MACROPHAGE STIMULATION BY BACTERIAL LIPOPOLYSACCHARIDES

II. Evidence for Differentiation Signals Delivered by Lipid A and by a Protein Rich Fraction of Lipopolysaccharides*

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Bacterial lipopolysaccharides (LPS)[1] stimulate mouse peritoneal macrophages to lyse tumor target cells in vitro (1–3). The mechanisms involved in the differentiation of macrophages into killer cells can now be explored by using the model described in the accompanying paper (3). In that study, purified LPS extracted from a rough mutant of *Salmonella minnesota* (R595) added to confluent monolayers of elicited macrophages stimulated the macrophages to lyse tumor target cells. The purpose of this paper is to show that macrophage stimulation is a generalized property of LPS and to probe the biochemical nature of the differentiation signal(s) delivered by LPS extracted from the cell walls of gram-negative bacteria.

The biological activities of LPS have been localized to either the lipid A moiety or to the polysaccharide portions (Fig. 1) (4). The lipid A moiety activates the classical complement cascade (5–7) and significantly enhances the kallikrein-initiated cleavage of Hageman factor thereby initiating the intrinsic clotting sequence (8). Lipid A also stimulates mitogenesis in B lymphocytes (9, 10). The polysaccharide component is responsible for activation of the properdin pathway of complement (7, 11) and bears the molecule's major antigenic determinants (4). Localization of the site of the stimulation signal on LPS may, therefore, provide important insights into the mechanism of macrophage stimulation.

LPS is well characterized biochemically and can be prepared in highly purified form (4). Moreover, considerable structural variation can be obtained

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[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; LAP, lipid A associated protein; LPS, lipopolysaccharide; MEM, minimal essential medium; NPP, native protoplasmic polysaccharide; PEC, peritoneal exudate cell; TCA, trichloroacetic acid.

by the use of mutant strains and by chemical modification of the LPS molecule (4). By using these preparations, we show in this paper that macrophage stimulation is a generalized property of LPS molecules and that the site of the stimulation signal on LPS resides in the lipid A moiety. In the course of these investigations, it became evident that variations in LPS preparative procedures resulted in the isolation of a protein rich component noncovalently bound to LPS, which we have called LAP (lipid A associated protein) although its precise biochemical nature is, as yet, undetermined. We report here that LAP itself delivers a stimulatory signal to differentiate macrophages into killer cells which is distinct from that produced by lipid A.

Materials and Methods

LPS Preparations. Eight sources of LPS were used in these experiments (Table I). *Escherichia coli* serotypes 0111:B4 (ATCC 12015), 055:B5 (ATC 12014), 075 (a gift of Dr. G. Schmidt, Max Planck Institut für Immunologie, Frieburg, West Germany), and lyophilized bacteria of the rough mutant strain *E. coli* B/r (donated by Dr. Wolfgang Bessler of Institut Microbiologie II, Universitat Tubingen, Tubingen, W. Germany) were extracted by one of two procedures; the phenol-water method of Westphal and Jann (12) as modified by Lieve et al. (13) or the butanol-water method of Morrison and Lieve (14). Preparations of LPS 0111:B4 chromatographed on Sepharose 4B yielded two distinct peaks; fraction 1 consisting of the whole LPS molecule and including approximately 10 "O" antigen polysaccharide subunits and fraction II differing from fraction I only by a shortened chain of approximately 2 "O" antigen subunits (Fig. 1). LPS from *E. coli* K235 prepared by a phenol water method (15) was a gift from Dr. F. McIntyre of the University of Colorado Medical Center, Denver, Colo. LPS from *E. coli* 0113 and its native protoplasmic polysaccharide (NPP) were generously provided by Dr. J. Rudbach of the University of Montana. Their preparation has been previously reported (16, 17). LPS from R595 *S. minnesota* (provided by Dr. J. Ryan, National Institutes of Health, Bethesda, Md.), was made by the phenol-chloroform-petroleum ether method of Galanos et al. (18). *Serratia marcescens* LPS lot 612017 (obtained from Difco Laboratories, Detroit, Mich.), was used after suspending the lyophilized LPS in sterile, isotonic saline. Lipid A was produced by acid hydrolysis of LPS from a phenol extract of *Salmonella typhimurium* R-60[2] and from *E. coli* 0111:B4 by using 1% glacial acetic acid at 100°C for 2 h (19). LAP was prepared by phenol extraction of *E. coli* 0111:B4 and 055:B5 LPS, previously extracted by butanol, and subsequently purified. LAP, which partitions into the phenol phase was lyophilized, resuspended in saline, and dialyzed. Base hydrolysis of LPS from *E. coli* 0111:B4 and of LAP was performed with 0.01 N NaOH in 90% ethanol at 37°C for 15 and 30 min (20). After neutralization with HCl, the preparations were dialyzed extensively against normal saline by using small pore dialysis tubing.

