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Biological Activities of *Brucella abortus* Lipopolysaccharides

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Purified lipopolysaccharide (LPS) from smooth (S) and rough (R) strains of *Brucella abortus* and lipid A isolated from S-LPS by mild acid hydrolysis were examined in several assays of biological activity. *Brucella* S- and R-LPSs and *Brucella* lipid A activated the complement cascade. Previously reported mitogenic activation by *Brucella* LPSs of spleen cells from endotoxin-resistant C3H/HeJ mice was confirmed and also produced by isolated *Brucella* lipid A. Mitogenicity was not inhibited by polymyxin B, and amino acid analysis showed no binding of polymyxin B to *Brucella* LPS under conditions in which mitogenicity of phenol-water-extracted *Escherichia coli* LPS was inhibited. S and R *Brucella* LPSs and lipid A all produced equivalent polyclonal stimulation of C3H/HeJ and C3H/HeAU spleen cells. Crude and purified LPS from S but not from R *B. abortus* was toxic for outbred mice, with 50% lethal doses approximately six times greater than that for *E. coli* LPS. S- and R-LPSs were abortifacient in pregnant outbred mice. S *Brucella* LPS was lethal for carrageenen-pretreated C3H/HeJ and C3H/HeAU mice, whereas only C3H/HeAU mice were killed by *E. coli* LPS. The data are consistent with the hypothesis that the unique fatty acid composition of *Brucella* lipid A is responsible for its biological activity in endotoxin-resistant C3H/HeJ mice. The participation of the protein strongly bound to the lipid A cannot be excluded, but its mode of action, if any, is different from that of the lipid A-associated protein of enterobacterial LPS.

A very large body of information has been developed over the past 20 years which relates biological activity and the structure of lipopolysaccharides (LPSs) of the *Enterobacteriaceae*. Most of the large constellation of biological effects which they exhibit have been associated with the lipid A moiety, which is quite uniform among the enterobacterial LPSs (5, 20). A number of lipid As have been recognized which have structural features different from those of enterobacterial lipid As, and they have also been shown to have unique biological properties (12, 36). Crude preparations of LPS from smooth (S) *Brucella* have been known for more than 15 years to share some of the biological properties of enterobacterial LPS and to be unique in other respects (8, 19), but systematic examination of chemical structure-biological activity relationships has been hampered by the difficulty in preparing highly purified and well-characterized LPSs from *Brucella*. We have recently described methods for purification of *Brucella* LPS and shown that it has a fatty acid composition quite distinct from that of enterobacterial LPS. Specifically, *Brucella* LPS lacks β-OH myristic acid, and it contains seven major fatty acids accounting for 85% of the total fatty acids, all of which have equivalent chain lengths of 16 or greater (24). In addition, S *Brucella* LPS has a strongly bound protein, accounting for approximately 6% of the purified preparations, which cannot be dissociated under conditions which remove the lipid A-associated protein (LAP) of enterobacterial LPS (23, 24). Purified rough (R) *Brucella* LPS, with the same fatty acid composition as S *Brucella* LPS, contains only 1.5% protein and has different physical properties. Purified S and R *Brucella* LPSs are both mitogenic for spleen cells of endotoxin-resistant C3H/HeJ mice, and activity is not proportional to protein content of the preparations. This led us to the conclusion that the unique fatty acid composition of the *Brucella* lipid A was probably the major factor responsible for some of the distinctive biological activities of *Brucella* LPS (23). We now report on experiments designed to define for the first time the activity of purified and well-characterized LPSs from S and R *B. abortus* and of the lipid A isolated from the S-LPS in several additional established endotoxin assays, and to examine further the possible role of the protein associated with *Brucella* lipid A in its biological activity.

MATERIALS AND METHODS

Mice. C3H/HeAU mice, originally obtained from Heston's laboratory in 1952, were provided by R. Auer-

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bach (Zoology Department, University of Wisconsin). Brother-sister mating of this strain has been continued in this department since 1977. BALB/c/BOM (nu/nu) mice, originally obtained from Ry, Denmark, were supplied by G. A. Splitter of this department. BALB/c (nu/nu) mice were purchased from the Gnotobiotic Laboratory, University of Wisconsin. C3H/HeJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. BALB/c and outbred ICR mice were obtained from this department's colony.

Bacterial cultures. The *Brucella* strains used and their characteristics and conditions of culture have been described previously (18).

