INFECTION AND IMMUNITY, Nov. 1990, p. 3743–3750
0019-9567/90/113743-08$02.00/0

Vol. 58, No. 11

Cytokine Induction by Lipopolysaccharide (LPS) Corresponds to Lethal Toxicity and Is Inhibited by Nontoxic *Rhodobacter capsulatus* LPS

HARALD LOPPNOW,[1†*] PETER LIBBY,[1,2] MARINA FREUDENBERG,[3] JÜRGEN H. KRAUSS,[3] JÜRGEN WECKESSER,[4] AND HUBERT MAYER[3]

Tufts University School of Medicine and New England Medical Center, 711 Washington Street, Boston, Massachusetts 02111[1]; Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115[2]; and Max-Planck-Institut für Immunobiologie, Stübeweg 51,[3] and Institut für Biologie II, Mikrobiologie, Schänzlestrasse 1,[4] D-7800 Freiburg i.Br., Federal Republic of Germany

Received 8 May 1990/Accepted 13 August 1990

Many pathological effects of gram-negative bacteria are produced by their cell wall-derived lipopolysaccharides (LPSs). Differing pathogenicity of gram-negative LPSs, however, may depend on their capacities to induce cytokines. Thus, we studied the lethal toxicity of four nonenterobacterial LPSs and compared it with their capacity to induce mononuclear cell (MNC)-derived interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF). Unstimulated MNC did not release these cytokines. LPS from the phototrophic strain *Rhodobacter capsulatus* 37b4 elaborated little toxicity in galactosamine-treated mice (10 μg of LPS per mouse was the 100% lethal dose [LD_{100}]) and induced IL-1 and IL-6 release only at high concentrations (10 to 50 μg of LPS per ml). *R. capsulatus* LPS failed to induce TNF activity even at the highest concentration tested (100 μg of LPS per ml). In contrast, LPS derived from *Pseudomonas diminuta* NCTC 8545 or the nodulating species *Bradyrhizobium lupini* DSM 30140 and *Rhizobium meliloti* 10406 expressed lethal toxicity (LD_{100}, 1,000, 100, and 10 ng per mouse, respectively) and induced IL-1 or IL-6 (10 to 100, 10, and 1 ng of LPS per ml, respectively) at concentrations 1,000- to 10,000-fold lower than effective levels of *R. capsulatus* LPS. LPSs from *P. diminuta*, *B. lupini*, and *R. meliloti* also stimulated TNF production and release. MNC accumulated cell-associated IL-1 activities under circumstances in which released activity was readily detected. The cells contained only scant IL-6 activity, indicating release of this mediator rather than intracellular accumulation. Antisera to the respective cytokines inactivated biological activities of the samples selectively. The *R. capsulatus* LPS inhibited cytokine induction by LPS from *P. diminuta*, *B. lupini*, and *R. meliloti* in coincubation experiments. These results show that the in vivo lethality of the LPSs tested correlates with the induction of monocyte-derived cytokines in vitro. The results of this study suggest that the different lethality of various LPSs from gram-negative bacteria may be due to the differential ability of these LPSs to induce cytokine production.

Lipopolysaccharides (LPSs) are important membrane components of gram-negative bacteria (60). They are also known as endotoxins, referring to their in vivo toxic effects without specifying the chemical structure. During bacterial infection, LPSs can induce host responses, such as fever, hypotension, circulatory abnormalities, multiorgan failure, and eventually death. Many of these responses, previously attributed to direct effects of activation with LPS, are probably mediated by cytokines derived from mononuclear phagocytes acting in part on vascular cells (29, 40). Two of these proteins, tumor necrosis factor (TNF) (5, 27, 43) and interleukin-1 (IL-1) (11, 44), are found in serum following LPS injection (9, 36, 39, 50, 53). Both are pyrogenic (12, 41) and might also be involved in generation of the acute-phase response (47, 48). Individual cytokines may preferentially mediate different aspects of LPS effects. For example, TNF might mediate lethality (15, 16, 55) or tissue injury (5), whereas IL-1 may activate lymphocytes (11, 13). Anti-IL-1- and anti-TNF antisera inhibited certain effects of LPS or the respective cytokine in experimental animals (6, 12, 36). LPS is also proposed to induce late acute-phase reactants like C-reactive protein or fibrinogen via early acute-phase reactants, such as IL-1, TNF, and gamma interferon (58). Another cytokine, interleukin-6 (IL-6), is a major inducer of acute-phase proteins (late-phase reactants) (19). Hence, IL-6 may be the major mediator of these latter LPS-induced activities. It might induce them either directly, in concert with, or in response to early-phase reactants (47). However, little is known regarding IL-6 induction by bacterial LPS or its lipid A moiety in monocytes (3) or vascular cells (33–35).

The availability of synthetic partial structures of LPS (23, 59) has increased our understanding of the structure-function relationship of LPS (49). The lipid A portion accounts for the endotoxic properties of LPS (18). This moiety is also responsible for the induction of immunoregulatory mediators, such as IL-1 (30, 31). The number of fatty acids within the lipid A, their position, probably their chain length, and the phosphate content appear to be critical determinants of the capacity of LPSs to induce IL-1 (30). Studies of other biological activities support this conclusion (8, 24). We reported previously that synthetic hexaacylated lipid A induced IL-1 activity, whereas synthetic tetraacylated lipid A precursor Ia (compound 406) lacked IL-1-inducing activity (31) and inhibited hexaacylated lipid A or LPS-derived IL-1 production (30).

Despite an array of available potent antibiotics, shock due to gram-negative sepsis remains an important clinical problem. Various strategies for inactivation of the toxic effects of

* Corresponding author.
† Present address: Forschungsinstitut Borstel, Parkallee 1-40, D-2061 Borstel, Federal Republic of Germany.

TABLE 1. Chemical characteristics of LPSs used

LPS source	Reference(s)	Lipid A constituents						
		Disaccharide backbone[a]		Phosphate linkage		Fatty acids		
		GlcN	DAG	Glycosidic	Ester	Amide bound	Ester bound	Total no.
Rhodobacter capsulatus 37b4	26	+	−	+	+	3-Oxo-14:0	3-OH-10:0, Δ^5-12:1[b]	4–5[b]
Pseudomonas diminuta NCTC 8545	25, 38	−	+	−	+	3-OH-12:0, 3-OH-14:0	14:0, unusual fatty acids	5–6[c]
Bradyrhizobium lupini DSM 30140	37[d]	−	+	+	−	3-OH-12:0, 3-OH-14:0	16:0, 18:1, 29-OH-30:0, 30-OH-31:0	NA[e]
Rhizobium meliloti	57	+	−	+	+	3-OH-16:0, 3-OH-18:0	3-OH-14:0, 27-OH-28:0	4–5[c]

[a] GlcN, Glucosamine; DAG, 2,3-diamino-2,3-dideoxy-D-glucose.
[b] Partially substituted by Δ^5-12:1.
[c] Nonstoichiometric substitution of oxyacyl residues by acyl groups.
[d] S. Basu and H. Mayer, unpublished.
[e] NA, Complete lipid A structures are not yet available.

