were as follows: albumin, 68,000 (68K); ovalbumin, 45,000; chymotrypsinogen, 25,000; myoglobin, 17,200; and cytochrome, 12,000. A 40-μg amount of each sample was applied to the gel.*

molecular weights of 35,000 and 10,000 to 11,000), there were major differences between *E. coli* EP and *S. typhosa* EP. The protein bands present at molecular weights of 17,000 and 12,000 in *E. coli* EP were present only as minor components in *S. typhosa* EP, whereas the 32,000- to 33,000-molecular-weight polypeptide seen in *S. typhosa* EP was not found in the EP preparation from *E. coli*.

Hydrolysis. To more fully characterize this material, the effect of alkali and acid hydrolysis on EP was studied. Figure 2 shows that *E. coli* EP was degraded by alkali treatment. The degradation was rapid; changes in the 35,000-molecular-weight band were apparent after 5 min of treatment. Early in alkali treatment, the low-molecular-weight polypeptides increased, but these too were eventually destroyed. The mitogenic activity of alkali-treated material was tested in lymphocytes from CBA/J mice and from LPS-nonresponder C3H/HeJ mice (Fig. 3). The mitogenicity of EP for lymphocytes from both mouse strains decreased rapidly. However, the stimulatory activity of EP for the C3H/HeJ cells was totally destroyed in 2 h, whereas it took 6 h to destroy the activity for the CBA/J lymphocytes. The greater resistance of the mitogenic moiety to degradation when assayed in the LPS-responsive lymphocytes is due to the resistance of lipid A to this treatment (11). Similar results were seen with *S. typhosa* EP (data not shown). These results demonstrate that EP is highly susceptible to alkali hydrolysis.

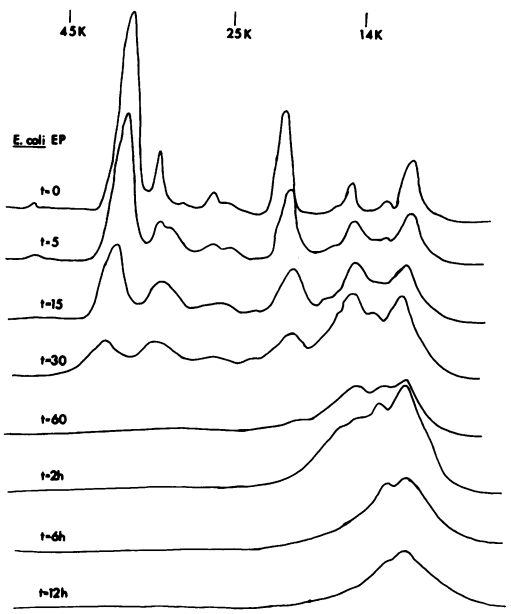

FIG. 2. *Densitometer tracings of alkali-treated E. coli EP. The treatment was for the times indicated, and the molecular weight markers were as follows: ovalbumin, 45,000 (45K); chymotrypsinogen, 25,000; and ribonuclease, 14,000.*

Acid hydrolysis has been used to liberate the fatty acids from the proteins associated with LPS (39, 41) as well as from EP. Although hydrolysis in 0.1 N HCl or in 10% acetic acid

reduced the density of the polypeptide bands (Fig. 4), it did not cause major qualitative changes in the polypeptide profile. In contrast to mild alkali treatment, which broke down the protein without initially affecting the fatty acid ester bond, acid treatment removed the fatty acids without qualitatively affecting the polypeptide bands or the activity of EP. Figure 5 shows that the acid-hydrolyzed EP was still mitogenic for C3H/HeJ lymphocytes. At 10 μg/ml, there was no difference in activity between acid-hydrolyzed EP and untreated EP, but at 50 μg/ml, the activities of the acetic acid hydrolysate and the HCl hydrolysate were reduced 50 and 33%, respectively. It is of interest that the HCl hydrolysate curve resembled the dose-response curve of the parent material, whereas the dose-response curve of the acetic acid-treated material was much sharper than that of the parent material. This sharper dose response was also seen when the mitogenic activity of acetic acid-hydrolyzed EP was analyzed on lymphocytes from C57Bl/6J mice. In contrast to the response of the C3H/HeJ lymphocytes, the response of C57Bl/6J cells to acetic acid- and HCl-treated EP was reduced 25% at 10 μg/ml and more than 50% at 50 μg/ml when compared with untreated EP (Fig. 6). These data indicate that EP is relatively stable to acid hydrolysis and that the decrease in activity seen in the C57Bl/6J spleen cells was most likely due to the loss of the lipid A component.