Polymyxin B Treatment. Polymyxin B sulphate obtained from Sigma Chemical Co., St. Louis, Mo. was incubated with the different preparations of LPS at the same concentration for 30 min at 37°C before addition to macrophage monolayers. Since LPS binds polymyxin B in a stoichiometric manner, these conditions provided mild polymyxin B excess (21). Control preparations contained polymyxin B alone.

Media and Cell Lines. Eagle's minimal essential medium (MEM) was supplemented by glutamine (2 mM), sodium pyruvate (1 mM), Hepes buffer (20 mM), sodium bicarbonate (0.14%), heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (Flow Laboratories, Inc., Rockville, Md.), penicillin (100,000 U/liter) and streptomycin (100,000 μg/liter) (Grand Island Biological Co., Grand Island, N.Y.) for use in tissue culture.

The DBA/2 mastocytoma cell line P815 was maintained in vitro by repeated passages in Hepes MEM supplemented by 10% FBS.

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[2] The lipid A from *Salmonella typhimurium* R-60 was prepared while D. C. Morrison was a guest in the laboratory of Professor Otto Lüderitz, Max Planck Institute für Immunobiologie, Freiburg, West Germany.

Macrophage Monolayers. C57 BL/6J female mice 8- to 10-wk of age were obtained from The Jackson Laboratory, Bar Harbor, Maine for use as the source of peritoneal macrophages which were elicited by injecting thioglycollate intraperitoneally as previously described (3). Macrophage monolayers consisting of approximately 1×10^6 cells were made in 16-mm diameter wells of Linbro plates (Flow Laboratories) as described in the accompanying paper (3). We have previously demonstrated that after extensive washing of peritoneal exudate cell (PEC) monolayers to remove nonadherent cells, such adherent cell monolayers comprise greater than 95% macrophages (3).

Macrophage Stimulation by LPS and LAP Preparations. Macrophage monolayers were incubated for 24 h in Hepes MEM containing 20% heated FBS and appropriate concentrations of LAP or LPS preparations before the addition of ^{51}Cr-labeled target cells.

Cytolytic Assay. The mastocytoma cell line P815 was labeled with Na ^{51}Cr (sp act 0.16 mCi/mg, New England Nuclear Corp., Boston, Mass.) as described (3). For control experiments, P815 cells were cultured in H-MEM containing 10% heat-inactivated FBS and 25 μg/ml of LPS-0113 or lipid A. After 24 h the cells were washed by centrifugation at 400 *g* and then resuspended in H-MEM for labeling by using Na ^{51}Cr. 5×10^4-labeled P815 cells were added to appropriately treated macrophage monolayers in Linbro plates and incubated at 37°C in 5% CO_2 in humidified air for periods of 8–24 h. The culture supernates were then harvested and centrifuged at 400 *g* for 10 min to remove cells and debris. Tubes containing aliquots of supernates were counted in a γ-scintillation counter (Searle Diagnostics Inc., Subsidiary of G. D. Searle & Co., Des Plaines, Ill). Results were calculated as mean ± 1 SD of four replicates and expressed as a percentage of radioactivity specifically released using the following calculation:

$$\% \text{ specific release} = \frac{\text{experimental release} - \text{macrophage control release}}{\text{total releasable counts} - \text{macrophage control release}} \times 100$$

where macrophage control release consisted of monolayers which were not treated with LPS.

