Brief Definitive Report

ISOLATION OF A LIPID A BOUND POLYPEPTIDE RESPONSIBLE FOR "LPS-INITIATED" MITOGENESIS OF C3H/HeJ SPLEEN CELLS*

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The experiments by Sultzer and Nilsson (1), and later by Watson and Riblet (2), established that spleen cells from the C3H/HeJ strain of mouse were refractory to the mitogenic effects of bacterial lipopolysaccharides (LPS). More recently, however, experiments from our laboratory (3) demonstrated that spleen cells from C3H/HeJ mice were in fact responsive to some preparations of LPS but not to others, and that the method of extraction played a critical role in determining activity. In particular, preparations of LPS prepared by extraction with aqueous butanol had potent mitogenic activity. Our data showed that the mitogenic activity of such positive preparations of LPS coisolated with the LPS during gel filtration chromatography and subsequent equilibrium banding on CsCl. In addition, lipid A isolated from positive preparations of LPS was also capable of stimulating C3H/HeJ spleen cells. Taken together, these experiments provided rather convincing data that it was the LPS (in particular the lipid A) itself, or some contaminant very tightly bound to the lipid A, which was responsible for its biological activity.

We further demonstrated that treatment of positive preparations of LPS with hot phenol rendered such preparations nonmitogenic for C3H/HeJ spleens, yet activity for other strains was only moderately decreased. These experiments would suggest either that the phenol treatment chemically alters the lipid A region of the LPS molecule or that such treatment removes the putative tightly bound contaminant responsible for C3H/HeJ mitogenesis.

In the experiments reported here, we have explored in greater detail the role of lipid A in the stimulation of C3H/HeJ spleen cells. For these experiments we have utilized our earlier observations that the antibiotic polymyxin B forms a highly stable molecular complex with the lipid A region of LPS (4), and that such polymyxin B-LPS complexes are unable to mitogenically stimulate B lymphocytes (5). In addition, we have attempted to distinguish between the two potential modes of action of phenol on LPS, namely, the chemical alteration of

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the lipid A or the removal of a tightly bound contaminant by phenol treatment. The results of the experiments we report here support the interpretation that mitogenic activity of positive preparations of LPS is associated with a low mol wt phenol soluble polypeptide of approximately 10,000 mol wt. After partial purification, this polypeptide initiates a significant mitogenic response at concentrations as low as 10 μg/ml. We conclude that the C3H/HeJ strain of mouse is a true nonresponder to the stimulatory effects of the lipid A region of LPS.

Materials and Methods

The LPS from *Escherichia coli* 0111:B4 was extracted either with butanol (B-LPS) (6) or with phenol (7) purified and fractionated into high lipid A (LPS-II) and low lipid A (LPS-I) (percent by weight) as previously described (6). All of the experiments reported here have utilized the LPS II fraction. The conditions used for inhibition of mitogenesis by polymyxin B were as described by Jacobs and Morrison (5). CBA/WEHI mice were bred at the Salk Institute, La Jolla, Calif.; C3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine; and C3H/St mice were obtained from Strong Laboratories, Sorrento Valley, Calif. Spleen cells were cultured at a concentration of 2.5×10^6 viable cells/ml in 200 μl of RPMI 1640 supplemented with glutamine, 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 95% air, 5% CO_2. After 1 or 2 days of culture, 0.5 μCi of [^{3}H]thymidine (10.6 Ci/mM, New England Nuclear, Boston, Mass.) or 0.05 μCi ^{125}I-UdR (sp act >200 Ci/mM, New England Nuclear, Boston, Mass.) were added and the cells incubated an additional 24 h. Cells were then harvested using a multiple automated sample harvester (Microbiological Associates, Bethesda, Md.) and assayed for incorporated radioactivity. All data represent the average of duplicate or triplicate samples. LPS concentrations were estimated by the assay for dideoxyhexoses using thiobarbituric acid (8), and protein was estimated by the Folin phenol procedure (9).