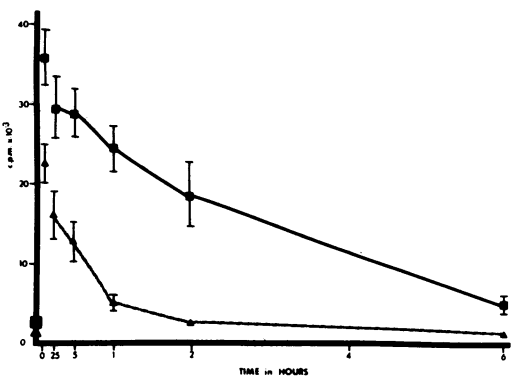

FIG. 3. *Activity of alkali-treated E. coli EP in lymphocytes from CBA/J (■) and C3H/HeJ (▲) mice. The EP was treated for the times indicated. A 50-μg amount of each sample was applied to the gel.*

Proteolysis. It has long been recognized that the protein moiety of LPS is resistant to trypsin degradation (10, 22, 23, 39). Therefore, we chose to examine the effect of various proteases on EP. The mitogenicity of both *E. coli* EP and *S. typhosa* EP, as measured in C3H/HeJ lymphocytes, was resistant to the proteolytic effects of trypsin, chymotrypsin, and pronase (Fig. 7 and 8). In contrast to the insensitivity of EP to these proteases, both preparations were degraded about 50% by papain. Because this degradation of activity was measured in lymphocytes from LPS-nonresponder C3H/HeJ mice, we are assured that the papain treatment was degrading the protein.

Bacterial culture conditions. During the last 2 years, a number of EP preparations were extracted from bacteria grown in shaker flasks and fermentation tanks. *E. coli* EP lot 1176, *S. typhosa* EP lots 1276 and 9578, and *S. typhimurium* EP lot 9778 were extracted from bac-

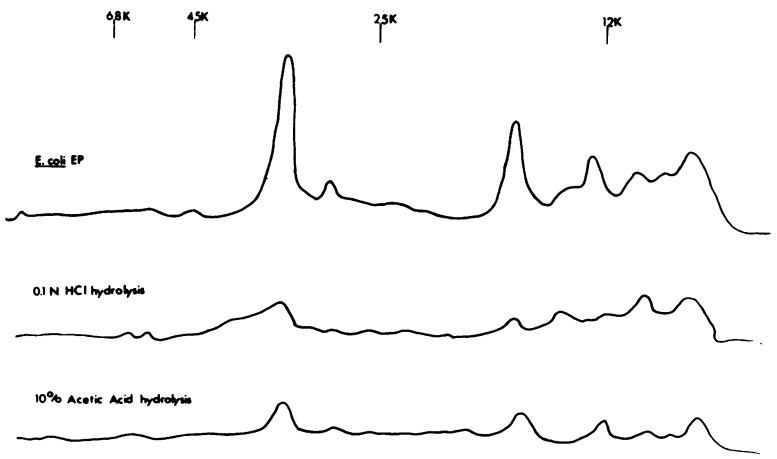

FIG. 4. *Densitometer tracings of acid-treated E. coli EP. The EP was hydrolyzed as described in the text. A 40-μg amount of each sample was applied to the gel. The molecular weight markers were albumin, ovalbumin, chymotrypsinogen and cytochrome c.*

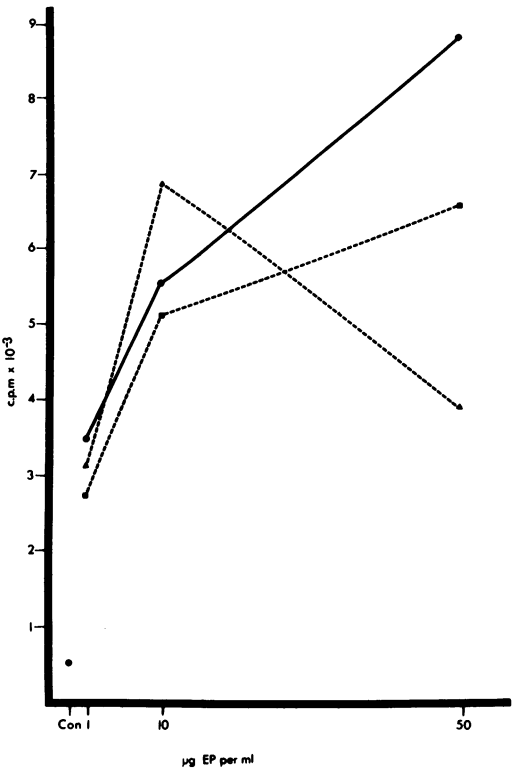

FIG. 5. *Activity of acid-hydrolyzed E. coli EP in C3H/HeJ lymphocytes. Symbols: ●, control EP; ▲, acetic acid; ■, HCl. Chloroform-extractable material had no activity at any concentration tested.*

teria grown in shaker flasks, whereas EP lots 1277, 1678, and 2578 were extracted from organisms grown in fermentation tanks. Table 3 shows that all of the EP preparations stimulated significant proliferation above background, as measured by [^{3}H]thymidine incorporation into acid-precipitable counts. Both *E. coli* EP preparations induced mitogenesis equal to more than 30 times background, as did *S. typhosa* EP lots 1276, 1678, and 2578. EP lots 9578 and 9778 showed slightly less activation, the stimulation index being equal to 13 to 17 times background; however, at the time that these preparations were tested, the stimulation indexes for other types of mitogens were lower as well. In any event, all of the stimulation indexes were significantly above background. Therefore, it may be concluded that the difference in bacterial culture conditions does not affect mitogenicity for mouse spleen cells. In contrast, EP lots 1277, 1678, and 2578 (fermentation tanks) can stimulate mitogenesis in human peripheral blood lymphocytes weakly, if at all; whereas lots 1176, 1276, 9578, and 9778 (shaker flasks) stimulate the proliferation of human B-cells (G. W. Goodman and B. M. Sultzer, J. Exp. Med., in press).