Source and characteristics of LPSs. The extraction by phenol-water, purification, chemical analyses, and characterization of S-LPS from *Brucella abortus* 1119-3 and R-LPS from *B. abortus* 45/20 have been described previously (24). The same chemical analytical methods were used for this work. Two different preparations of S-LPS and one of R-LPS were used: (i) crude S-LPS (f5), (ii) purified S-LPS (f5p), (iii) purified R-LPS (R3a). In addition, endotoxin from *B. abortus* 1119-3 was extracted with trichloroacetic acid (3).

National Reference Standard Endotoxin from *Escherichia coli* O113 prepared by phenol-water extraction (PW-LPS) was obtained from the Bureau of Biologics, Food and Drug Administration. The characteristics of this preparation have been described by Rudbach et al. (31). Endotoxin from *E. coli* O128:B12 extracted with trichloroacetic acid (TCA-LPS) was purchased from Difco Laboratories, Detroit, Mich. *E. coli* LPS from serotype O128:B12, extracted with phenol-water (Difco), was purified by ultracentrifugation as described by Westphal and Jann (37). In addition, *E. coli* LPS from serotype O111:B4, extracted with trichloroacetic acid, and S-LPS from *Salmonella typhimurium*, extracted with phenol-water from Difco, were used for assay of anticomplementary action. Analyses for protein were not made.

Preparation of lipid A and specific degraded polysaccharide from LPS. *E. coli* lipid A was obtained by hydrolysis of *E. coli* O111:B4 PW-LPS with 1% acetic acid at 100°C for 1 h (11, 16). *Brucella* lipid A and the water-soluble degraded polysaccharide (AH) were obtained by the same method from f5p, except that hydrolysis for 5 h was required to release all of the lipid A. AH was purified by column chromatography on G-50 Sephadex with pyridine buffer, dialyzed, and lyophilized as described by Jann et al. (16).

The precipitate formed after acid hydrolysis, which was composed mainly of lipid A, was washed six times in double-distilled water, suspended in 5 ml of double-distilled water, and dissolved with 10 μl of triethylamine. The insoluble residue was sedimented at 10,000 × *g*; the supernatant fluids were removed, and the sediment was suspended in triethylamine as described above. The supernatant fluids were pooled, and the procedure was repeated an additional time. The excess triethylamine was removed from the pooled supernatant fluids containing solubilized lipid A by dialysis against double-distilled water for 2 weeks. Part of the soluble lipid A was stored at 4°C until used. The rest was lyophilized and either used for chemical analysis or adsorbed to bovine serum albumin (lipid A-BSA). *Brucella* lipid A contained 51% fatty acid amide plus fatty acid ester, 15% protein, and 12% carbohydrate; 2-keto-3-deoxyoctonate was not detectable by thiobarbituric acid assay (24).

Preparation of poly B. Crude polysaccharide B (poly B), the "native cytoplasmic polysaccharide" of R *B. melitensis* B115, was extracted with trichloroacetic acid by a modification of the method of Boivin and Mesrobeanu (3, 7). It contained 83% carbohydrate, 50% nucleic acids, and 5% protein. Poly B was freed of nucleic acid and protein by the following method: a suspension of 75 mg of lyophilized poly B in 9 ml of phosphate-buffered saline was treated with 0.75 mg of ribonuclease and 0.75 mg of deoxyribonuclease (both from Sigma), and the mixture was stirred for 18 h at room temperature and finally heated at 100°C for 2 h. The preparation was clarified by centrifugation at 800 × *g* for 15 min, followed by centrifugation at 106,000 × *g* for 6 h, and the supernatant fluid was finally lyophilized. It contained 96% carbohydrate, approximately 2% nucleic acids and <1% protein. 2-Keto-3-deoxyoctonate and lipid were not detectable.

Treatment of LPS with polymyxin B. LPS preparations were treated with polymyxin B sulfate (Sigma) as described by Morrison and Jacobs (26). Fifteen milligrams of polymyxin B was added to 1 ml of LPS solution at a concentration of 5 mg/ml. After 30 min at room temperature, the solution was dialyzed extensively against double-distilled water. Binding of polymyxin B to LPS was determined by automated analysis of hydrolysates, using ninhydrin reagent in a Durrum D-500 (Dionex) amino acid analyzer. The conditions of acid hydrolysis used permitted the detection of 5 nmol of each amino acid per sample, including α,γ-aminobutyric acid present in the polymyxin B standard (22).