Results

Our initial experiments attempted to define further the participation of lipid A in the mitogenic response of both responder (CBA/WEHI) and nonresponder (C3H/HeJ) spleen cells to both positive (B-LPS) and negative (P-LPS) preparations of LPS. To do this, spleen cells were cultured in the presence of both preparations of LPS in the presence and absence of the lipid A inhibitor, polymyxin B. The T-cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) and another B-cell mitogen purified protein derivative (PPD) were used to control for nonspecific effects of polymyxin B. The results of one such experiment are shown in Table I and demonstrate several salient points. First, as we have shown previously (3), B-LPS has the capacity to stimulate spleen cells from both strains of mice, but P-LPS can stimulate only the responder cells. Second, the P-LPS response, as would be predicted (5), is completely abrogated in the presence of polymyxin B. Third, polymyxin B has very little effect on other B- or T-cell mitogens, including Con A, PHA, and PPD, insuring that the polymyxin B effect is not nonspecific. Finally, however, and perhaps somewhat surprisingly, polymyxin B has, in addition, virtually no effect on the mitogenic response of spleen cells to B-LPS. Extraction of B-LPS with phenol (data not shown) gave results identical to those obtained with P-LPS. These data, therefore, indicate either that it is not the lipid A in B-LPS which is responsible for the mitogenic activity or that polymyxin B, for some reason, does not interact with the lipid A of B-LPS.

To examine this latter possibility in greater detail, we have utilized our earlier observation that polymyxin B-LPS complexes have significantly higher

TABLE I

*Effect of Polymyxin B on the Incorporation of ^{125}I-UdR into Murine Spleen Cells Stimulated by Various Mitogens**

		CBA/WEHI		C3H/HeJ	
		−PB	+PB‡	−PB	+PB
		cpm × 10^{-2}			
Con A	(0.25 μg)	199.9	180.6	142.9	149.7
PHA	(0.12 μg)	170.0	145.1	153.0	126.8
P-LPS	(1.0 μg)	53.7	1.7	3.5	1.6
B-LPS	(1.0 μg)	169.8	138.8	20.9	23.7
PPD	(20 μg)	27.1	30.8	8.4	8.5
Control		1.2	1.7	1.3	2.3

* 5.0×10^5 spleen cells in 200 μl of media were cultured for 48 h in the presence of the indicated amounts of the various mitogens, after which 0.05 μCi of ^{125}I-UdR was added and the cells incubated an additional 24 h.

‡ 1.0 μg of polymyxin B (PB) was added to the cultures.

molecular weights and may be distinguished from untreated LPS by gel filtration chromatography on Sepharose 4B (4). An equal weight of polymyxin B was incubated with either B-LPS or P-LPS for 30 min at 37°C, after which the LPS was chromatographed. Control LPS was incubated in the absence of polymyxin B. As shown in Fig. 1, addition of polymyxin B to P-LPS caused the characteristic shift to higher apparent molecular weight (4). In contrast, addition of polymyxin B to B-LPS had no detectable effect on this LPS, whose chromatographic profile was identical to control B-LPS. (Again, treatment of B-LPS with phenol gave results identical to those obtained with P-LPS.) These data, in addition to the mitogen data described above, suggest that the lipid A of B-LPS is blocked in its ability to interact with polymyxin B.

A consideration of several pertinent observations may provide further information on the nature of the interaction of polymyxin B to interact with LPS prepared by different extraction procedures. First, we have shown that polymyxin B binds to the lipid A region of phenol-extracted LPS and causes a decrease in the isopycnic density of such complexes (4). Second, polymyxin B does not bind to butanol-extracted LPS (Fig. 1). Third, re-extraction of butanol-extracted LPS with phenol causes an increase in isopycnic density (6) approximately equal to the shift in density seen upon addition of polymyxin B to phenol LPS. These observations are consistent with an interpretation of a distinct biologically active moiety associated with the lipid A of B-LPS. This biologically active moiety may bind to the same region of the lipid A as polymyxin B would, be responsible for C3H/HeJ mitogenesis, and be removed by treatment with hot aqueous phenol.