LPS in vivo appear to be possible. Antisera or monoclonal antibodies could neutralize LPS directly. However, this approach may not potently inhibit LPS effects (10), and cross-protection might not be available (4). The effects of LPS are mediated at least in part by cytokines; thus, antibodies to these mediators could decrease the harmful effects of LPS. However, this approach might also interfere with beneficial cytokine functions. Toxic effects of LPS might be prevented by administration of inactive synthetic lipid A analogs, but the production of synthetic compounds is laborious and expensive. This holds true also for modified LPS (monophosphoryl lipid A) of toxic LPSs (21, 46). Thus, the use of readily isolated natural products, which lack toxic effects and can compete with toxic LPSs, might provide an attractive alternative in prevention of the lethal effects of LPS.

Lipid A variants with little or no lethality are encountered in the LPSs of nonenterobacterial species like *Rhodobacter capsulatus*. In contrast to enterobacteria, the lipid A of *R. capsulatus* contains two amide-linked 3-oxo-myristic acids and two ester-linked 3-OH-10:0. One of the latter can be partially substituted by Δ^5-dodecenoic acid (26). The lethal toxicity and cytokine induction capacity of *R. capsulatus* LPS and various other nonenterobacterial LPSs has not been established, and therefore we compared these biological activities and also performed competition experiments. We show that the potency of the LPSs to induce cytokines in vitro and their lethal toxicity for mice in vivo correlated well and that the *R. capsulatus* LPS inhibited cytokine induction by three LPSs in cultured mononuclear cells. We propose that nontoxic LPSs like that of *R. capsulatus* might provide a useful tool for the study of LPS binding and that they might provide a therapeutic adjunct in treating life-threatening gram-negative bacterial infection.

MATERIALS AND METHODS

Bacterial strains and LPSs. The nonenterobacterial species selected for this study and their main structural elements are shown in Table 1. They were chosen because their lipid A differs from the usual well-known enterobacterial lipid A. Structure-function relationship investigations of these LPSs might help in understanding the structural necessities of cytokine induction by LPS. In detail, they possess unusual fatty acids, such as 3-oxo-14:0, or long-chain hydroxylated fatty acids, such as 27-OH-28:0, in addition to 3-OH fatty acids, as usually found in enterobacterial lipid A. Others contain lipid A with 2,3-diamino-2,3-dideoxy-D-glucose instead of glucosamine as the backbone sugar. Some lack phosphate or contain only ester-linked phosphate and lack glycosidically linked phosphate groups attached to the backbone.

The origin of the strains, their mass cultivation, and the isolation and purification of their LPSs have been reported previously (see references in Table 1). The presence of protein or nucleic acid contamination (ribose, RNA) in the LPSs tested was excluded by amino acid analysis and gas-liquid chromatographic examination, respectively. The synthetic hexaacylated *Escherichia coli* lipid A (compound 506) was a kind gift of Daiichi Pure Chemicals (Tokyo, Japan), and the heat-killed *Staphylococcus epidermidis* were kindly provided by C. A. Dinarello.

Detection of LPS toxicity in galactosamine-treated mice. Lethal toxicity was determined in galactosamine-sensitized mice (C57BL/6) as described previously (17, 52). Briefly, 10- to 16-week-old C57BL/6 mice were injected intravenously with a mixture of various concentrations of LPS and D-galactosamine (20 mg/ml) in 0.2-ml volumes. Toxicity was expressed as 100% lethal dose (LD_{100}, ng of LPS per mouse).

Stimulation of cytokine production and release by MNC. Mononuclear cells (MNC) from healthy donors were isolated by density gradient on Ficoll-Hypaque. The MNC had no contact with serum throughout the procedure. Five million cells per ml were incubated without serum in 100-μl cultures in round-bottomed microtiter plates for 24 h. They were suspended in RPMI 1640 with L-glutamine, antibiotics, and the appropriate stimulus. For dose-response experiments, 50 μl of serial 10-fold dilutions of the stimulus (double concentrated) was added to 50 μl of double concentrated cells. Parallel cultures of MNC were stimulated with various 10-fold dilutions of the respective LPS, and the supernatants were analyzed in one biological assay as described below. If not mentioned otherwise, results of representative experiments are shown.

Preparation of intracellular cytokines. The intracellular (cell-associated) cytokine activity of MNC was determined after freezing and thawing the cells (28, 30). MNC (5×10^6/ml) were stimulated in 1 ml of serum-free medium in 24-well culture plates for 24 h. Then the supernatants were collected, 2% fetal calf serum (FCS) was added, and super-

TABLE 2. Lethal toxicity of various LPSs

LPS source	Lethality (no. dead/no. tested) of various LPSs in galactosamine-treated mice at concn (ng of LPS/mouse):				
	10,000	1,000	100	10	1
R. capsulatus	3/3	2/10			
P. diminuta		3/3	0/3		
B. lupini			4/4	0/4	
R. meliloti			4/4	8/8	0/4
S. abortus-equi				6/6	2/8

natants were centrifuged to pellet residual cells. The pellet and remaining adherent cells, which were detached with a rubber policeman, were suspended in culture medium. Cells were collected, centrifuged, and resuspended twice in culture medium. Finally, for freezing (−20°C), the cells were suspended in RPMI 1640 containing 2% FCS, antibiotics, and L-glutamine. After at least 2 h at −20°C, the suspension was thawed, and cell-associated cytokine activity was determined in the respective biological assay.

Detection of cytokine activities. Cytokine activities were measured in biological assays. The samples were analyzed in serial fourfold dilutions, the mean of triplicate cultures was determined, and biological activity was calculated by probit analysis in reference to recombinant standard preparations, according to the method described for IL-2 (20). The data are usually presented as picograms of recombinant cytokine per milliliter. In some experiments, however, the thymidine incorporation of B9 or D10S cells (dpm per culture ± SD) is shown.

IL-1 was detected in the human dermal fibroblast assay as described previously (32, 51). This cytokine was also determined with the murine cell line D10S (45), kindly provided by C. A. Dinarello. IL-6 was measured by proliferation of murine B9 hybridoma cells, kindly provided by L. A. Aarden (1, 2). TNF was detected with the murine tumorigenic fibroblast line L929 by staining with crystal violet (14).