Solubilization of R3a and lipid As. R3a and the lipid As were each solubilized by adsorption to BSA (R3a-BSA or lipid A-BSA) as described by Galanos et al. (11).

***Limulus* lysate gelation activity.** The *Limulus* lysate gelation assay was carried out as described by Sullivan and Watson (32). Activity was expressed as the lowest concentration in nanograms per milliliter needed to form a solid gel.

Anticomplementary activity. The test for the interaction of LPS and LPS-derived products with complement was performed by a modification of the method of Galanos and Lüderitz (10). Fresh guinea pig serum from eight animals was pooled and absorbed with sheep erythrocytes at 0°C, distributed in 1-ml quantities, and stored at −60°C. Amounts of LPS or product ranging from 1 to 100 μg in 20 μl of pyrogen-free distilled water were incubated with 100 μl of fresh guinea pig serum diluted 1:2 with barbital buffer (pH 7.4) at 37°C for 30 min. The samples were then diluted with 400 μl of buffer, and 20 μl of diluted sample was added to 1.5 ml of barbital buffer, containing 2.5×10^8 sensitized sheep erythrocytes, and incubated at 37°C for 1 h. The samples were centrifuged at 4°C, and hemoglobin release was recorded as absorbance by the supernatant fluids at 546 nm. Under these conditions, the hemolytic activity of the complement without LPS corresponded to lysis of 40 to 50% of the standard

erythrocyte suspension. Anticomplementary activity was recorded as the percentage decrease in lysis from the control level of 40 to 50%.

Assays for mitogenic and polyclonal activation. RPMI 1640 (GIBCO, Grand Island, N.Y.) was the culture medium used in all experiments. The conditions of culture for mitogenic activation have been described previously (23). For assay of polyclonal activity, 5×10^6 murine spleen cells in 1 ml of medium were cultured with the appropriate concentration of LPS or lipid A at 37°C in an atmosphere of 5% CO_2 in air for 48 h. Controls were cultured in medium alone. The cells were washed two times at 4°C with Dulbecco balanced salt solution (29) and suspended in this solution to a final concentration of 10^7 cells per ml. A volume of 0.1 ml of the cell suspension (10^6 cells) was assayed for direct plaque-forming cells against trinitrophenylated sheep erythrocytes (17).

Toxicity for mice. The minimum lethal dose was determined by intravenous injection of inbred mice, weighing 20 to 22 g, with 0.1 ml of the appropriate LPS concentration.

The 50% lethal dose for outbred ICR mice was determined by a modification of the method of Becker and Rudbach (1). Mice weighing 20 to 22 g were injected intraperitoneally with 0.5 ml of a solution of carrageenan (Sigma) at a concentration of 20 mg/ml in pyrogen-free phosphate-buffered saline. After 1 h, 0.2-ml volumes of different concentrations of LPS were injected intraperitoneally. Deaths were recorded at 12, 24, 48, and 72 h, and the 50% lethal dose was determined by the probit method (4).

Abortions were induced by LPS in female mice in week 3 of pregnancy. The mice were injected intravenously with 0.1 ml of the appropriate LPS concentration. Deaths of dams, abortions, death of the fetus in utero, or live births were recorded each 6 h after injection.

RESULTS

The protein content and *Limulus* lysate gelation activity of the LPS preparations, lipid A, poly B, and AH are shown in Table 1.

Anticomplementary activity. The anticomplementary activities of different LPSs and LPS-derived products are presented in Table 2. Lipid A-BSA from *E. coli* and LPSs from *E. coli* and *S. typhimurium* had more anticomplementary activity than the *Brucella* preparations. Two exceptions were observed: National Standard Endotoxin from *E. coli* (PW-LPS), which had anticomplementary activity similar to that of *Brucella* R-LPS, and soluble lipid A from S *B. abortus* 1119-3, which had anticomplementary activity similar to those of enterobacterial LPSs from commercial sources. *Brucella* R-LPS had more anticomplementary activity than *Brucella* S-LPS. Practically no anticomplementary activity was observed with either AH or poly B.

The maximum level of anticomplementary activity for *Brucella* preparations was reached at concentrations of 50 μg; however, for *E. coli* and *S. typhimurium* LPSs, as well as lipid A-BSA from *E. coli*, the maximum level was at concentrations between 10 and 50 μg (Fig. 1). In agreement with the observations of Galanos and Luderitz (10), higher concentrations of LPS or lipid A-BSA produced spontaneous hemolysis of the erythrocytes.