Analysis of the EP preparations by PAGE revealed that the *E. coli* EP preparations were quite similar, regardless of the method of growth (Fig. 9). However, major differences appeared in the *S. typhosa* EP preparations. Although EP lot 1678 (fermentation tank) contained many of the same polypeptides as did EP lot 1276 (shaker flask), the bands were stained less intensely, resulting in smaller, flatter peaks on the densitometer tracings. Also, EP lot 2578 (fermentation tank) had a gel pattern completely different from that of either of the other preparations. The 35,000- and 32,000- to 33,000-molecular-weight components were fused into one band, and the low-molecular-weight bands were shifted to a molecular weight below 10,000. Subsequent cultures of *S. typhosa* O-901 were grown in shaker flasks in our laboratory, and the EP preparation obtained (lot 9578) was characteristic of the original EP (lot 1276). EP lot 9578 contained the 35,000- and 32,000- to 33,000-molecular-weight doublet as well as the two lower-molecular-weight bands. In addition, minor polypeptide bands similar to those seen in EP lot 1276 could be detected (data not shown). *S.*

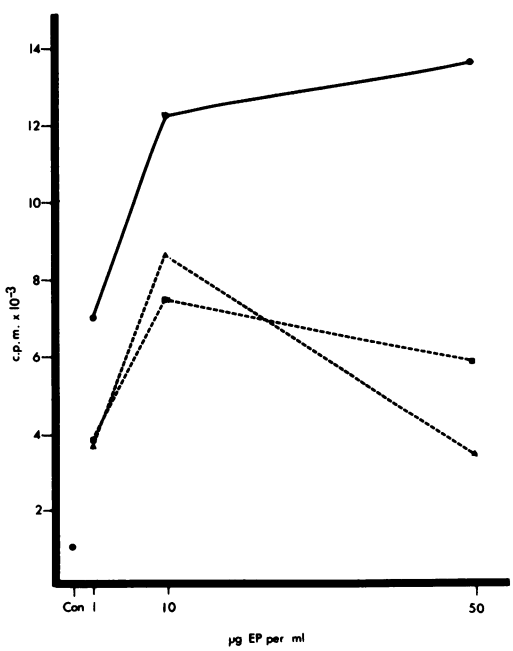

FIG. 6. *Activity of acid-hydrolyzed E. coli EP in C57Bl/6J lymphocytes. See legend to Fig. 5. Chloroform-extractable material had no activity at any concentration tested.*

typhimurium EP lot 9778 was also prepared from organisms grown in shaker flasks. This EP preparation was composed of a singlet at a molecular weight of 35,000, minor polypeptide bands at molecular weights above and below 35,000, and two low-molecular-weight proteins at approximately 14,000 and 12,000, which is similar to *S. typhosa* EP shown in Fig. 9.

Separation of the polypeptides. To determine which of the polypeptides were responsible for the activation of murine spleen cells, EP preparations were separated by micropreparative PAGE. *E. coli* EP (25 mg) was separated into four fractions which showed absorption at 280 nm. Each fraction was dialyzed to remove unbound sodium dodecyl sulfate, lyophilized, and tested at low concentrations in spleen cell cultures to avoid toxicity due to any residual

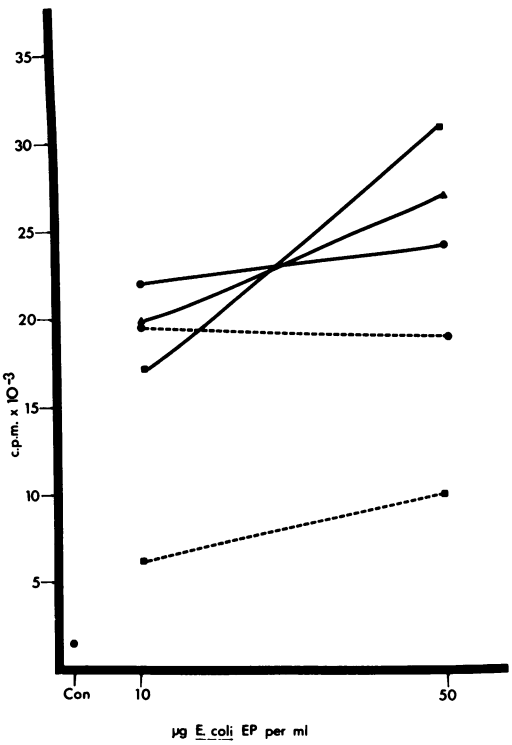

FIG. 7. *Activity of protease-treated E. coli EP in C3H/HeJ lymphocytes. The proteases alone had nc activity, and the ethylenediaminetetraacetic acid and cysteine used to activate papain did not inhibit the EP response. Symbols: ●——●, control; ●-----●, trypsin; ▲——▲, chymotrypsin; ■——■, pronase; ■-----■, papain.*

