Brief Definitive Report

ENDOTOXIN PROTEIN: A B-CELL MITOGEN AND POLYCLONAL ACTIVATOR OF C3H/HeJ LYMPHOCYTES*

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Bacterial endotoxins have been characterized as lipopolysaccharides (LPS) and for all intents and purposes these two terms have become synonymous. Boivin-type endotoxins, however, contain significant amounts of cell wall protein complexed to the LPS (1). Removal of this protein by chemical and enzymatic means has little effect on the basic biological properties of LPS (2). Consequently, it has been generally accepted that the protein component is by and large superfluous and functions primarily as an inert carrier of the other biologically active components (3).

In our earlier investigations of the endotoxin-resistant C3H/HeJ mouse a Boivin endotoxin from *Escherichia coli* 0127:B8 was used for the genetic analysis of endotoxemia and the cellular responses to endotoxin. This preparation was reported to activate DNA synthesis in C3H/HeJ spleen cells but to a lesser extent than cells from endotoxin-susceptible mice (4). Subsequent studies with essentially protein-free LPS from *E. coli* 0127:B8 revealed that spleen cells of C3H/HeJ mice are essentially unresponsive or at the most low responders to the mitogenic effects of LPS (5), a finding which has been confirmed in other laboratories (6–9). Further examination of various endotoxin preparations, all of which contained protein, revealed that C3H/HeJ spleen cells could be activated to DNA synthesis. The possibility existed that the presumably inert protein carrier might enhance the mitogenic response of mouse lymphocytes and at least partially restore the response of C3H/HeJ B cells to the LPS moiety. The experiments reported here show that not only do endotoxins composed of protein complexes activate C3H/HeJ lymphocytes but that the protein fraction isolated from this complex can act as a B-cell mitogen and polyclonal activator for this strain.

Materials and Methods

Animals. CBA/J and C3H/HeJ mice, originally obtained from The Jackson Laboratory, Bar Harbor, Maine, were bred in our laboratory. Congenitally athymic "nude" (nu/nu, BALB/c) mice were obtained from stocks maintained in our institution. Mice of both sexes were used for lymphocyte cultures at 2–4 mo of age, unless otherwise noted.

Mitogens. LPS preparations from *E. coli* 0127:B8 and *Salmonella typhosa* 0-901 were made by the phenol-water method of Westphal et al. (10). Boivin endotoxins from the same organisms were made by the TCA method previously described (11). Also a Boivin preparation of *S. typhosa* 0-901 was obtained from Difco Laboratories, Detroit, Mich. Concanavalin A (Con A) was obtained from

* This work was supported in part by U. S. Public Health Service grant AI-07816.

Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Tuberculin (PPD-RT32) was obtained from Statens Serum Institute, Copenhagen, Denmark. Protein-free LPS and endotoxin with protein from *E. coli* 12408 were kindly provided by Dr. J. C. Stewart, Twyford Laboratories, Ltd., London, England. These materials were prepared by methods reported previously (12).

Endotoxin Protein. TCA-extracted endotoxin was used as the starting material. 500 mg was dissolved in 50 ml of nonpyrogenic water and heated to 65°C. An equal volume of preheated 90% phenol was added to the endotoxin solution and the mixture incubated for 45 min at 65°C with occasional shaking. After cooling, the phenol and aqueous phases were separated by centrifugation. The phenol phase was then re-extracted with nonpyrogenic water as described above. Addition of 300 ml of cold anhydrous ethanol to the separated phenol layer produced a precitate which was stored overnight at −20°C. The precipitate was collected by centrifugation, washed five times with cold anhydrous ethanol, resuspended in nonpyrogenic water, dialyzed, and lyophilized. A typical yield obtained was 36 mg or approximately 7% of the starting endotoxin. The pooled aqueous phases were treated in a similar manner. The phenol-phase fraction was found to be sparingly soluble in water so that stock solutions were solubilized by suspending 2 mg in 0.95 ml of nonpyrogenic water and adding 0.05 ml of 0.1 N NaOH. This solution was diluted in cell culture medium to a concentration of 1 mg/ml and kept at −20° until used. Protein was analyzed by the Lowry procedure (13), total nitrogen by the microkjeldahl-Ninhydrin method (14), glucosamine by the method of Rondle-Morgan (15), and 2-keto-3-dexoxyoctanate (KDO) by the method of Osborn (16). Representative results of these analysis indicate the phenol-phase fraction to contain approximately 81% protein, 12.5% nitrogen, 1.6% glucosamine, and less than 1% KDO. Analysis provided by Dr. J. C. Stewart indicated the *E. coli* 12408 endotoxin contained 7.2% protein, 13.7% heptose, and 6% glucosamine, whereas the *E. coli* 12408 LPS contained no protein, 16.5% heptose, and 8.4% glucosamine.