Detergent-polyacrylamide electrophoresis. We investigated the individual chemical character of the oligosaccharide portion (smooth, semirough, or rough) of the various LPSs by their electrophoretic migration pattern in deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE) (26). The stacking gel consisted of 4% and the running gel of 13% acrylamide. Following preelectrophoresis at 25 mA without samples, 5 or 10 μg of the LPSs was applied to separate lanes and run at 20 mA. The gels were silver-stained by the method of Tsai and Frasch (56).

RESULTS

Lethal toxicity of various nonenterobacterial lipopolysaccharides. We analyzed the lethal toxicity of the various LPSs in galactosamine-treated mice (Table 2). In control experiments, a concentration of 1 to 10 ng of *Salmonella abortus-equi* LPS per mouse was lethal. The LPS of *Rhizobium meliloti* exhibited the highest toxic activity of the LPSs tested, a dose of 10 ng per mouse causing 100% lethality. In comparison, the toxicity of the LPS of *Bradyrhizobium lupini* was about 10 times (100 ng per mouse) and that of *Pseudomonas diminuta* was 100 times (1,000 ng per mouse) lower. The LPS of *R. capsulatus* was the least active. A dose of 10 μg per mouse had to be administered to obtain 100% lethality. This LPS was designated nontoxic.

Nontoxic *R. capsulatus* LPS induces cytokines less potently than toxic LPSs. To determine the capacity of the various LPSs to induce IL-1, IL-6, and TNF, mononuclear cells were incubated with the respective LPSs at concentrations of 1 pg/ml to 50 μg/ml. The supernatants were analyzed in biological assays. In a representative experiment with one blood donor, unstimulated MNC released no IL-1, IL-6, or TNF activity (Table 3). The nontoxic *R. capsulatus* LPS caused release of IL-1 and IL-6 activity only at very high concentrations (10 or ≥50 μg/ml) and did not induce TNF under these experimental conditions (see also Table 4). The other LPSs, however, induced IL-1 and IL-6 even at low concentrations (1 to 100 ng/ml). The latter LPSs also stimulated TNF production, but at higher LPS concentrations (0.1 to 10 μg of LPS per ml) than were active for IL-1 or IL-6 induction. In previous studies we showed that (depending on the blood donor) 1 to 100 pg of various LPSs per ml and 10 to 10,000 pg of synthetic lipid A's per ml induced IL-1 (30). The results of the two independent IL-1 assays with the D10S cells and the fibroblasts correlated very well ($y = 19847x - 1865.5$; $R = 0.975$). The IL-1 and IL-6 activities generally paralleled each other.

TABLE 3. Induction of cytokines by toxic and nontoxic LPSs[a]

Measured cytokine and LPS source	Cytokine activity (pg/ml) in supernatants of LPS-stimulated MNC at LPS concn (pg/ml):						
	5×10^7	10^7	10^6	10^5	10^4	10^3	10^2
IL-1 (D10S cells)							
R. capsulatus	8,123	110	0	0	0	0	0
R. meliloti	171,484	34,832	3,078	670	335	42	0
B. lupini	56,569	69,644	12,311	884	32	0	0
P. diminuta	6,156	4,061	8,123	3,299	32	0	0
IL-1 (fibroblasts)							
R. capsulatus	3,080	70	0	0	0	0	0
R. meliloti	80,010	22,980	2,170	1,650	540	180	0
B. lupini	30,320	45,960	7,580	1,250	60	0	0
P. diminuta	5,740	3,300	1,900	1,340	60	0	0
IL-6 (B9 cells)							
R. capsulatus	64	2	0	0	ND	0	0
R. meliloti	453	1,810	226	787	171	14	0
B. lupini	4,159	3,152	2,744	640	28	0	0
P. diminuta	226	3,620	1,576	279	3	0	0
TNF (L929 cells)							
R. capsulatus	0	0	0	0	0	0	0
R. meliloti	625	156	0	0	0	0	0
B. lupini	625	625	156	39	0	0	0
P. diminuta	39	0	0	0	0	0	0

[a] Cytokine activities were measured by probit analysis with respect to 10-ng/ml recombinant standards. The detection limits were 5 pg/ml in D10S, 50 pg/ml in fibroblasts, 10 pg/ml in B9, and 50 pg/ml in L929 cells. Supernatants of unstimulated or synthetic lipid A (1 μg/ml)-stimulated MNC contained 0 or 65 pg of IL-1 per ml (D10S), 0 or 890 pg of IL-1 per ml (fibroblasts), and 0 or 1,436 pg of IL-6 per ml, respectively. Unstimulated MNC did not release TNF activity.

Comparison of cell-associated and released cytokine activities following LPS stimulation. Mononuclear cells activated with certain stimuli might accumulate cytokines intracellularly rather than release them into the culture supernatant. Thus, we studied the activities of cell-associated cytokines following LPS stimulation and compared them to the cytokine activities released into the supernatants of the same cells. Unstimulated MNC neither contained nor released biologically active cytokines (Table 4). *R. capsulatus* LPS-activated MNC accumulated cell-associated IL-1 and released scant IL-1 and IL-6 but no TNF. Following stimulation with the other LPSs, the cells retained more cell-associated IL-1 activity than they released into the supernatant. In contrast, the cells secreted IL-6 mainly into

TABLE 4. Comparison of released and cell-associated cytokine activities following stimulation with various LPSs

Stimulus	Concn (μg/ml)	Cytokine activity[a] (pg/ml)					
		IL-1		IL-6		TNF	
		SN	IC	SN	IC	SN	IC
Medium		0	0	0	0	0	0
R. capsulatus LPS	100	1	15	15	0	0	0
	50	0	0	0	0	0	0
	10	0	0	0	0	0	0
R. meliloti LPS	50	1,895	5,743	14,143	110	3,100	3,100
	10	359	947	4,353	17	0	0
B. lupini LPS	50	3,789	8,123	32,490	272	50,000	3,100
	10	1,166	5,743	5,744	52	12,500	0
P. diminuta LPS	50	237	8,706	20,000	313	12,500	0
	10	103	3,299	11,486	68	0	0

[a] IL-1, IL-6, and TNF activities were measured in D10S, B9, and L929 cells, respectively. Activity was determined with respect to recombinant standards by probit analysis. SN, Supernatant; IC, cell lysate.

the supernatant and retained only small amounts of this mediator intracellularly. The MNC released TNF activity only following stimulation with high concentrations of LPS from *B. lupini*, *R. meliloti*, or *P. diminuta*. The release of TNF correlated to some extent with the accumulation of IL-1 activity or the release of IL-6 activity. Cell-associated TNF, however, was only found in cells stimulated with 50 μg of *R. meliloti* or *B. lupini* LPS per ml.