Results

Macrophage Stimulation is a General Property of LPS. As shown in the previous paper, LPS derived from the cell wall of the rough mutant strain *S. minnesota* (R595) stimulates macrophages from a PEC population to lyse tumor cells in vitro (3). It was important therefore to establish that this was a general property of LPS derived from any gram-negative bacterium and not a property peculiar to LPS-R595.

Macrophages preincubated for 24 h with LPS from eight different sources representing both rough and smooth strains and prepared by a number of extraction procedures lysed tumor target cells in vitro (Table I). Since LPS derived from a wide variety of bacterial strains stimulate peritoneal macrophages to become killer cells, this property appears to be shared by all bacterial LPS.

Control studies similar to those shown for LPS-R595 in the preceding paper (3), demonstrated that LPS, lipid A, or LAP preparations did not affect the viability of ^{51}Cr-labeled P815 target cells over the 24-h incubation period of the cytotoxicity assay. Additional control experiments included the preincubation of P815 target cells with LPS-0113 or with lipid A for 24 h before ^{51}Cr labeling. When these treated target cells were added to macrophage monolayers, no target cell lysis occurred providing further evidence that LPS and lipid A stimulation of macrophages is an active process involving a direct effect of LPS on macrophages (3).

Role of Lipid A in Macrophage Stimulation. The lipid A component of LPS appears to be structurally identical or similar in all strains of Enterobacteria-

TABLE I
Ability of different LPS Preparations to Stimulate MΦ Cytotylic Effect

	Method of extraction	Specific release at 24 h*
		%
E. coli 0111:B4 FI	Phenol-water	59
E. coli 0111:B4 FII	Phenol-water	58
E. coli 0111:B4 FI	Butanol-water	59
E. coli 0111:B4 FII	Butanol-water	64
E. coli K235	Phenol-water	44
E. coli 055:B5	Phenol-water	42
E. coli 0113	Phenol-water	67
E. coli 075	Phenol-water	43
E. coli B/r	Phenol-water	87
S. minnesota R595	Phenol-chloroform-petroleum ether	75
S. marcescens	Boivin	44
Lipid A from *S. typhimurium* R60	Acid hydrolysis of phenol preparations	69
Lipid A from *E. coli* 0111:B4		75

* Representative results from at least three separate experiments.

ceae examined (22, 23) while the core polysaccharide defines the genus and the O antigens determine strain specificity (4). Since macrophages were stimulated by all the eight different preparations of LPS which share only lipid A as a common component (Table I), these results suggest that lipid A bears the stimulatory signal. Therefore, lipid A purified from LPS 0111:B4 and R:60 was tested for its capacity to stimulate macrophages. As shown in Table I, purified lipid A alone induced the macrophages to become cytolytic confirming that this LPS moiety delivers a stimulation signal to macrophages.

Evidence that the Polysaccharide Moiety Containing the O Antigens is not Involved in Macrophage Stimulation. To determine whether the lipid A moiety was the only part of the LPS molecule that stimulated macrophages or whether the polysaccharide components were also involved, we used three approaches. First, we compared the stimulatory capacity of LPS preparations containing differing amounts of carbohydrate (Fig. 1). Although there were slight quantitative differences in the specific release of radioactivity induced by these LPS preparations when compared on a weight basis, each preparation stimulated significant cytolysis with similar kinetics (Fig. 2). The second approach involved incubating the different LPS preparations with polymyxin B, a cationic polypeptide which forms a stable molecular complex with lipid A (21). The complexing of polymyxin B to lipid A abolishes many of the biological activities of LPS including the mitogenic stimulation of B lymphocytes (24) and its capacity to initiate the classical complement pathway (25). Incubation of phenol extracted LPS from *E. coli* 0111:B4 fractions I and II, *S. minnesota* R595, and lipid A with polymyxin B resulted in almost complete disappearance of stimulatory activity, demonstrating that for these preparations of LPS, the signal to stimulate the macrophage is localized entirely to the lipid A moiety. Third, we investigated the role of the O antigen polysaccharide chain in the stimulation or modulation of macrophages by LPS. For this purpose, the *E. coli*