Lymphocyte Cultures. The methods of culture and measurement of DNA synthesis by [^{3}H]thymidine uptake have been previously described (5, 17). Spleen cells were used at a concentration of 1×10^6 cells/ml, whereas lymph node and thymus cells were used at 2×10^6 cells/ml. Cortisone-resistant thymocytes were obtained by the method of Anderson and Blomgren (18). All cultures were done in triplicate and replicate values were within 10% of the mean. For polyclonal activation spleen cell cultures were prepared at cell concentrations of 10×10^6 ml. 1 ml of the suspension was incubated in 35 mm plastic Petri dishes at 37°C in an atmosphere of 83% N_2, 10% CO_2, and 7% O_2 with or without stimulants for a period of 48 h as previously described (19). Antibody-forming cells were enumerated by a modified hemolytic plaque assay (19) using trinitrophenyl (TNP)-conjugated sheep erythrocytes (SRBC). TNP-SRBC were prepared by the method of Rittenberg and Pratt (20).

Results

A comparison of the mitogenic activity of four lipopolysaccharide preparations made by phenol extraction on cultured spleen cells is shown in Table I. In all cases little or no response was obtained with C3H/HeJ cells at concentrations of 1–100 μg. On the other hand CBA/J cultures were highly stimulated within the same concentration range, although the optimal concentration varied between the preparations tested. However, C3H/HeJ spleen cell cultures can be stimulated into DNA synthesis, when endotoxin preparation are made by TCA extraction. As shown in Table I, four different TCA preparations stimulated [^{3}H]thymidine uptake in these cultures in the dose range of 1–100 μg with maximum responses obtained at 10 or 100 μg, depending on the preparation tested. It should be noted that CBA/J cell DNA synthesis was about two to three times higher than that obtained with C3H/HeJ cells, although cells from both mouse strains exhibited the same optimal mitogen concentration for these TCA endotoxins.

Phenol-water extraction of TCA endotoxin has been shown to separate LPS in the aqueous phase, whereas the protein of the endotoxin is solubilized in the