Cytokine activities released following stimulation with LPS are inhibited by corresponding antisera. To verify the identity of the cytokines measured in the biological assays, we examined the ability of specific anticytokine antisera to neutralize these activities. A mixture of recombinant IL-1α and IL-1β antibodies (1:500 dilution each) inhibited the D10S stimulatory activity completely (Fig. 1). Note that in this particular experiment, 100 μg (*CAPS) of *R. capsulatus* LPS per ml was necessary to induce IL-1 activity rather than the 50 μg/ml used for the other stimuli (Fig. 1). The anti-recombinant IL-6 antibody inhibited B9 stimulatory activity (Fig. 2). Since the *R. capsulatus* LPS did not induce IL-6 at the concentration tested (10 μg/ml), an additional sample (100 μg/ml; *CAPS) and lower assay dilution was used in the experiment depicted in Fig. 2. The IL-6 activity in the supernatants of MNC stimulated with LPS from *R. meliloti*, *B. lupini*, or *P. diminuta* was not inhibited completely, since the IL-6 activities in these supernatants were much higher than those of the 506- or *R. capsulatus* LPS-induced supernatants (data not shown; compare Tables 3 and 4). Note that the graph simply shows thymidine incorporation of the assay cells and not the results of a probit analysis (picograms of IL-6). TNF antibody reversed the L929 lytic activity in the supernatants (data not shown).

Nontoxic *R. capsulatus* LPS inhibits toxic LPS-induced cytokine production. Further experiments analyzed whether the nontoxic *R. capsulatus* LPS could antagonize cytokine induction by LPS from *B. lupini*, *R. meliloti*, or *P. diminuta*. Coincubation with *R. capsulatus* LPS abolished the induction of IL-1 and IL-6 or of TNF by the other LPSs (Table 5). The nontoxic LPS did not alter induction of cytokines by the gram-positive *S. epidermidis*, indicating the specificity of the inhibition.

Structure-function relationship of the tested LPSs. LPS consists of two structural regions, the lipid moiety, described in Table 1, and the sugar part (O-chains and R-core). We performed DOC-PAGE to analyze the individual migration patterns of the LPSs. The LPSs showed different patterns (Fig. 3) depending on the extent of R-core substitution by O-chains and on different O-chain composition and length. *R. capsulatus* and *R. meliloti* LPSs showed only

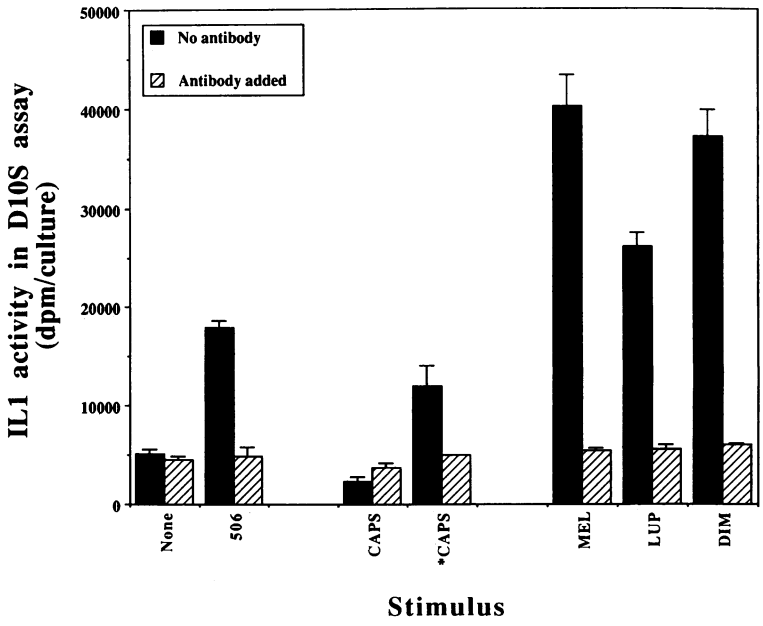

FIG. 1. Inhibition of IL-1 activity. MNC were cultured for 24 h with various stimuli (506, synthetic *E. coli* type lipid A; CAPS, *R. capsulatus* LPS; MEL, *R. meliloti* LPS; LUP, *B. lupini* LPS; DIM, *P. diminuta* LPS). The supernatants were harvested and incubated (final dilution, 1:10) with a mixture of monospecific rabbit anti-recombinant IL-1α and IL-1β antisera (final dilutions, 1:500) for 1 h at 37°C. D10S cells were added, and IL-1 activity was measured by [^{3}H]thymidine incorporation (dpm per culture ± SD). Concentrations of stimuli: 506, 1 μg/ml; *CAPS, 100 μg/ml; other LPSs, 50 μg/ml.

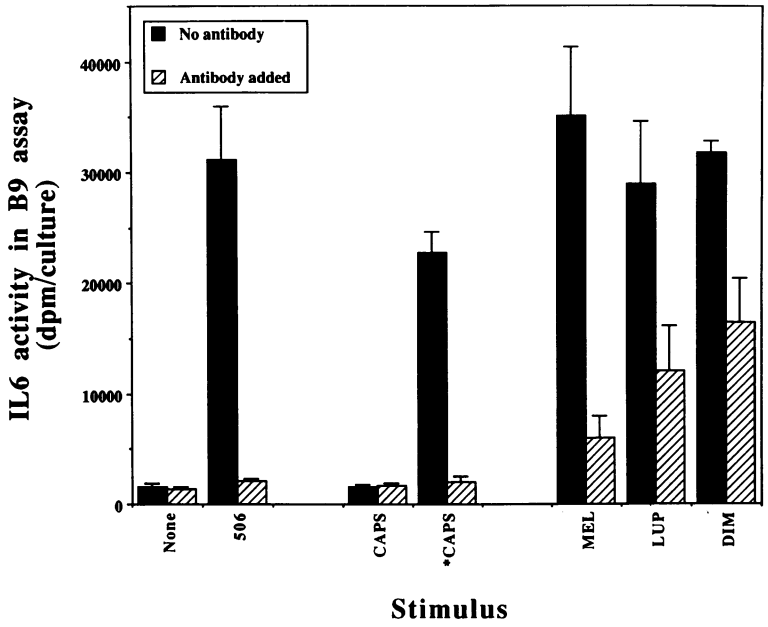

FIG. 2. Inhibition of IL-6 activity. MNC were cultured for 24 h with various stimuli (10 μg/ml; for abbreviations, see Fig. 1 legend). The supernatants were harvested and incubated (final dilution, 1:1000) with monospecific rabbit anti-recombinant IL-6 antiserum (final dilution, 1:1000) for 1 h at 37°C. B9 cells were added, and IL-6 activity was measured by [^{3}H]thymidine incorporation (dpm per culture ± SD). A second supernatant of MNC stimulated with a higher concentration of *R. capsulatus* LPS (*CAPS, 100 μg/ml) was tested at a final dilution of 1:100.