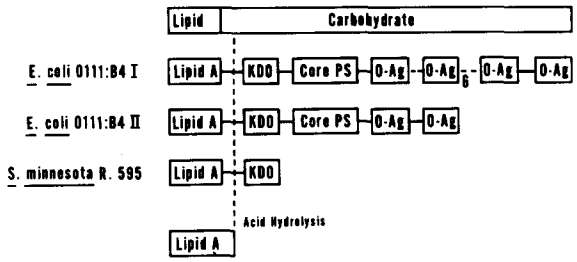

FIG. 1. Diagram illustrating the three regions of contrasting chemical and biological properties in protein-free bacterial lipopolysaccharides. The O polysaccharide consists of repeating subunits bearing the antigenic specificity for each strain, the core polysaccharide is common within each genus of bacteria and is linked by a trisaccharide of 2-keto-3-deoxy-octulosonate to the lipid A moiety which is structurally identical, or very similar, in all Enterobacteriaciae.

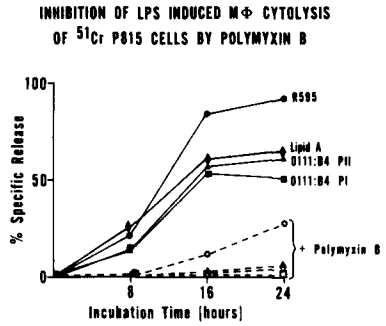

FIG. 2. Kinetics of cytolysis of ^{51}Cr-labeled P815 mastocytoma cells by macrophages preincubated with 25 μg/ml of LPS R595 from *S. minnesota* (●), lipid A from *S. typhosa* R60 (■), and LPS 0111:B4 fractions I (▲) and II (■) from *E. coli* represented by continuous lines. Inhibition of the cytolytic effect induced by these LPS preparations by preincubation with polymyxin B at 25 μg/ml is represented by the open symbols joined by interrupted lines.

strain 0113 which produces an excess of polysaccharide which is antigenically identical to the O antigen polysaccharide of the complete LPS-0113 was used (26). The effects of several concentrations of complete LPS 0113 and its polysaccharide NPP on macrophage stimulation are shown in Fig. 3. Although as little as 40 ng/ml of complete LPS was highly active, NPP produced no discernible lysis at concentrations up to 25 μg/ml, demonstrating that the polysaccharide portion of 0113 does not induce a cytolytic capacity in macrophages.

To determine whether the polysaccharide portion of LPS could modulate the lipid A signal of complete LPS, macrophage monolayers were preincubated with NPP for 1 h at concentrations from 0.04 to 25 μg/ml before adding 5 μg/ml of complete LPS 0113. After 24 h, labeled P815 cells were added as targets. No blocking or modulation of cytolysis was observed (Fig. 4).

Evidence for a Macrophage Signal Distinct from Lipid A in some LPS Preparations. In the course of our studies, it became apparent that polymyxin B did not block the activity of all the LPS extracts examined. When LPS was extracted from *E. coli* 0111:B4 by a butanol procedure (14), both fractions I and

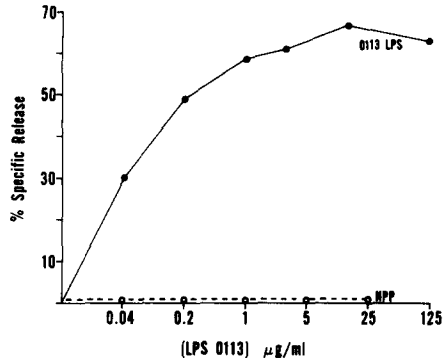

FIG. 3. Dose-response profile for complete LPS from *E. coli* 0113 (●, continuous line) and native protoplasmic polysaccharide, NPP (○, interrupted lines). Stimulation of macrophages assayed by their cytolytic effect on ^{51}Cr-labeled P815 mastocytoma cells at 24 h. All concentrations of LPS-0113 and NPP were preincubated with macrophages for 24 h before adding the target cells.