TABLE I
Mitogenic Activity of Endotoxin on Mouse Spleen Cells

Endotoxin	[^{3}H]thymidine uptake mean net cpm* (SI)‡					
	CBA/J			C3H/HeJ		
	100 μg/ml	10 μg/ml	1 μg/ml	100 μg/ml	10 μg/ml	1 μg/ml
Phenol						
E. coli 12408	−2,170 (0.3)	56,769 (18.1)	37,496 (12.7)	−1,422 (0.4)	417 (1.2)	−285 (0.9)
S. typhosa 0-901 (L3)§	47,509 (15.8)	40,985 (13.8)	41,278 (13.9)	736 (1.3)	308 (1.1)	−366 (0.9)
E. coli 0127:B8	2,298 (1.7)	50,440 (16.2)	48,732 (16.2)	−2,288 (0.1)	534 (1.2)	106 (1.0)
S. typhosa 0-901 (L21)§	64,678 (21.2)	54,658 (18.1)	46,033 (15.4)	7,241 (3.9)	1,568 (1.6)	−379 (0.8)
TCA						
E. coli 12408	25,508 (9.0)	115,624 (37.1)	59,552 (19.6)	14,447 (6.7)	43,142 (18.1)	10,983 (5.3)
S. typhosa 0-901 (Difco)	38,955 (13.2)	153,798 (49.0)	73,215 (23.8)	20,889 (9.3)	53,787 (22.3)	21,524 (8.5)
E. coli 0127:B8	122,573 (39.2)	104,731 (33.7)	39,182 (13.2)	54,682 (22.6)	34,881 (14.8)	7,979 (4.2)
S. typhosa 0.901 (L21)	141,951 (45.3)	98,530 (31.7)	65,341 (21.4)	64,100 (26.4)	39,281 (16.5)	24,916 (10.9)

* Stimulated counts per minute minus control counts per minute.
‡ Stimulation index equals total stimulated cpm/control cpm.
§ L3, L21 represents different preparations.

TABLE II
Mitogenic Activity of Endotoxin Fractions on Mouse Spleen Cells*

Fraction	[^{3}H]thymidine uptake mean net cpm (SI)‡		
	CBA/J	C_3H/HeJ	"Nude"
μg/ml			
Aqueous phase, 100	106,317 (22.0)	5,476 (2.6)	40,570 (14.0)
Aqueous phase, 50	107,678 (22.3)	4,268 (2.3)	33,252 (11.6)
Aqueous phase, 10	99,534 (20.7)	2,704 (1.8)	25,804 (9.3)
Phenol phase, 100	192,671 (39.2)	102,389 (31.0)	105,219 (34.7)
Phenol phase, 50	194,936 (39.6)	108,762 (32.9)	104,056 (34.3)
Phenol phase, 10	190,634 (38.8)	90,060 (27.4)	83,220 (27.6)

* Endotoxin extracted from *E. coli* 0127:B8 by the TCA method.
‡ See Table I.

phenol phase (2). As shown in Table II the aqueous-phase LPS is mitogenic for CBA/J and "nude" spleen cells but only to a trivial extent for C3H/HeJ cells. On the other hand, the phenol-phase material which we have designated endotoxin protein (EP) is a potent mitogen for spleen cells of all three strains. EP is also mitogenic for neonatal spleen cells and adult lymph node cells of C3H/HeJ mice (Table III). However, EP is inactive for C3H/HeJ thymocytes or cortisone-resistant thymocytes (Table III).

These data suggest that EP is stimulating the proliferation of B lymphocytes. Since all B-cell mitogens have been shown to activate B lymphocytes to nonspecific antibody production, we tested EP as a polyclonal activator. As shown in Fig. 1, in vitro cultures of C3H/HeJ spleen cells were stimulated by EP to produce significant plaque-forming cells against TNP-SRBC and uncoated SRBC, whereas LPS was relatively ineffective.

Discussion

Our results serve to re-emphasize the specificity of the defect of C3H/HeJ B lymphocytes for LPS or lipid A (17). Furthermore, the data demonstrate that cell wall protein ordinarily complexed to the LPS of gram-negative bacteria is

TABLE III

Mitogenic Activity of Endotoxin Protein on Various C_3H/HeJ Cell Types

Mitogen		Neonatal spleen cells net cpm (SI)*	Pooled lymph node cells net cpm (SI)	Thymocytes net cpm (SI)	Cortisone-resistant thymocytes net cpm (SI)
	μg/ml				
EP	500	25,438 (24.0)	–	106 (1.2)	−226 (0.55)
	100	29,657 (27.8)	22,020 (96.3)	81 (1.1)	508 (1.84)
	10	32,184 (30.1)	16,934 (74.3)	−177 (0.67)	203 (1.33)
	1	26,019 (24.5)	4,410 (20.1)	–	–
PPD	100	25,327 (23.9)	–	–	–
Con A	1.25	–	–	3,890 (8.0)	–
	0.625	–	–	21,089 (39.2)	51,213 (86.5)
	0.3125	–	–	9,390 (18.0)	–
LPS	100	–	–	–	−232 (0.6)
	10	–	–	–	−24 (0.96)

* [^{3}H]Thymidine uptake, see Table I.