three or two bands (long arrows), respectively, indicating short O-specific chains. The LPS of *P. diminuta* had a high rough-type content (arrowhead) and only a single main band in the smooth region (long broken arrow), whereas *B. lupini* (short arrows) contained significant amounts of smooth LPS. The minimal concentrations active for cytokine induction and the toxicity (LD_{100}) of the LPSs tested indicate that expression of biological activities is not related to the type of LPS (smooth or rough).

DISCUSSION

The importance of LPS as an inducer of cytokines that mediate certain systemic responses to bacterial infections is increasingly apparent. However, little is known about the correlation of cytokine induction capacity and lethal toxicity of nonenterobacterial LPSs. The present report compares these important biological functions and shows that nontoxic *R. capsulatus* LPS is 1,000- or 10,000-fold less potent as an inducer of MNC-derived cytokines than the other LPSs investigated here. Furthermore, the *R. capsulatus* LPS reduced or abolished cytokine induction following stimulation of MNC with the other LPSs. The overall structure of the lipid A backbone, the number and type of fatty acids, their position, and complete phosphorylation all appear to be important for the expression of biological activities.

LPSs of several *Rhizobiaceae*, including *B. lupini* and *R. meliloti*, as well as *P. diminuta* LPS induced all cytokines tested. The lowest concentration (minimal dose) still active for IL-1 and IL-6 induction was 1 ng/ml for *R. meliloti* LPS, 10 ng/ml for *B. lupini* LPS, and 10 to 100 ng/ml for *P. diminuta* LPS. The most active TNF inducer was *B. lupini* LPS (100 ng/ml), followed by *R. meliloti* LPS (10 μg/ml) and *P. diminuta* LPS (50 μg/ml). Thus, the rank order of these LPSs for IL-1 and IL-6 induction differed from that for TNF induction, although all three LPSs induced IL-1, IL-6, and TNF production. In contrast, nontoxic *R. capsulatus* LPS

TABLE 5. *R. capsulatus* LPS inhibits toxic LPS-induced cytokine release

Stimulus	Cytokine activity (pg/ml) in supernatants of stimulated MNC[a]					
	IL-1		IL-6		TNF	
	No CAPS	CAPS added	No CAPS	CAPS added	No CAPS	CAPS added
Medium	0	2	313	1,767	0	1,700
R. meliloti LPS	12,311	22 (99)[b]	211,000	8,705 (96)	22,700	2,100 (91)
B. lupini LPS	15,157	2,500 (83)	343,000	130,000 (62)	139,700	1,000 (93)
P. diminuta LPS	32,490	253 (99)	520,000	42,870 (92)	10,050	54,900 (45)
S. epidermidis	1,895	1,895 (0)	60,628	60,628 (0)	21,600	24,500 (0)

[a] See Table 4, footnote *a*. The induction of TNF was measured in a separate set of parallel cultures of MNC stimulated under the various conditions. Nontoxic *R. capsulatus* LPS (CAPS) was used at 5 μg/ml, the other LPSs were used at 2 μg/ml, and *S. epidermidis* was applied at 23,000 bacteria per ml.

[b] The percent inhibition by addition of *R. capsulatus* LPS is shown in parentheses. Inhibition of *S. abortus-equi* LPS was 80 and 90% for IL-1 and IL-6 induction, respectively.

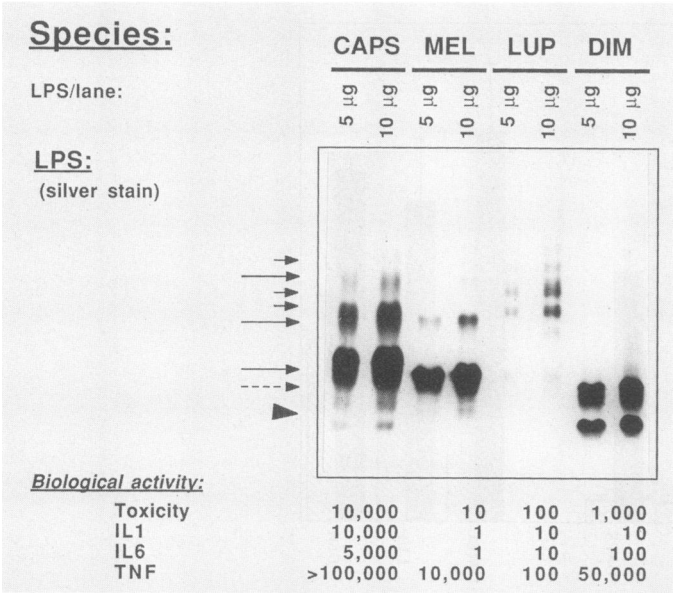

FIG. 3. Comparison of chemical character and biological activities of the various LPSs. The migration pattern was analyzed in DOC-PAGE. Subsequently, 5 or 10 µg of the same LPS was applied to each lane and silver stained following the run. The arrows indicate the various S (smooth) and R (rough) type bands as follows: long arrows, short O-chains of *R. capsulatus* and *R. meliloti* LPSs; long broken arrow, single main band of *P. diminuta* LPS in the semirough region; short arrows, long O-chains of *B. lupini* LPS; arrowhead, single main band in the rough region of *P. diminuta* LPS. The minimal doses active for cytokine induction (nanograms per milliliter) and the lethal toxicity (LD_{100}, nanograms per mouse), summarized from the data shown above, are arranged below the DOC-PAGE gel. See Fig. 1 legend for abbreviations.

induced IL-1 or IL-6 activity only poorly and did not elaborate TNF activity (up to 100 µg/ml). A recent report showed that TNF is the endogenous mediator of the lethal effects of LPS in galactosamine-sensitized mice (16). Therefore, it was not surprising that the nontoxic LPS of *R. capsulatus* did not induce TNF formation in vitro and that the relatively low-toxicity *P. diminuta* LPS stimulated only a little TNF production. The TNF activity induced by the two other LPSs did not correlate with their lethal activity, as the LPS of *B. lupini* was a stronger inducer of TNF but less toxic than *R. meliloti* LPS. The use of different species for the in vitro (human MNC) and in vivo (mouse) experiments and/or different sensitivities of the biological assays might explain these results.