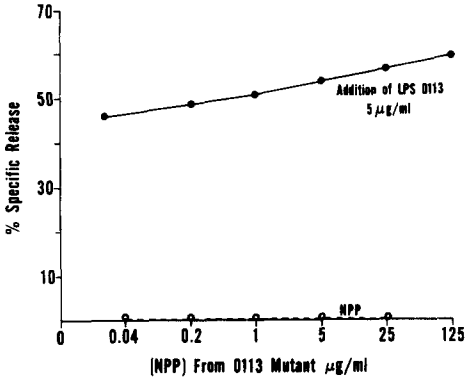

FIG. 4. Effect of adding 5 μg/ml of complete LPS 0113 to monolayers of 1×10^6 macrophages preincubated for 30 min with different concentrations of the NPP from *E. coli* 0113 (●, continuous line) assayed by macrophage cytolytic effect on ^{51}Cr-labeled P815 cells. Dose-response profile for NPP (○ interrupted line) is shown as a control.

II fully stimulated macrophages to lyse tumor cells (Fig. 5). After incubation with polymyxin B, however, only the activity of fraction I of the butanol prepared LPS was blocked but the activity of fraction II was entirely unaffected (Fig. 5), suggesting that polymyxin B does not bind to butanol LPS fraction II. Moreover, since polymyxin B binds to the lipid A region of the LPS molecule (21), these results indicate that fraction II of butanol LPS 0111:B4 stimulates macrophages by a mechanism independent of lipid A.

Recent reports from this (27) and other laboratories (28, 29) have shown that LPS extracted using butanol (14), or trichloroacetic acid (TCA) (30) methods consists of a complex of LPS and a protein rich component which is firmly bound to the lipid A moiety and variously named simple protein, (28), LAP (27), and endotoxin protein (29). We therefore examined the ability of the isolated protein rich component, hereafter referred to as LAP, to stimulate a lytic capacity in macrophages. Preparations of LAP from *E. coli* 0111:B4 and 055:B5, in concen-

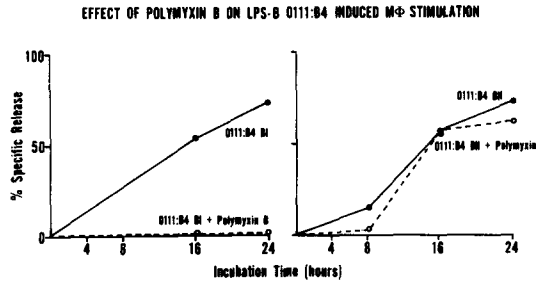

FIG. 5. Kinetics of cytolysis of ^{51}Cr-labeled P815 cells by macrophages stimulated with 25 μg/ml 0111:B4 LPS fractions I and II without (●, continuous line) and with (○, interrupted line) preincubation of LPS with polymyxin B at 25 μg/ml.

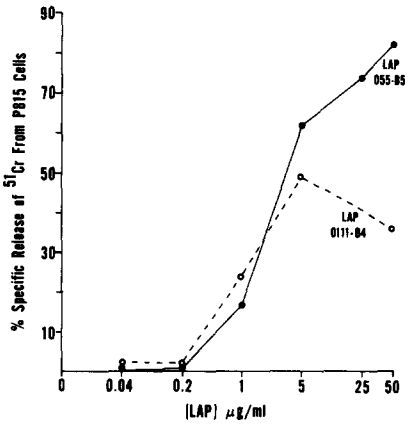

FIG. 6. Dose-response profile for stimulatory effect of lipid A associated protein LAP isolated from *E. coli* 055:B5 (●, continuous line) and 0111:B4 (○, interrupted line) on macrophage monolayers assayed by cytolysis of ^{51}Cr-labeled P815 mastocytoma cells.

TABLE II
Effect of Mild Base Hydrolysis of LPS and LAP on their Capacity to Stimulate Macrophages

MΦ stimulated by:	% Specific release from ^{51}Cr P815 cells			
	Base-hydrolyzed*		Non base-hydrolyzed	
	5 μg/ml	25 μg/ml	5 μg/ml	25 μg/ml
LPS 0111:B4	45	51	30	54
LAP	0	0	30	77

* 15 min at 37°C with 0.01 N NaOH in 90% ethanol.

trations varying from 0.04 to 50 μg/ml stimulated macrophage monolayers to lyse tumor cells in vitro (Fig. 6) indicating that LAP delivers a signal for macrophage stimulation independent from that delivered by lipid A.