Mitogenicity. Since polymyxin B has been shown to inhibit the mitogenicity of *E. coli* PW-

TABLE 1. *Protein content and Limulus lysate gelation activity of LPS and lipid A preparations*

Prepn	Protein (%)	*Limulus* lysate gelation activity (ng/ml)[a]
B. abortus f5	24.0	1.0
B. abortus f5p	6.3	0.1
B. abortus TCA-LPS	19.0	ND[b]
B. abortus R3a	1.5	1.0
B. abortus lipid A[c]	15.0	0.2
B. abortus lipid A[d]	ND	1.0
B. abortus AH	2.0	10^4
B. melitensis poly B	<1.0	10^4
E. coli O113 PW-LPS	5.4	0.1
E. coli O128:B12 PW-LPS	4.4	ND
E. coli O128:B12 TCA-LPS	8.2	ND

[a] Amount of preparation required for a solid gel.
[b] ND, Not determined.
[c] Soluble lipid A.
[d] Lipid A-BSA expressed as lipid A.

TABLE 2. *Anticomplementary activity of LPSs and LPS-derived products*

Prepn (50 μg)	% Loss of hemolytic activity ± SE[a]
E. coli PW lipid A-BSA[b]	100 ± 0
S. typhimurium PW S-LPS	88.2 ± 5.2
E. coli S TCA-LPS	80.0 ± 5.8
B. abortus soluble lipid A	65.7 ± 7.6
B. abortus S TCA-LPS	31.4 ± 14.9
B. abortus R3a-BSA[b]	31.1 ± 3.1
B. abortus R3a-BSA[b]	29.5 ± 9.0
E. coli PW S-LPS	28.4 ± 2.1
B. abortus R3a-BSA[b]	20.6 ± 7.2
B. abortus S TCA-LPS	20.5 ± 7.5
B. abortus f5	18.6 ± 7.0
B. abortus lipid A-BSA[b]	15.6 ± 2.0
B. abortus f5p	14.6 ± 1.0
B. abortus f5p	12.8 ± 2.5
B. abortus f5p	12.0 ± 0.8
B. abortus f5	11.3 ± 1.9
B. abortus f5p	11.0 ± 2.2
B. abortus lipid A-BSA[b]	9.2 ± 3.3
B. melitensis poly B	5.3 ± 6.0
B. abortus AH	2.2 ± 3.0

[a] SE, Standard error, calculated from 12 different assays.
[b] Expressed as 50 μg of lipid A or R3a.

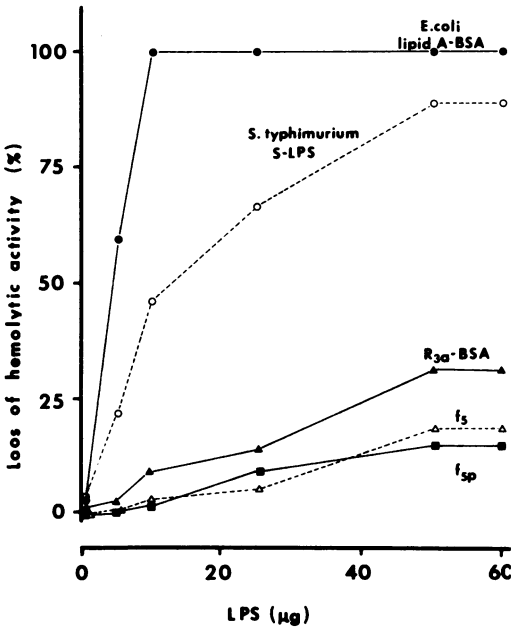

FIG. 1. *Anticomplementary activity of LPSs and E. coli lipid A-BSA. Increasing amounts of each preparation were incubated with guinea pig complement, and percentage loss of hemolytic activity was measured. E. coli lipid A-BSA* (●), *S. typhimurium S-LPS* (○), *B. abortus R3a-BSA* (▲), *B. abortus f5* (△), *and B. abortus f5p* (■).

LPS but not of TCA-LPS (25; S. J. Betz, PhD Thesis, University of Arizona, Tucson, 1978), its effects on the mitogenicity of *Brucella* LPS preparations were investigated. The results obtained in a single typical experiment with spleen cells of C3H/HeAU and C3H/HeJ mice are shown in Fig. 2. Polymyxin B was able to inhibit mitogenic induction of C3H/HeAU cells by *E. coli* PW-LPS completely. Although the mitogenic activity of *E. coli* TCA-LPS was reduced considerably, it was not inhibited completely. The mitogenicity of f5 or f5p of *B. abortus* was not affected by treatment with polymyxin B. R3a-BSA was insoluble in the presence of polymyxin B, so it was not possible to test the effect of the antibiotic on its mitogenicity.