As the mitogenic activity for C3H/HeJ spleen cells is abrogated by treatment with phenol, the putative polypeptide bound to the lipid A of B-LPS is either chemically destroyed or is insoluble in aqueous solvents. In an attempt to distinguish between these two possibilities, we have extracted purified B-LPS II with phenol as described by Westphal and Jann (7). However, in these experiments we have focused upon the phenol phase. Approximately 30 mg of B-LPS II in 6 ml of H_2O were extracted with an equal vol of 90% phenol for 15 min at 68 ± 1°C. After cooling and centrifugation, the aqueous phase was removed, and the resultant phenol phase and interface re-extracted two times as described above.

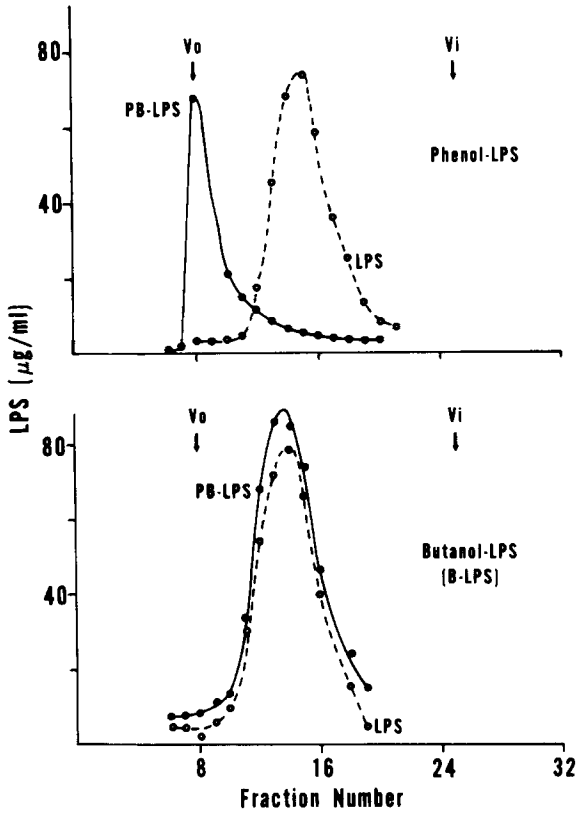

FIG. 1. Effect of polymyxin B on the chromatographic properties of preparations of LPS. Approximately 500 μg of LPS prepared by either butanol or phenol extraction were incubated with or without an equal weight of polymyxin B in a final vol of 500 μl of saline as described in the text. The LPS was then chromatographed on a 1.5 × 32 cm column of Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.) equilibrated with 0.1 M Tris, pH 8.0. Portions of volume fractions were assayed for the presence of LPS by the thiobarbituric acid assay for dideoxyhexoses (8).

The phenol phase was then carefully concentrated by flash evaporation at 30°C under reduced pressure and finally lyophylized. A yield of approximately 5 mg of material resulted. The material contained no detectable LPS, as measured by the dideoxyhexose assay for colitose (8), and, as estimated by the Folin phenol procedure (9), the extracted residue was greater than 95% protein.

When examined for its ability to stimulate spleen cells from both responder and nonresponder mice, this material was shown to have potent mitogenic properties with as little as 0.2 μg initiating a significant increase in [^{3}H]thymidine incorporation over background in both strains. Representative data from one such experiment is shown in Table II. It is interesting to note that the phenol extract appears to have a similar specific activity as does the butanol LPS. Whether this represents some deleterious effect of the phenol on the subsequent biological activity of the extract is not, at present, clear. We have, in addition, demonstrated that, as predicted, the phenol-soluble extract is not inhibited by polymyxin B.