To show that the LAP activity was not due to contamination of these preparations by lipid A but rather represents a functionally distinct entity, we took advantage of the exquisite sensitivity of LAP to very mild alkaline hydrolysis (31). Under such conditions, the capacity of LAP to stimulate

macrophages was completely abolished (Table II). By contrast, phenol LPS 0111:B4 activity was undiminished by exposure to identical base hydrolysis conditions (Table II). These results suggest that there is a distinct macrophage stimulation signal delivered by the LAP-LPS complex extracted from gram-negative bacterial cell walls. The evidence suggests that this signal is localized in LAP and is independent of, and separable from, that delivered by the lipid A moiety.

Discussion

These studies delineate the relationship between the structural characteristics of LPS from different sources and their ability to stimulate macrophages to lyse tumor target cells in vitro. Stimulation of macrophages appears to be a general property of LPS extracted from gram-negative bacterial cell walls. The lipid A component of LPS, which is structurally identical in nearly all LPS derived from many bacterial strains (22, 23), bears the stimulatory signal for LPS. The polysaccharide moiety containing the repeating O antigen subunits is neither stimulatory itself nor does it block the activity of complete LPS on macrophages. Extraction with butanol (14) or TCA (30) results in preparations of LPS complexed to a protein-rich component, LAP which alone acts as a distinct stimulator of macrophages.

LPS extracted from a wide variety of gram-negative organisms including *Escherichia*, *Salmonella*, and *Serratia* stimulate macrophages in vitro. This property is shared by complete LPS and by LPS from rough mutant strains in which a heptose deficiency results in a truncated LPS molecule consisting of lipid A plus the core trisaccharide, 2-keto-3-deoxyoctulosonate but lacking the long polysaccharide O antigen chain (4). Although the assay reported here measures a cytolytic effect on tumor target cells, evidence for a macrophage cytostatic effect induced by LPS from Shigella (32) and of the generation of a soluble cytolytic factor by macrophages treated with *Salmonella typhosa* LPS (2) have been reported. Thus, macrophage stimulation by LPS, apparently the consequence of direct interaction between LPS and macrophages (3), is a property common to all LPS tested suggesting that macrophages recognize at least one component that is structurally identical in nearly all LPS molecules, i.e., lipid A.

Several experimental approaches were used to confirm the localization of the LPS stimulation signal to lipid A. First, isolated lipid A alone induces a macrophage cytolytic effect. Second, our results demonstrate that when the activity of the lipid A moiety of protein free LPS is blocked by polymyxin B, no macrophage stimulation occurs indicating that only the lipid A component is involved in macrophage stimulation by LPS. The localization of this property to the lipid A moiety adds another to the list of biological actions of this component of LPS which include binding of $C1_q$ (33), initiation of the classical complement cascade (5–7), enhancement of the kallekrein-initiated cleavage of Hageman factor (factor XII) resulting in activation of the intrinsic clotting sequence (8), and delivery of a mitogenic signal to B lymphocytes (9, 10). That LPS or its lipid A moiety directly stimulates macrophages, as shown both here and in the companion paper (3), suggests that these cells may have a membrane receptor for lipid A.

Although the polysaccharide portion of LPS has potent biological properties including initiation of the properdin pathway of complement (7, 11), and the ability to act as a T lymphocyte independent immunogen (26), it does not appear to play a significant role in macrophage stimulation either as a stimulator of macrophages or as a modulator of the LPS effect. When the lipid A moiety of LPS is rendered inactive by being complexed to polymyxin B, the LPS molecule cannot stimulate macrophages in vitro. Moreover, the polysaccharide NPP, although antigenically indistinguishable from the O antigens of LPS 0113, has no stimulatory activity for macrophages nor does it block the activity of complete LPS.