The relative amounts and amino acid composition of f5p and R3a before and after treatment with polymyxin B were determined in order to establish whether its lack of effect on mitogenicity was the result of failure of polymyxin B to bind. None of the preparations contained α,γ-diaminobutyric acid, the characteristic amino acid of polymyxin B, nor were there significant changes in the relative amounts of the other amino acids.

The mitogenic activity of soluble *Brucella* lipid A and lipid A-BSA at different concentrations is shown in Fig. 3. Higher doses of these lipid A preparations were not tested because of the large quantities required. The responses of the C3H/HeJ cells to *E. coli* TCA-LPS were unusually low in this experiment as compared with responses usually seen in comparable experiments (Fig. 2 and reference 23), but the responses were significantly greater than those seen in control cultures. Mitogenic activity was not observed with AH or poly B at concentrations of 50 to 100 μg/ml.

Polyclonal activation. Data from a typical experiment on polyclonal activation of spleen cells of C3H/HeAU, C3H/HeJ, and nude mice by f5p, R3a-BSA, soluble *Brucella* lipid A, and *E. coli* PW-LPS are presented in Table 3. Maximum polyclonal activation was observed at concentrations of 50 and 100 μg of LPS or soluble lipid A per 5×10^6 cells in 1 ml of medium.

Toxicity. Lambda carrageenan was the most powerful of the three types tested in potentiating the toxicity of LPS. However, it was also the most toxic, killing 25 to 50% of outbred ICR control mice within 48 h, at concentrations between 0.8 and 1 mg of carrageenan per mouse. Kappa carrageenan had little toxicity for mice and little potentiating activity for the LPSs used. Iota carrageenan was the most useful potentiating agent. It had low toxicity for control mice and relatively good potentiating activity, especially for *E. coli* LPS; 10 mg of iota carrageenan per mouse potentiated the toxicity of *E. coli* LPS 50- to 100-fold. A combination of 0.5 mg of lambda carrageenan with 9.5 mg of iota carrageenan per mouse was found to potentiate the toxic activity of *E. coli* and *Brucella* LPSs optimally (Table 4). Although appropriate doses of *E. coli* LPS killed 100% of the mice pretreated with the combination of carrageenans, *Brucella* S-LPS did not. In groups of mice given as much as 200 to 400 μg of *Brucella* f5 or f5p, all showed signs of toxicity and up to 90% died, but there were always some survivors. Similarly, *Brucella* lipid A, injected as lipid A-BSA in concentrations between 100 and 200 μg per mouse, induced signs of toxicity in most of the carrageenan-pretreated animals, but only 3 of 10 mice injected with 200 μg of lipid A-BSA died.

The minimum lethal dose of f5 and f5p varied depending upon the strain of mice used. BALB/c mice required about 1 mg of f5 per mouse, but C3H/HeJ and C3H/HeAU mice required about 1.5 mg per mouse. With f5p, C3H/HeJ mice required approximately 0.5 mg more than the other two inbred strains (Table 4). The minimum lethal dose with *E. coli* PW-LPS in C3H/HeAU mice was about 0.8 mg per mouse. Con-

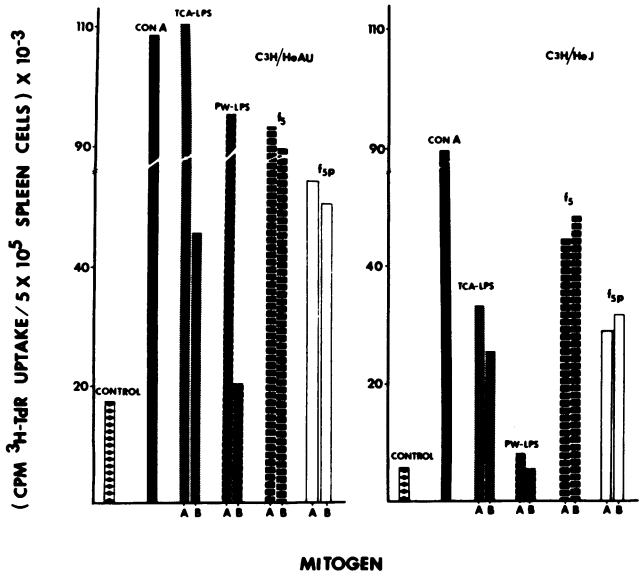

FIG. 2. *Effect of polymyxin B on mitogenic induction by different preparations of LPS. Spleen cells (5 × 10^5) were cultured in presence of 100 μg of LPS. Results are expressed as mean counts per minute (cpm) of [^{3}H]thymidine (^{3}H-TdR). Standard error with each LPS preparation was less than 10%. (A) LPS and (B) LPS treated with polymyxin B.*