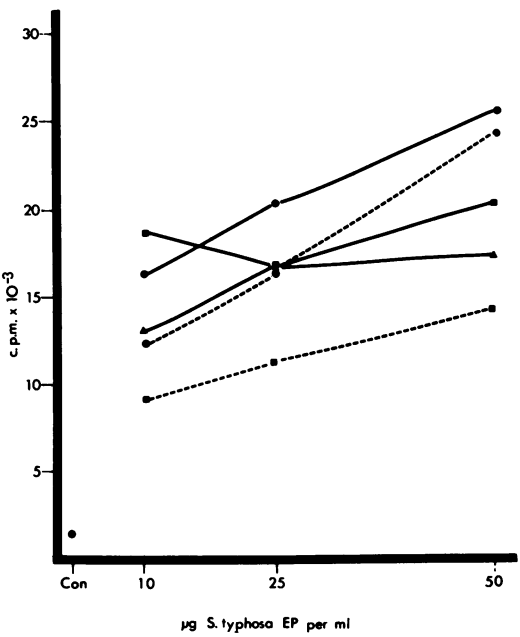

FIG. 8. *Activity of protease-treated S. typhosa EP in C3H/HeJ lymphocytes. See legend to Fig. 7.*

TABLE 3. *Mitogenicity of several EP preparations for C3H/HeJ lymphocytes*

Mitogen (μg/ml)	[^{3}H]thymidine uptake (mean cpm)						
	E. coli EP lot:		*S. typhosa* EP lot:				
	1176	1277	1276	1678	2578	9578	*S. typhimurium* EP lot 9778
Control	1,215	691	587	691	691	1,069	1,069
0.1	4,277 (3.5)[a]	3,857 (5.6)	1,652 (2.8)	1,097 (1.6)	7,202 (10.4)	ND[b]	ND
1	16,997 (14.0)	8,673 (12.1)	5,977 (10.2)	4,266 (6.2)	12,631 (18.3)	9,339 (8.7)	9,689 (9.1)
10	47,778 (39.3)	16,593 (24.0)	9,664 (16.5)	13,235 (19.2)	21,390 (31.0)	14,351 (13.4)	18,031 (16.9)
25	60,796 (50.4)	21,580 (31.2)	9,622 (16.4)	22,053 (31.9)	29,197 (42.3)	13,881 (13.0)	18,279 (17.1)
50	52,912 (43.6)	21,402 (31.0)	19,470 (33.2)	23,869 (34.5)	29,918 (43.3)	13,886 (13.0)	16,882 (15.8)
100	58,208 (47.9)	17,658 (25.6)	23,144 (39.4)	24,038 (34.8)	28,090 (40.7)	12,794 (12.0)	16,461 (15.4)

[a] Numbers within parentheses indicate stimulation indexes.
[b] ND, Not done.

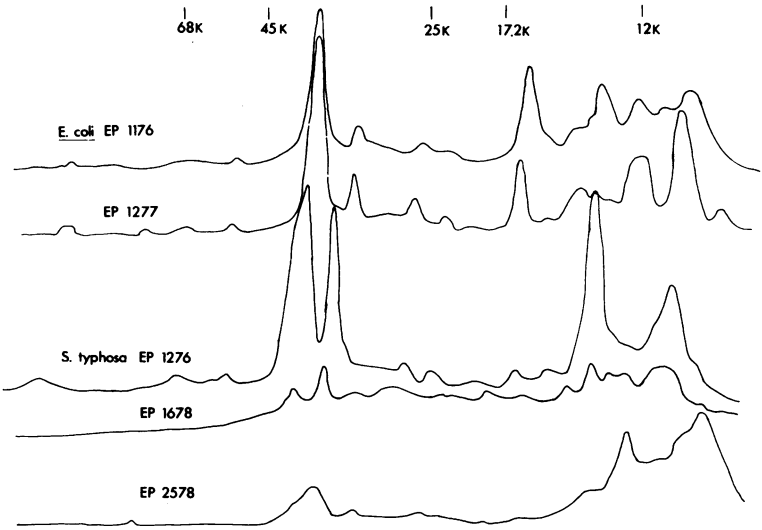

FIG. 9. *Densitometer tracings of different EP preparations. A 40-μg amount of each sample was applied to the gel. Albumin, ovalbumin, chymotrypsinogen, myoglobin, and cytochrome c were the molecular weight markers.*

bound sodium dodecyl sulfate. In addition, samples were run in the analytical PAGE system described above. As shown in Fig. 10, fractions A and B contained no identifiable polypeptides and were inactive mitogenically (Table 4). However, fraction C, which consisted of the three relatively low-molecular-weight polypeptides (17,000 and less), was active in stimulating deoxyribonucleic acid synthesis and plaque-forming cells. Likewise, fraction D, which contained polypeptides with molecular weights of 17,000 and greater, was also active in both assays. In a similar manner, *S. typhosa* EP (25 mg) was separated into multiple 280-nm fractions A to F (Fig. 11 and Table 5). The first fraction, A, which contained none of the major polypeptides, was inactive. Fraction B, which consisted chiefly of the 10,000-molecular-weight polypeptide, was active. Fractions C and D, which contained polypeptides ranging in molecular weights from 10,000 to about 17,000, stimulated both polyclonal activation and proliferation. However, fractions E and F, which contained the polypeptides of higher molecular weight and their degradation products, did not stimulate deoxyribonucleic acid synthesis and were weakly active, if at all, in stimulating polyclonal activation. It should be noted that in the micropreparative PAGE system, the high-molecular-weight, slower-migrating polypeptides were exposed to an alkaline pH for several hours. As described above, these polypeptides were particularly susceptible to degradation by these conditions, so