The structure-function relationship of the LPSs tested supports the concept that none of the different lipid A constituents represents the toxophore group by itself. It is more likely that expression of biological activities depends on the overall structure of the lipid A and that structural similarity to enterobacterial lipid A is important. The influence of the type of acylation within the lipid A backbone is documented by the results obtained with the *R. capsulatus* and *R. meliloti* LPSs. Replacement of the amide-bound 3-OH fatty acids of *R. meliloti* by 3-oxo fatty acids, as found in the *R. capsulatus* lipid A, in concert with the presence of shorter and unsaturated fatty acids results in a 1,000-fold decrease in toxicity and 1,000- to 10,000-fold decrease in cytokine-inducing capacity. A similar observation was made earlier when the toxicity of *Rhodopseudomonas sphaeroides* LPS containing amide-bound 3-oxo fatty acids and enterobacterial lipid A containing amide-bound 3-OH fatty acids was compared (52). Two of the LPSs tested (*B. lupini* and *R. meliloti*) contain long-chain fatty acids of a new, recently discovered structural type (22, 57). These fatty acids are twice as long as those usually present in enterobacterial lipid A. Consequently, they contribute to a high hydrophobicity of the respective lipid A and may compensate for the low degree of acylation (4 to 5 fatty acids per molecule) of *B. lupini* and *R. meliloti* LPS.

Study of synthetic lipid A partial structures has shown that the fatty acid composition influences the ability of various synthetic lipid A's to induce IL-1 (30). Induction of IL-1 by LPS or lipid A could be antagonized by non-IL-1-inducing tetraacylated precursor Ia, indicating specificity of binding, which, however, does not suffice to induce lymphokine production. Since the *R. capsulatus* LPS only killed galactosamine-treated mice and induced cytokines at high concentrations, we analyzed whether this LPS inhibited induction of cytokine production by toxic LPSs. The *R. capsulatus* LPS did inhibit or decrease (by 45 to 99%) the lymphokine induction by all LPSs tested. These results support our previous findings and extend them to natural substances. The present investigations agree well with findings that lipid A obtained from the LPS of *Rhodopseudomonas sphaeroides*, structurally similar to *R. capsulatus* LPS, inhibited TNF production by toxic LPS in RAW 264.7 cells (54) and that lipid IVA inhibited TNF production in whole blood ex vivo (25a).

The regulatory network of immune response in bacterial infection leading to septic shock is incompletely understood. However, more than one mediator may cause the lethal effects of bacterial endotoxin under certain circumstances. Concentrations of IL-1 and TNF that alone are innocuous produced a shocklike state in rabbits when administered in combination (42), an indication that these two mediators act synergistically (7, 42). Since our data show induction of three major cytokines by toxic LPSs, correlating with the rank order of lethal toxicity in mice, it appears possible that development of septic shock is not restricted to interaction of the two mediators IL-1 and TNF.

Our observation that cytokine induction in cultured cells parallels in vivo lethality suggests an in vitro assay for the toxic properties of a given LPS that avoids the use of animals. Furthermore, the finding that the toxic potential of various LPSs correlates well with in vitro stimulation of cytokines supports the pathogenic role of these mediators in development of septic shock. In this regard, the immediate therapy of acute septic shock with nontoxic LPS of *R. capsulatus* in combination with appropriate antibiotic therapy might mitigate the fatal effects of gram-negative infection.

ACKNOWLEDGMENTS

The B9 cell line and recombinant IL-6 were kindly provided by L. Aarden. Recombinant IL-1β, IL-6, antisera, and the D10S cell line were a kind gift of C. A. Dinarello. Recombinant IL-1α was kindly provided by P. Lomedico. The technical assistance of Marisia Muszynski, Cindy Galin, Elissa Simon-Morrisey, and Dung Sean Ha, as well as the secretarial and administrative assistance of Joan Leonard and Lisa Joseph, is gratefully acknowledged.

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (Lo 385/1-1; H. Loppnow) and a Public Health Service grant from the National Institutes of Health (HL 34636; P. Libby). Dr. Libby is an Established Investigator of the American Heart Association.