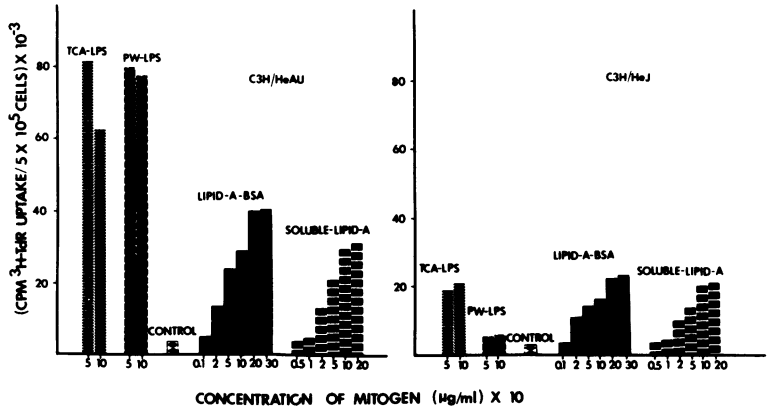

FIG. 3. *Mitogenicity of LPS preparations from E. coli and soluble lipid A and lipid A-BSA from B. abortus f5p for spleen cells from C3H/HeAU and C3H/HeJ mice. Spleen cells (5 × 10^5) were cultured in the presence of various concentrations of mitogen. Results are expressed as mean counts per minute (cpm) of [^{3}H]thymidine (^{3}H-TdR). Standard error with each preparation was less than 10%.*

sistent with the reports of others (28, 33), 2 mg of LAP-free *E. coli* LPS (PW-LPS) did not kill C3H/HeJ mice.

Table 5 shows the abortifacient and toxic effects of *E. coli* TCA-LPS, f5, f5p, and R3a-BSA in pregnant ICR mice. *E. coli* TCA-LPS was more toxic than any of the *Brucella* LPSs. R3a-BSA, which was not toxic in inbred mice pretreated with carrageenan at concentrations of 800 μg of R3a-BSA per mouse (Table 4), did induce abortions in pregnant ICR mice at concentrations of 2 mg of R3a-BSA per mouse.

DISCUSSION

LPS from *Enterobacteriaceae* can activate the complement cascade by both the alternate and classical pathways, with lipid A responsible for activation of the classical pathway and the polysaccharide moiety responsible for activation of the alternate pathway (6, 27). It has also been demonstrated (9, 10) that the degree of aggregation and the solubility of LPS are important factors in its anticomplementary activity. The unique fatty acid and carbohydrate composition

of *Brucella* LPS, together with its very limited solubility in water, may be factors which contribute to its low anticomplementary activity compared with enterobacterial LPS. Differences in degree of aggregation may also explain why the highly purified, phenol-water-extracted National Standard endotoxin from *E. coli* (31) had less anticomplementary activity than the less purified commercial preparations of *E. coli* and *S. typhimurium* LPS. This also may explain why the *Brucella* R3a-BSA had more anticomplementary activity than several lots of f5 and f5p, as well as the striking differences in anticomplementary activity produced by soluble *Brucella* lipid A and lipid A-BSA.

The difficulty of establishing the immunostimulatory role of the protein strongly bound to *Brucella* LPS and lipid A is shown by the experiments on mitogenicity and polyclonal antibody production. Jacobs and Morrison (15, 25) concluded that polymyxin B inhibits mitogenic activity of LAP-free LPS from *E. coli* or *Salmonella* by binding to the lipid A–2-keto-3-deoxyoctonate moiety of the LPS molecule. Betz and Morrison (2) also showed that polymyxin B did not inhibit the mitogenicity of isolated LAP, and they proposed this as a useful differential characteristic. The failure of polymyxin B to reduce the mitogenic activity of *Brucella* LPSs is in sharp contrast with the complete inhibition of mitogenic activity of *E. coli* PW-LPS and the reduction of activity of *E. coli* TCA-LPS (Fig. 2). The inability of polymyxin B to bind to f5 and f5p, as shown by amino acid analysis, constitutes additional evidence for the unique struc-