Although butanol extracted LPS fractions I and II from *E. coli* 0111:B4 are fully active as stimulators of macrophages, only the activity of butanol LPS fraction I was blocked by incubation with polymyxin B. By contrast, polymyxin B neither bound to butanol LPS fraction II nor did it interfere with the ability of this LPS to stimulate macrophages indicating that this LPS fraction produces a stimulation signal independent of lipid A. When LAP was isolated by phenol extraction of butanol LPS 0111:B4 into the phenol phase, isolated LAP from this and other bacterial strains was a potent macrophage stimulator suggesting this component contained a separate and functionally distinct stimulation signal for macrophages. The LAP preparations used in these studies are heterogeneous as judged by SDS-polyacrylamide gel electrophoresis and isopycnic density ultracentrifugation in CsCl (S. J. Betz, unpublished results). The material responsible for LAP-initiated macrophage activation has not as yet been characterized. However, several pieces of evidence suggest that such activity is independent of the low levels of LPS which may contaminate such preparations. A majority of isolated *E. coli* 0111:B4 LAP bands at an average equilibrium density of 1.33 to 1.34 g/cm^3 (31) (isolated LPS exhibits a significantly higher density under identical conditions). This lower density material retains the ability to activate macrophage cytolytic capacity after isolation from CsCl gradients (data not shown). Furthermore, we have shown that LAP activity is sensitive to mild alkaline hydrolysis under conditions which do not affect the ability of LPS to stimulate macrophages. Nor is the stimulatory capacity of LAP inhibited by polymyxin B. Finally, both the 0111:B4 and 055:B5 LAP preparations used in these studies are mitogenic for C3H/HeJ lymphocytes (31). The *E. coli* 055:B5 LAP has also been demonstrated to induce the expression of Ia antigens on bone marrow cells from C3H/HeJ mice (34, 35). Preliminary studies with C3H/HeJ peritoneal macrophages suggest that these cells are also responsive to LAP but do not respond to protein-free LPS.[3] Thus, our evidence strongly suggests that two separate constituents of the cell walls of gram-negative bacteria, lipid A and LAP, deliver distinct signals to differentiate macrophages into killer cells.

As well as establishing an in vitro model system allowing the biochemical dissection of macrophage stimulation, we believe that the findings reported in this and in the previous paper have a wider biological significance. Since the macrophage appears to be the central effector cell in the lesion of chronic inflammation (36), LPS could potentially initiate or perpetuate chronic inflam-

[3] W. F. Doe and P. M. Henson. Manuscript in preparation.

mation in the intestinal tract where gram-negative organisms commensal in the intestinal lumen, and presumably their breakdown products, gain access to the submucosa after a breach in mucosal integrity. In addition, our findings reveal that there are at least two structurally distinct constituents of cell walls of gram-negative bacteria (LPS and LAP) which can act as stimulators of macrophages. Their differences in structure, size, and solubility may broaden the environments in which gram-negative organisms can express their biological activity in vivo. Moreover, LPS has been shown to boost the in vitro cytotoxic capacity of murine macrophages obtained directly from solid tumors (37) and to induce regression of solid tumors in mice in vivo (38). Therefore, the prospect exists of inducing tumor regression in humans by using LAP which has less potential for toxic side effects than the complete LPS molecule, the toxicity of which hampers its use as an inducer of tumor regression.

Summary

Stimulation of macrophages to lyse tumor cells is a property common to lipopolysaccharide (LPS) extracted from a variety of smooth and rough bacterial strains by several different preparative procedures. The relationship between macrophage stimulation and the structural characteristics of LPS is defined. In protein-free LPS, lipid A bears the stimulatory signal which results in the differentiation of elicited macrophages into killer cells. The polysaccharide moiety is neither stimulatory itself nor does it block the activity of complete LPS on macrophages. Extraction of LPS by the butanol or Boivin procedures produces preparations in which LPS is complexed through its lipid A moiety to a protein rich component, LAP. Isolated LAP delivers a macrophage differentiation signal which is independent of lipid A. The presence of these two structurally distinct constituents in the cell walls of gram-negative bacteria broadens the biological environments in which they can stimulate macrophages in vivo.

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