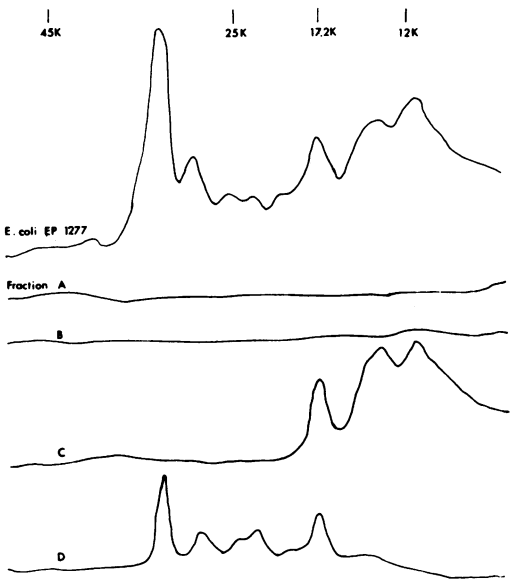

FIG. 10. *Densitometer tracings of E. coli EP fractions separated by micropreparative PAGE. A 40-μg amount of each sample was applied to the gel. The molecular weight markers were albumin, ovalbumin, chymotrypsinogen, myoglobin, and cytochrome c.*

that other separation procedures must be used before any firm conclusions can be drawn about the activity of these high-molecular-weight components. Indeed, more definitive separation is necessary before activity can be assigned to any

TABLE 4. *Activity of E. coli EP fractions on C3H/HeJ lymphocytes*

Prepn	μg/ml	[^{3}H]thymidine uptake (mean cpm)	Mean plaque-forming cells per 10^6 viable cells
Control		909	0
E. coli EP	0.1	1,616	2
	1	6,727	6
	10	13,898	29
	50	16,875	73
Fraction A[a]	0.1[b]	623	8
	1[c]	510	3
Fraction B[a]	0.1[b]	408	2
	1[c]	347	2
Fraction C[a]	0.1	4,926	80
	1	252	79
Fraction D[a]	0.1	5,574	58
	1	2,862	1

[a] Fraction A contained 0.0 mg of protein per ml; fraction B contained 0.4 mg of protein per ml; fraction C contained 1.6 mg of protein per ml; fraction D contained 1.95 mg of protein per ml.

[b] 1:1,000 dilution of stock.

[c] 1:100 dilution of stock.

one polypeptide, but it is clear that the intermediate- and low-molecular-weight polypeptides of the *S. typhosa* EP are active on mouse lymphocytes.

DISCUSSION

Characterization of the basic properties of EP revealed that it is soluble in dilute alkali and in phenol, but not in water or acidic solutions, indicating that EP is a hydrophobic, acidic protein. These qualities were first attributed to the protein moiety of LPS by Morgan and Partridge (22, 23), Freeman and Anderson (8), and, more recently, Homma (14). More specifically, EP is composed of approximately 84% protein and 2.5% glucosamine (depending upon the bacteria from which the EP was extracted). None of the preparations contains KDO, which binds lipid A to the polysaccharide, indicating that EP is not contaminated with the core polysaccharide component of LPS.

The presence of glucosamine, which is the backbone of lipid A, indicated that EP was closely associated with this component of LPS. The close relationship between the protein and lipid A moieties was implied by the extraction of toxic proteins by Binkley et al. (2). A short time later, Westphal and Lüderitz (37) suggested that the protein moiety might be bound to the endotoxin through lipid A and later proposed that the toxic protein was an alkali-sensitive lipid A-protein complex (20). Evidence presented by Wober and Alaupovic' (39, 40) and by Wu and Heath (41) has demonstrated the association of lipid A with the protein. In addition to glucosamine, analysis of the fatty acid in EP by gas chromatography has revealed the presence of β-hydroxymyristate. The presence of this fatty acid is substantial evidence of the association of EP with lipid A, and the absence of KDO shows that the presence of lipid A is not due to LPS contamination. Although a material similar to EP has been described as lipid A-associated protein (24), this is the first time that lipid A has been demonstrated to be associated with the mitogenic protein extracted from LPS.