TABLE 3. *Polyclonal activation of murine spleen cells by LPS preparations and lipid A*

Prepn (100 μg/ml)	Mean plaque-forming cells per 10^6 spleen cells from mouse strain[a]:		
	C3H/HeAU	C3H/HeJ	BALB/c (nu/nu)
Control	24 ± 9	12 ± 1	20 ± 6
f5p	113 ± 19	181 ± 10	234 ± 34
R3a-BSA[b]	113 ± 13	137 ± 9	173 ± 27
Soluble lipid A	134 ± 5	187 ± 11	174 ± 5
E. coli PW-LPS	125 ± 4	10 ± 5	183 ± 16

[a] Numbers are mean number of plaque-forming cells per 10^6 viable spleen cells ± standard error from triplicate assays.
[b] Expressed as 100 μg of R3a per ml.

TABLE 4. *Lethal toxicity of LPSs for mice*[a]

Prepn	LD_{50} (μg)[b] for ICR mice	Minimum lethal dose (mg) for mouse strain:		
		C3H/HeAU	C3H/HeJ	BALB/c
E. coli PW-LPS	9	0.8	>2.0	0.8
E. coli TCA-LPS	9	ND[c]	ND	ND
B. abortus f5	52	1.5	1.5	1.0
B. abortus f5p[d]	70	ND	ND	ND
B. abortus f5p[e]	50	1.5	2.0	1.5
B. abortus R3a-BSA[f]	>800	>2.0	>2.0	>2.0
B. abortus AH	>500	>500	ND	ND
B. melitensis poly B	>500	>500	ND	ND

[a] Ten mice (20 to 22 g) per dose of LPS.
[b] LD_{50}, 50% lethal dose, determined from mice preinjected intraperitoneally with 0.5 mg of lambda carrageenan + 9.5 mg of iota carrageenan.
[c] ND, Not done.
[d] Lot 1, 7% protein.
[e] Lot 2, 6.3% protein.
[f] Expressed as R3a-BSA.

TABLE 5. *Effects of intravenous injection of LPS in pregnant ICR mice*

LPS prepn	Dose (mg)	No. of mice				
		Aborted	Aborted and died	Died	Fetal death	Live birth
E. coli TCA-LPS	0.25	3	5	3	1	0
B. abortus f5	0.25	2	1	1	4	2
	0.5	4	4	2	0	0
B. abortus f5p (lot 1)	0.25	0	0	1	6	3
	0.5	2	1	0	2	5
B. abortus f5p (lot 2)	0.5	5	1	0	0	0
B. abortus R3a-BSA[a]	2.0	4	0	0	1	4

[a] Expressed as R3a.

ture of the lipid A of *Brucella* LPS.

Betz and Morrison (2) showed that isolated enterobacterial LAP preparations had a characteristic dose-response profile in mitogenicity assays with both C3H/HeSt and C3H/HeJ cells. Optimal stimulation was at a concentration of approximately 10 μg of LAP per ml; higher concentrations were toxic and resulted in sharply less [^{3}H]thymidine incorporation. They presented evidence for slight inhibition of mitogenicity at high doses of LAP-LPS complex, but not with high doses of LAP-free LPS. Similar results were reported by Izui et al. (14). In our experiments, the dose-response profile of the mitogenic response induced by *Brucella* lipid A-BSA in spleen cells of C3H/HeAU and C3H/HeJ mice was similar to that previously observed with f5p but not with R3a-BSA or *E. coli* PW-LPS (23). That is, concentrations of lipid A-BSA or f5p greater than 100 μg/ml did not depress the mitogenic response of the cells, whereas the mitogenic response to R3a-BSA, *E. coli* PW-LPS, or *E. coli* TCA-LPS at concentrations greater than 100 μg/ml was regularly depressed below that observed with 100 μg/ml. Thus, there was no clear relationship between the protein content of the *Brucella* LPS or lipid A preparations and their dose-response profiles in the mitogenicity assay.