LITERATURE CITED

1. **Aarden, L. A., E. R. De Groot, O. L. Schaap, and P. M. Lansdorp.** 1987. Production of hybridoma growth factor by human monocytes. Eur. J. Immunol. **17**:1411–1416.
2. **Aarden, L. A., P. M. Lansdorp, and E. R. De Groot.** 1985. A growth factor for B cell hybridomas produced by human monocytes. Lymphokines **10**:175–185.
3. **Bauer, J., U. Ganter, T. Geiger, U. Jacobshagen, T. Hirano, T. Matsuda, T. Kishimoto, T. Andus, W. Acs, W. Gerok, and G. Ciliberto.** 1988. Regulation of interleukin 6 expression in cultured human blood monocytes and monocyte-derived macrophages. Blood **72**:1134–1140.
4. **Baumgartner, J. D., D. Heumann, J. Gerain, P. Weinbreck, G. E. Grau, and M. P. Glauser.** 1990. Association between protective efficacy of anti-lipopolysaccharide (LPS) antibodies and suppression of LPS-induced tumor necrosis factor alpha and interleukin 6. J. Exp. Med. **171**:889–896.
5. **Beutler, B., and A. Cerami.** 1986. Cachectin and tumour necrosis factor as two sides of the same biological coin. Nature (London) **320**:584–588.
6. **Beutler, B., I. W. Milsark, and A. C. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **29**:869–871.
7. **Boswell, J. M., M. A. Yui, D. W. Burt, and V. E. Kelley.** 1988. Increased tumor necrosis factor and IL1β gene expression in the kidneys of mice with lupus nephritis. J. Immunol. **141**:3050–3054.
8. **Cady, A. B., S. Kotani, T. Shiba, S. Kusumoto, and J. M. Krueger.** 1989. Somnogenic activities of synthetic lipid A. Infect. Immun. **57**:396–403.
9. **Cannon, J. G., and C. A. Dinarello.** 1985. Increased plasma interleukin 1 activity in woman after ovulation. Science **227**: 1247–1250.
10. **Chia, J. K. S., M. Pollack, G. Guelde, N. L. Koles, M. Miller, and M. E. Evans.** 1989. Lipopolysaccharide (LPS)-reactive monoclonal antibodies fail to inhibit LPS-induced tumor necrosis factor secretion by mouse-derived macrophages. J. Infect. Dis. **159**:872–880.
11. **Dinarello, C. A.** 1989. Interleukin 1 and its biologically related cytokines. Adv. Immunol. **44**:153–205.
12. **Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, Jr., and J. V. O'Connor.** 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med. **163**:1433–1450.
13. **Durum, S. K., J. A. Schmidt, and J. J. Oppenheim.** 1985. Interleukin 1: an immunological perspective. Annu. Rev. Immunol. **3**:263–287.
14. **Flick, D. A., and G. E. Gifford.** 1984. Comparison of in vitro cell toxicity assays for tumor necrosis factor. J. Immunol. Methods **68**:167–177.
15. **Freudenberg, M. A., D. Keppler, and C. Galanos.** 1986. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect. Immun. **51**:891–895.
16. **Galanos, C., and M. A. Freudenberg.** 1990. Tumor necrosis factor mediates endotoxin shock: the protective effects of antibodies and cortisone, p. 187–193. *In* B. Bonavida and G. Granger (ed.), Tumor necrosis factor: structure, mechanism of action, role in disease and therapy. Karger, Basel.
17. **Galanos, C., M. Freudenberg, and W. Reutter.** 1979. Galactosamine-induced sensitization of the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA **76**:5939–5943.
18. **Galanos, C., O. Lüderitz, E. T. Rietschel, O. Westphal, H. Brade, L. Brade, M. Freudenberg, U. Schade, M. Imoto, H. Yoshimura, S. Kusumoto, and T. Shiba.** 1985. Synthetic and natural *E. coli* free lipid A express identical endotoxic activities. Eur. J. Biochem. **148**:1–5.
19. **Gauldie, J., C. Richards, D. Harnish, P. Lansdorp, and H. Baumann.** 1987. Interferon-β_2/BSF-2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute-phase protein response in liver cells. Proc. Natl. Acad. Sci. USA **84**:7251–7255.
20. **Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith.** 1978. T-cell growth factor: parameters for production and a quantitative microassay for activity. J. Immunol. **120**:2027–2032.
21. **Hiernaux, J. R., P. W. Stashak, J. L. Cantrell, J. A. Rudbach, and P. J. Baker.** 1989. Immunomodulatory activity of monophosphoryl lipid A in C3H/HeJ and C3H/HeSnJ mice. Infect. Immun. **57**:1483–1490.
22. **Hollingsworth, R. I., and R. W. Carlson.** 1989. 27-Hydroxyoctacosanoic acid is a major structural fatty acyl component of the LPS of *Rhizobium trifolii* ANU 843. J. Biol. Chem. **264**:9300–9303.
23. **Imoto, M., N. Yoshimura, T. Shimamoto, N. Sakaguchi, S. Kusumoto, and T. Shiba.** 1987. Total synthesis of *E. coli* lipid A, the endotoxic principle of cell-surface lipopolysaccharide. Bull. Chem. Soc. Jpn. **60**:2205–2214.
24. **Kanegasaki, S., K. Tanamoto, T. Yasuda, J. Y. Homma, M. Matsuura, M. Nakatsuka, Y. Kumazawa, A. Yamamoto, T. Shiba, S. Kusumoto, M. Imoto, H. Yoshimura, and T. Shimamoto.** 1986. Structure-activity relationship of lipid A: comparison of biological activities of natural and synthetic lipid A's with different fatty acid composition. J. Biochem. **99**:1203–1210.
25. **Kasai, N., S. Arata, J. Mashimo, Y. Akiyama, C. Tanaka, K. Egawa, and S. Tanaka.** 1987. *Pseudomonas diminuta* LPS with a new endotoxic lipid A structure. Biochem. Biophys. Res. Commun. **142**:972–978.

25a. **Kovach, N. L., E. Yee, R. S. Munford, C. R. H. Raetz, and J. M. Harlan.** 1990. Lipid IVa inhibits synthesis and release of tumor necrosis factor induced by lipopolysaccharide in human blood ex vivo. J. Exp. Med. **172**:77–84.

26. **Krauss, J. H., U. Seydel, J. Weckesser, and H. Mayer.** 1989. Structural analysis of the nontoxic lipid A of *Rhodobacter capsulatus* 37b4. Eur. J. Biochem. **180**:519–526.
27. **Le, J., and J. Vilcek.** 1987. Biology of disease: tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab. Invest. **56**:234–248.
28. **Lepe-Zuniga, J. L., J. S. Zigler, Jr., M. L. Zimmermann, and I. Gery.** 1985. Differences between intra- and extracellular interleukin 1. Mol. Immunol. **22**:1387–1392.
29. **Libby, P., H. Loppnow, and J. Girard.** 1989. The roles of endothelial cells in septic shock, p. 25–31. *In* F. Beaufils and J. C. Mercier (ed.), Séminaires Robert Debré: réanimation, urgences, pédiatriques, chocs septiques en pédiatrie. Arnette, Paris.
30. **Loppnow, H., H. Brade, I. Dürrbaum, C. A. Dinarello, S. Kusumoto, E. T. Rietschel, and H.-D. Flad.** 1989. IL1 induction capacity of defined lipopolysaccharide partial structures. J. Immunol. **142**:3229–3238.
31. **Loppnow, H., L. Brade, H. Brade, E. T. Rietschel, S. Kusumoto, T. Shiba, and H.-D. Flad.** 1986. Induction of human interleukin-1 by bacterial and synthetic lipid. A. Eur. J. Immunol. **16**:1263–1267.
32. **Loppnow, H., H.-D. Flad, I. Dürrbaum, M. Joachim, R. Fetting, A. J. Ulmer, H. Herzbeck, and E. Brandt.** 1989. Detection of interleukin 1 with human dermal fibroblasts. Immunobiology **179**:283–291.
33. **Loppnow, H., and P. Libby.** 1989. Adult human vascular endothelial cells express the IL6 gene differentially in response to LPS or IL1. Cell. Immunol. **122**:493–503.
34. **Loppnow, H., and P. Libby.** 1989. Comparative analysis of cytokine induction in human vascular endothelial and smooth muscle cells. Lymphokine Res. **8**:293–299.
35. **Loppnow, H., and P. Libby.** 1990. Proliferating or interleukin 1-activated human vascular smooth muscle cells secrete copious interleukin 6. J. Clin. Invest. **85**:731–738.
36. **Mathison, J. C., E. Wolfson, and R. J. Ulevitch.** 1988. Participation of tumor necrosis factor in the mediation of gram-negative bacterial lipopolysaccharide-induced injury in rabbits. J. Clin. Invest. **81**:1925–1937.
37. **Mayer, H., J. H. Krauss, T. Urbanik-Sypniewska, V. Puvanesarajah, G. Stacey, and G. Auling.** 1989. Lipid A with 2,3-diamino-2,3-dideoxyglucose in lipopolysaccharides from slow-growing