Another major outer membrane protein, murein lipoprotein, is also a B-cell mitogen and has been characterized as containing palmitic acid as one-half of the total fatty acids present (3, 4-6, 13, 21). The insignificant amount of palmitic acid in the fatty acids isolated from EP is strong

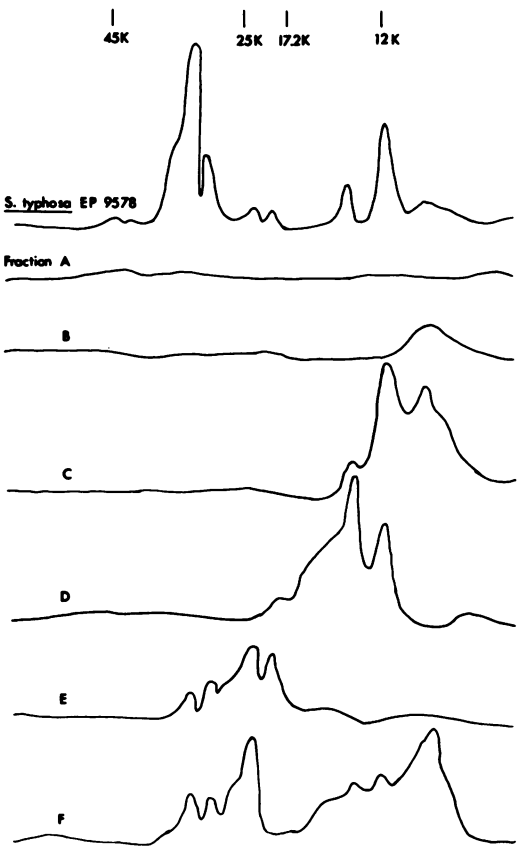

FIG. 11. *Densitometer tracings of S. typhosa EP fractions separated by micropreparative PAGE. A 40-μg amount of each sample was applied to the gel. The molecular weight markers were ovalbumin, chymotrypsinogen, myoglobin, and cytochrome c.*

TABLE 5. *Activity of S. typhosa EP fractions on C3H/HeJ lymphocytes*

Prepn	μg/ml	[^{3}H]thymidine uptake (mean cpm)	Mean plaque-forming cells per 10^6 viable cells
Control		1,082	7
S. typhosa EP	0.1	8,210	28
	1	11,495	33
	10	15,276	67
	50	17,690	81
Fraction A[a]	0.1[b]	1,216	ND[c]
	1[d]	769	ND
Fraction B[a]	0.1[b]	6,066	27
	1[d]	652	ND
Fraction C[a]	0.1	4,127	39
	1	2,178	2
Fraction D[a]	0.1	3,880	79
	1	165	63
Fraction E[a]	0.1	464	10
	1	306	14
Fraction F[a]	0.1	719	17
	1	219	13

[a] Fraction A contained 0.0 mg of protein per ml; fraction B contained 0.1 mg of protein per ml; fraction C contained 1.54 mg of protein per ml; fraction D contained 1.92 mg of protein per ml; fraction E contained 1.58 mg of protein per ml; fraction F contained 1.65 mg of protein per ml.

[b] 1:1,000 dilution of stock.

[c] ND, No data.

[d] 1:1,000 dilution of stock.

evidence that EP and murein lipoprotein are not the same. An additional difference between EP and murein lipoprotein is that whereas murein lipoprotein is a homogeneous polypeptide with a molecular weight of 7,500 (3, 6), EP is a heterogeneous material when analyzed by PAGE. EP is composed of four (*S. typhosa* EP) or five (*E. coli* EP) major proteins. These proteins vary in molecular weight from 10,000 to 35,000, although the minor proteins may have molecular weights as high as 80,000. The polypeptides in EP differ between lots, and many of these differences can be attributed to species differences and culture conditions, which have been reported to be typical of outer membrane proteins from gram-negative bacteria (32).

The sensitivity of EP to alkaline hydrolysis has been shown under conditions in which activity of LPS is enhanced (11). These mild conditions do not cleave the fatty acids from the lipid backbone (11), but the mitogenicity of EP for lymphocytes from LPS-nonresponsive C3H/HeJ mice is rapidly destroyed. The alkali sensitivity of the lipid A-protein complex was proposed by Lüderitz et al. (20); in addition, lipid A-associated protein has been demonstrated to be alkali sensitive (1). In contrast to its alkali sensitivity, EP is relatively resistant to acid hydrolysis. Acid treatment removes the fatty acids from EP, but leaves the proteins in EP intact functionally, as determined by mitogenicity assays.

The resistance of the protein moiety of LPS to protease digestion has been documented (2, 9, 10, 23). Wober and Alaupovic' found that trypsin treatment did not substantially alter their "simple protein" and that there was a large pronase-insensitive core (39). EP is resistant to trypsin, chymotrypsin, and pronase degradation when assayed for mitogenicity. This resistance may be due, in part, to the hydrophobicity of EP. In contrast to the other proteases, papain partially decreases the mitogenicity of EP. Since the activity is reduced by 50% in lymphocytes from both LPS-nonresponsive C3H/HeJ mice and C57Bl/6J mice, it may be concluded that papain degrades the protein. Kim and Watson observed that the secondary toxicity of endotoxin was abolished after papain treatment, and although they acknowledged the esterase and amidase activity of papain, they believed that papain was degrading a peptide in LPS (16). Because of the presence of the proteases in the reaction mixture, it is technically difficult to assay the effect that the enzymes might have on the different polypeptides in EP by PAGE. Therefore, it is necessary to purify the protein away from the enzymes to analyze the possible changes in the polypeptides. Because EP is absorbed onto the carrier material, Sepharose- or Sephadex-insoluble enzymes cannot be used (G. W. Goodman, unpublished data).