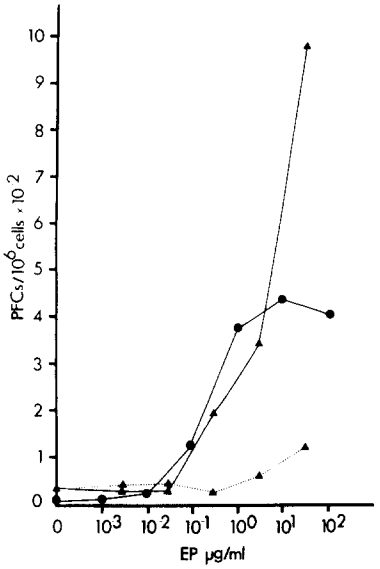

FIG. 1. Plaque-forming cells (PFC) against TNP-SRBC in C3H/HeJ cell cultures stimulated with EP (▲–▲) or LPS (▲---▲), and PFC against SRBC in C3H/HeJ cell cultures stimulated with EP (●–●).

more than an immunogen which may contribute to the biological properties of endotoxins by means of hypersensitivity reactions (21). A reasonable interpretation of the data is that EP is inherently a mitogen and polyclonal activator of B cells. That a residual of lipid A may be present in EP can be inferred from its solubility characteristics and the small amount (1.6%) of glucosamine detected which is a constituent of lipid A. The glucosamine content is consistent with that found by Wober and Alaupović who reported 1.3% glucosamine in the "simple protein" they isolated from an *E. coli* TCA endotoxin by aqueous phenol (2). Furthermore, from proteolytic enzyme treatment and acid hydrolysis data,

these workers proposed that in intact endotoxin lipid A is covalently linked to the protein moiety and that there exists a phenol-sensitive linkage within the molecule of lipid A resulting in protein preparations which contain a small segment of lipid A. While full characterization of EP is yet to be done before firm conclusions may be drawn, at the moment it would appear that EP and the protein of Wober and Alaupović are probably one and the same. Other workers have also isolated proteins or lipoproteins from various gram-negative organisms (22–24), however, the activity of these materials on lymphocytes has not been tested. More recently Melchers et al. (25) found that murein-associated lipoprotein isolated by Braun and Rehn (26) to be a mitogen for C3H/HeJ lymphocytes. The lipid component of this material is not lipid A, suggesting that the protein is not identical to EP. However, further studies are needed to clarify this point. In addition, as reported elsewhere in this issue, Morrison and coworkers have also isolated a protein bound to lipid A from ethanol-extracted LPS by phenol treatment (27). Their material is mitogenic for C3H/HeJ lymphocytes as well and is most likely similar to or identical to EP.

In any event, it is apparent now that there are a number of molecular components of the cell walls of gram-negative bacteria including lipid A, EP, and the lipoprotein linked to the murein layer in the wall which are B-cell mitogens and polyclonal activators. Use of the C3H/HeJ genetic model and structural analysis of these materials may contribute to a greater understanding of the mechanism of B-cell activation.

Summary

A cell wall protein that is ordinarily complexed to the lipopolysaccharide endotoxin in gram-negative bacteria has been separated by the use of aqueous phenol. The protein is active as a B-cell mitogen and polyclonal activator of murine lymphocytes including the C3H/HeJ strain which is a nonresponder to lipopolysaccharide or lipid A.

The authors thank John Butke for technical assistance and Maria Ferrera for her assistance in the preparation of the manuscript.

Received for publication 9 June 1976.

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