TABLE II
*Mitogenic Response of Spleen Cells to Phenol-Soluble Extract of Butanol-Purified LPS**

		C3H/St	C3H/HeJ
		cpm × 10^{-3}	
P-LPS	20 μg	16.1	1.5
	2 μg	10.4	1.2
	0.2 μg	8.8	1.3
B-LPS	20 μg	43.9	22.1
	2 μg	20.7	11.5
	0.2 μg	12.7	3.0
Phenol-soluble extract	20 μg	38.5	23.6
	2 μg	19.1	12.4
	0.2 μg	11.0	3.6
Control		4.1	1.1

* Spleen cells were cultured as described in the legend to Table I except that cells were pulsed with 0.50 μCi of [^{3}H]thymidine after 24 h of incubation and harvested after 24 h to maximize the C3H/HeJ response (reference 3).

TABLE III
*Mitogenic Activity of Sephadex G-75 Purified Low Molecular Weight Polypeptide from B-LPS**

		C3H/St	C3H/HeJ
		cpm × 10^{-2}	
P-LPS	2 μg	28.6	7.4
	0.2 μg	14.7	5.0
B-LPS	2 μg	69.8	23.5
	0.2 μg	27.8	5.5
Polypeptide	2 μg	17.4	12.7
	0.2 μg	9.4	5.6
Control		4.5	2.1

* Spleen cells were treated as described in the legend to Table II.

The extract was then solubilized in 10% formic acid and subjected to chromatography on Sephadex G-75 superfine. An analysis of the protein elution profile demonstrated that most of the protein eluted as a single peak slightly preceding the column inclusion volume at a mol wt of about 10,000. The column fractions containing this major peak were pooled, lyophylized, resuspended in a small volume of saline, and assayed for capacity to stimulate spleen cells. As shown in Table III, this low molecular weight polypeptide itself initiated a significant response above background in both responder and nonresponder spleen cells.

Discussion

Our data thus provide evidence that the mitogenic activity of positive preparations of LPS for spleen cells from the C3H/HeJ mouse is associated with a low molecular weight polypeptide which on the basis of our earlier observations (3) appears to be associated with the lipid A region of the LPS. Proteins associated with LPS have earlier been described by Leive et al. (10) after treatment of *E.*

coli 0111:B4 with the divalent chelator ethylenediaminetetraacetic acid. More recently, Wu and Heath (11) have demonstrated a low molecular weight protein associated with the LPS of *E. coli* 0111:B4 after extraction of the bacteria with sodium dodecyl sulfate. Their report of an approximate mol wt of about 12,000 is in agreement with our data reported here. Finally, Sultzer and Goodman (12) have elsewhere in this issue described a protein associated with LPS after extraction by mild procedures, such as trichloroacetic acid, which is both mitogenic and a polyclonal activator of C3H/HeJ spleen cells. It is probable that all of these proteins represent the same LPS-bound polypeptide.

Braun and co-workers (13, 14) have isolated and characterized a low molecular weight lipoprotein which is associated with the murein layer of the cell wall of gram negative bacteria. More recently, Melchers et al. (15) have demonstrated that this lipoprotein is a potent mitogen for lymphocytes from C3H/HeJ as well as other strains. The relationship between this lipoprotein and the one we and Wu and Heath (11) and Sultzer and Goodman (12) describe here at present is unclear. The fact that their protein is associated with the murein layer, whereas ours appears tightly bound to LPS, the lipid A region of the LPS, might suggest that they are not identical.

What emerges from these and other studies is the rather exciting observation that many of the potent B-cell mitogens now isolated and partially characterized have as their origin the bacterial cell wall, including PPD (1), peptidoglycans (16), lipid A (17), the murein-binding lipoprotein (15), and the lipid A-binding polypeptide described here. A consideration of the physical-chemical properties of these various mitogens should provide further insight into the mechanisms of the initiation of B-cell mitogenesis. In any case, it seems relatively certain that the initial description of Sultzer (1) of the C3H/HeJ mouse as being a true nonresponder to the mitogenic effects of LPS (lipid A) was indeed an accurate one.

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