members of Rhizobiaceae and from "*Pseudomonas carboxydovorans*." Arch. Microbiol. **151**:111–116.
38. **Mayer, H., and J. Weckesser.** 1984. Unusual lipid A's: structures, taxonomical relevance and potential value for endotoxin research, p. 221–227. *In* E. T. Rietschel (ed.), Handbook of endotoxin, vol. 1: chemistry of endotoxin. Elsevier, Amsterdam.
39. **Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, C. A. Dinarello, A. Cerami, S. M. Wolff, and D. W. Wilmore.** 1988. Detection of circulating tumor necrosis factor after endotoxin administration. N. Engl. J. Med. **318**:1481–1486.
40. **Movat, H. Z., M. I. Cybulsky, I. G. Colditz, M. K. W. Chan, and C. A. Dinarello.** 1987. Acute inflammation in gram-negative infection: endotoxin, interleukin 1, tumor necrosis factor, and neutrophils. Fed. Proc. **46**:97–104.
41. **Murphy, P. A., P. L. Simon, and W. F. Willoughby.** 1980. Endogenous pyrogens made by rabbit peritoneal exudate cells are identical with LAF's made by rabbit alveolar macrophages. J. Immunol. **124**:2498–2501.
42. **Okusawa, S., J. A. Gelfand, T. Ikejima, R. J. Connolly, and C. A. Dinarello.** 1988. Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. J. Clin. Invest. **81**:1162–1172.
43. **Old, L. J.** 1985. Tumor necrosis factor. Science **230**:630–633.
44. **Oppenheim, J. J., E. J. Kovacs, K. Matsushima, and S. K. Durum.** 1986. There is more than one interleukin 1. Immunol. Today **7**:45–56.
45. **Orencole, S. F., and C. A. Dinarello.** 1990. Characterization of a subclone (D10S) of the D10.G4.1 helper T-cell line which proliferates to attomolar concentrations of interleukin 1 in the absence of mitogens. Cytokines **1**:14–22.
46. **Rackow, E. C., M. E. Astiz, Y. B. Kim, and M. H. Weil.** 1989. Monophosphoryl lipid A blocks the hemodynamic effects of lethal endotoxemia. J. Lab. Clin. Med. **113**:112–117.
47. **Ramadori, G., J. V. Damme, H. Rieder, and K. Meyer zum Buschenfeld.** 1988. Interleukin 6, the third mediator of acute-phase reaction, modulates hepatic protein synthesis in human and mouse. Comparison with interleukin 1 beta and tumor necrosis factor-alpha. Eur. J. Immunol. **18**:1259–1264.
48. **Ramadori, G., J. D. Sipe, C. A. Dinarello, S. Mizel, and H. R. Colten.** 1985. Pretranslational modulation of acute phase hepatic protein synthesis by murine recombinant interleukin 1 (IL1) and purified human IL1. J. Exp. Med. **162**:930–942.
49. **Rietschel, E. T., L. Brade, K. Brandenburg, H.-D. Flad, J. De Jong-Leuveninck, K. Kawahara, B. Lindner, H. Loppnow, T. Lüderitz, U. Schade, U. Seydel, Z. Sidorczyk, A. Tacken, U. Zähringer, and H. Brade.** 1987. Chemical structure and biologic activity of bacterial and synthetic lipid A. Rev. Infect. Dis. **95**:527–536.
50. **Ruff, M. R., and G. E. Gifford.** 1980. Purification and physicochemical characterization of rabbit tumor necrosis factor. J. Immunol. **125**:1671–1677.
51. **Schmidt, J. A., S. B. Mizel, D. Cohen, and I. Green.** 1982. Interleukin 1, a potential regulator of fibroblast proliferation. J. Immunol. **128**:2177–2182.
52. **Strittmatter, W., J. Weckesser, P. V. Salimath, and C. Galanos.** 1983. Nontoxic lipopolysaccharide from *Rhodopseudomonas sphaeroides* ATCC 17023. J. Bacteriol. **155**:153–158.
53. **Tabor, D. R., S. K. Burchett, and R. F. Jacobs.** 1988. Enhanced production of monokines by canine alveolar macrophages in response to endotoxin-induced shock. Proc. Soc. Exp. Biol. Med. **187**:408–415.
54. **Takayama, K., N. Qureshi, B. Beutler, and T. N. Kirkland.** 1989. Diphosphoryl lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023 blocks induction of cachectin in macrophages by lipopolysaccharide. Infect. Immun. **57**:1336–1338.
55. **Tracey, J. J., B. Beutler, F. Lowry, J. Merryweather, S. Wolpe, W. Milsark, R. J. Hariri, T. J. Fahey III, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami.** 1986. Shock and tissue injury induced by recombinant human cachectin. Science **234**: 470–474.
56. **Tsai, C. N., and C. E. Frasch.** 1982. A sensitive silver stain for detecting LPS in polyacrylamide gels. Anal. Biochem. **119**:115–119.
57. **Urbanik-Sypniewska, T., U. Seydel, M. Greck, J. Weckesser, and H. Mayer.** 1989. Chemical studies on the lipopolysaccharide of *Rhizobium meliloti*. Arch. Microbiol. **152**:527–532.
58. **Vogel, S. N., E. N. Kaufmann, M. D. Tate, and R. Neta.** 1988. Recombinant interleukin 1a and recombinant tumor necrosis factor synergize in vivo to induce early endotoxin tolerance and associated hematopoietic changes. Infect. Immun. **56**:2650–2657.
59. **Waldstätten, P., R. Christian, G. Schulz, F. M. Unger, P. Kosma, and H. Paulsen.** 1983. Synthesis of oligosaccharides containing 3-deoxy-D-manno-2-octulopyranosylono (KDO) residues, p. 121. *In* L. M. Anderson and F. M. Unger (ed.), Bacterial lipopolysaccharides: structure, synthesis, and biological activities. American Chemical Society, Washington, D.C.
60. **Westphal, O., O. Lüderitz, C. Galanos, H. Mayer, and E. T. Rietschel.** 1985. The story of bacterial endotoxin, p. 13–34. *In* L. Chedid, J. W. Hadden, F. Spreafico, P. Dukor, and D. Willoughby (ed.), Advances in immunopharmacology (Proceedings of the Third International Conference on Immunopharmacology, Florence, Italy, 6–9 May 1985. Pergamon Press, New York.