Although this work has demonstrated that EP has many of the same chemical properties as the LPS-associated proteins described in the last 35 years, the fact that EP has significant biological activity as a mitogen and polyclonal activator for lymphocytes from many species, including humans (G. W. Goodman and B. M. Sultzer, J. Immunol., in press; G. W. Goodman and B. M. Sultzer, J. Exp. Med., in press), increases the need for further characterization of this material. For example, a more definitive separation and purification of the polypeptides to determine which is the mitogen(s) for human B-cells are needed. In our laboratory, we isolated EP from *E. coli*, *S. typhosa*, and *S. typhimurium*. Other investigators have found the LPS-associated protein in *Shigella* spp. (2, 10, 22), *Serratia marcescens* (39, 40), *P. aeruginosa* (14), and *E. coli* (24, 39, 40). These results suggest that these proteins are ubiquitous, in contrast to the murein lipoprotein which is not found in *Proteus* spp. or *Pseudomonas fluorescens* (4). In addition, we have shown that the protein moiety

differs depending upon bacterial culture conditions, an observation also made by Schnaitman (32). It may be possible, therefore, to increase the yields of active EP by more suitable culture conditions and/or improved extraction procedures. Nevertheless, we believe that the EP described so far holds promise as a new mitogen for investigating the functional activities of human B-lymphocytes.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service Biomedical Research grant 5SO74405401 from the Division of Research Resources, National Institutes of Health.

We thank Leslie Hull, T. C. Werner, and Robert Scheafer of Union College for the use of the gas chromatograph and for their helpful discussions. In addition, we thank Toby Eisenstein for the culture of *S. typhimurium* W118-2 and John Butke for technical assistance.

LITERATURE CITED

1. **Betz, S. J., and D. C. Morrison.** 1977. Chemical and biological properties of a protein-rich fraction of bacterial lipopolysaccharides. I. The *in vitro* murine lymphocyte response. J. Immunol. **119**:1475–1481.
2. **Binkley, F., W. F. Goebel, and E. Perlman.** 1945. Studies on the flexner group of dysentery bacilli. II. The chemical degradation of the specific antigen of type Z *Shigella paradysenteriae* (Flexner). J. Exp. Med. **81**: 331–347.
3. **Boivin, A., I. Mesrobeanu, and L. Mesrobeanu.** 1933. Extraction d'un complex toxique et antigenique a partir du bacille d'aertrycke. C. R. Soc. Biol. **114**:307–310.
4. **Braun, V., and V. Bosch.** 1972. Sequence of the murein lipoprotein and the attachment site of the lipid. Eur. J. Biochem. **28**:51–69.
5. **Braun, V., K. Rehn, and H. Wolff.** 1970. Supramolecular structure of the rigid layer of the cell wall of *Salmonella, Serratia, Proteus* and *Pseudomonas fluorescens*. Number of lipoprotein molecules in a membrane layer. Biochemistry **9**:5041–5049.
6. **Braun, V., and H. Wolff.** 1970. The murein lipoprotein linkage in the cell wall of *Escherichia coli*. Eur. J. Biochem. **14**:387–391.
7. **Freedman, H. H., A. E. Fox, R. S. Willis, and B. S. Schwartz.** 1967. Role of protein component of endotoxin in modification of host reactivity. Proc. Soc. Exp. Biol. Med. **125**:1316–1320.
8. **Freeman, G. G., and T. H. Anderson.** 1941. The hydrolytic degradation of the antigenic complex of *Bact. typhosum* Ty2. Biochem. J. **35**:564–577.
9. **Freeman, G. G., S. W. Challinor, and J. Wilson.** 1940. The use of a synthetic medium in the isolation of the osmatic antigens of *Bact. typhi-murium* and *Bact. typhosum*. Biochem. J. **34**:307–324.
10. **Goebel, W. F., F. Binkley, and E. Perlman.** 1945. Studies on the flexner group of dysentery bacilli. I. The specific antigens of *Shigella paradysenteriae* (Flexner). J. Exp. Med. **81**:315–330.
11. **Goodman, G. W., and B. M. Sultzer.** 1977. Mild alkaline hydrolysis of lipopolysaccharide endotoxin enhances its mitogenicity for murine B cells. Infect. Immun. **17**:205–214.
12. **Halegoua, S., A. Hirashima, and M. Inouye.** 1974. Existence of free form of a specific membrane lipoprotein in gram-negative bacteria. J. Bacteriol. **120**:1204–1208.
13. **Hirashima, A., H. C. Wu, P. S. Venkateswaran, and M. Inouye.** 1973. Two forms of a structural lipoprotein in the envelope of *Escherichia coli*. Further characterization of the free form. J. Biol. Chem. **248**:5654–5659.
14. **Homma, J. Y.** 1968. The protein moiety of the endotoxin of *Pseudomonas aeruginosa*. Z. Allg. Mikrobiol. **8**:227–248.
15. **Kasai, N.** 1966. Chemical studies on the lipid component of endotoxin, with special emphasis on its relation to biological activities. Ann. N.Y. Acad. Sci. **133**:486–507.
16. **Kim, Y. B., and D. W. Watson.** 1964. Inactivation of Gram-negative bacterial endotoxins by papain. Proc. Soc. Exp. Biol. Med. **115**:140–142.
17. **Kim, Y. B., and D. W. Watson.** 1966. Role of antibodies in reactions to Gram-negative endotoxins. Ann. N.Y. Acad. Sci. **133**:727–743.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Lüderitz, O., K. Jann, and R. Wheat.** 1968. Somatic and capsular antigens of Gram-negative bacteria. Comp. Biochem. Physiol. A **26**:105–228.
21. **Melchers, F., V. Braun, and C. Galanos.** 1975. The lipoprotein of the outer membrane of *Escherichia coli*. A B-lymphocyte mitogen. J. Exp. Med. **142**:473–482.
22. **Morgan, W. T. J., and S. M. Partridge.** 1940. Studies in immunochemistry. 4. The fractionation and nature of antigenic material isolated from *Bact. Dysenteriae* (shiga). Biochem. J. **34**:169–191.
23. **Morgan, W. T. J., and S. M. Partridge.** 1942. An examination of the O antigenic complex of *Bact. typhosum*. Br. J. Exp. Pathol. **23**:151–165.
24. **Morrison, D. C., S. J. Betz, and D. M. Jacobs.** 1976. Isolation of a Lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C3H/HeJ spleen cells. J. Exp. Med. **144**:840–846.
25. **Nilsson, B. S., B. M. Sultzer, and W. W. Bullock.** 1973. Purified protein derivative of tuberculin induces immunoglobulin production in normal mouse spleen cells. J. Exp. Med. **137**:127–139.
26. **Nowotny, A.** 1961. Chemical structure of a phosphomucolipid and its occurrence in some strains of Salmonella. J. Am. Chem. Soc. **83**:501–503.
27. **Nowotny, A. M., S. Thomas, O. S. Duron, and A. Nowotny.** 1963. Relation of structure to function in bacterial O antigens. I. Isolation methods. J. Bacteriol. **85**:418–426.
28. **Osborn, M. J.** 1963. Studies on the Gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. **50**:499–506.
29. **Rank, W. R., U. Flugge, and R. DiPauli.** 1969. Inheritance of the Lipid A-induced 19S plaque-forming cell response in mice. Evidence for three antigen recognition mechanisms. Behringwerk Mitt. **49**:222–229.
30. **Rittenberg, M. B., and K. L. Pratt.** 1969. Antitrinitrophenyl (TNP) plaque assay primary response of Balb/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med. **132**:575–581.
31. **Rondle, C. J. M., and W. T. J. Morgan.** 1955. The determination of glucosamine and galactosamine. Biochem. J. **61**:586–589.
32. **Schnaitman, C. A.** 1974. Outer membrane proteins of *Escherichia coli*. IV. Differences in outer membrane proteins due to strain and cultural differences. J. Bacteriol. **118**:454–464.
33. **Sultzer, B. M., and G. W. Goodman.** 1976. Endotoxin protein: a B-cell mitogen and polyclonal activator of C3H/HeJ lymphocytes. J. Exp. Med. **144**:821–827.
34. **Sultzer, B. M., and B. S. Nilsson.** 1972. PPD-tuberculin—a B-cell mitogen. Nature (London) New Biol. **240**: 198–200.

35. **Tsang, J. C., C. S. Wang, and P. Alaupovic'.** 1974. Degradative effect of phenol on endotoxin and lipopolysaccharide preparations from *Serratia marcescens*. J. Bacteriol. **117**:786–795.
36. **Westphal, O.** 1975. Bacterial endotoxins. Int. Arch. Allergy Appl. Immunol. **49**:1–43.
37. **Westphal, O., and O. Lüderitz.** 1954. Chemische Erforschung von Lipopolysaccharide Gram-negative Bakterien. Angew. Chem. **66**:407–417.
38. **Westphal, O., O. Lüderitz, and F. Bister.** 1952. Uber die Extracktion von Bacterien mit Phenol-Wasser. Z. Naturforsch. Teil B **7**:148–155.
39. **Wober, W., and P. Alaupovic'.** 1971. Studies on the protein moiety of endotoxin from Gram-negative bacteria. Characterization of the protein moiety isolated by phenol treatment of endotoxin from *Serratia marcescens* O8 and *Escherichia coli* O141:K85B. Eur. J. Biochem. **19**:340–356.
40. **Wober, W., and P. Alaupovic'.** 1971. Studies on the protein moiety of endotoxin from Gram-negative bacteria. Characterization of the protein moiety isolated by acetic acid hydrolysis of endotoxin from *Serratia marcescens* O8. Eur. J. Biochem. **19**:357–367.
41. **Wu, M.-C., and E. C. Heath.** 1973. Isolation and characterization of lipopolysaccharide protein from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **70**:2572–2576.