Similarly, even though polyclonal activation of the C3H/HeJ spleen cells by *Brucella* f5p, its lipid A, and R3a was not proportional to their respective protein content (Table 3), we cannot rigorously exclude the possibility that an LAP-like protein participates in these in vitro reactions. Although there is a close biological linkage between in vitro mitogenic activation and polyclonal activation (13), they are the result of similar but not identical phenomena (21). Furthermore, several investigators have shown that there is a lack of correlation between in vitro B-lymphocyte proliferative responses and in vivo immunostimulatory responses to enterobacterial LPS and LPS-LAP complex. For example, LPS-LAP complex, but not LPS, functioned as a mitogen and polyclonal B cell activator for C3H/HeJ cells in vitro, but neither was an effective adjuvant when injected after aggregated human gamma globulin into C3H/HeJ mice (13). It has also been shown that although LAP might contribute to some of the in vivo responses of rabbits or mice to enterobacterial endotoxin effects, large doses of either LPS-LAP complex or LPS were not toxic for C3H/HeJ mice (28).

The demonstration, in the experiments reported here, that f5 and f5p are toxic for both C3H/HeAU and C3H/HeJ mice is the strongest evidence in support of our hypothesis that the unique fatty acid composition of *Brucella* lipid A is responsible for its activity on cells of C3H/HeJ mice in vitro as well as in vivo. At the very least, these results indicate that if the strongly bound protein of *Brucella* LPS does contribute to its endotoxin activity, its mode of action is apparently different from that of enterobacterial LAP.

Becker and Rudbach (1) reported that mice pretreated with carrageenan were between 1,000- and 2,000-fold more sensitive to the toxic effects of *E. coli* LPS than normal mice. We observed only a 50- to 100-fold increase in sensitivity to both *Brucella* and *E. coli* LPSs, including the *E. coli* PW-LPS which was prepared by Rudbach et al. (31). There is no obvious explanation for the failure of high concentrations of *Brucella* f5 or f5p to kill 100% of the carrageenan-pretreated mice. This finding is not unique since the same phenomenon has been observed with other endotoxin preparations from *Brucella* and with acetone-killed *Brucella* cells (18). The lack of toxicity of *Brucella* endotoxin for chicken embryos and rabbits, its very low pyrogenicity (8, 19), and the generally lower toxicity of *Brucella* S-LPS for mice are all consistent with the hypothesis that the mediation of its toxicity may be unique.

In agreement with the reports of Urbaschek (34, 35), crude *Brucella* S-LPS (f5) was abortifacient for pregnant mice, as was f5p. The difference in activity observed between the two lots of f5p may be due to the particular preparations used, since in these experiments the fractions came from different batches of f5.

Our finding that R3a did not kill mice was unexpected, as it had previously been shown in this laboratory that a hot-saline extract of *Brucella ovis* containing R-LPS and approximately 70% protein was toxic for mice (18). In contrast, R3a, with 1.5% protein, 27% lipid, and high *Limulus* lysate gelation activity, and which was a mitogen and a potent polyclonal activator for mouse spleen cells, did not kill mice. This result is apparently in conflict with our hypothesis that both mitogenicity and toxicity are related to the unique fatty acid composition of the lipid As from S and R *Brucella* LPSs. However, *Brucella* lipid A (15% protein) was less toxic than the f5p (6.3% protein) from which it was prepared, killing only 3 of 10 carrageenan-pretreated mice at a dose of 100 μg of lipid A. It is likely that the state of aggregation of the LPSs and lipid A is modified by the presence or absence of O polysaccharide or of particular proteins and that this influences its endotoxic activity (30), as is also the case with anticomplementary activity (10).

To resolve the unsettled questions, it will be necessary to do further work, including experiments on adjuvant activity and effects on toler-

ance, with preparations of *Brucella* lipid A obtained from R3a with its low protein content, as well as with reconstituted complexes of f5p or R3a with proteins isolated from the crude LPS extracts. Isolation and characterization of *Brucella* LAP are likely to be much more difficult than has been true of enterobacterial LAP, since its binding to the LPS and to lipid A resists conditions which dissociate enterobacterial LAP-LPS complexes (24).

The unusual behavior of *Brucella* LPS in the endotoxin-resistant C3H/HeJ mice opens new possibilities in understanding the immunoregulatory activity of this class of bacterial macromolecules.

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LITERATURE CITED

1. **Becker, L. J., and J. A. Rudbach.** 1978. Potentiation of endotoxicity by carrageenan. Infect. Immun. **19**:1099–1100.
2. **Betz, S. J., and D. C. Morrison.** 1977. Chemical and biologic properties of a protein-rich fraction of bacterial lipopolysaccharides. I. The *in vitro* murine lymphocyte response. J. Immunol. **119**:1475–1481.
3. **Boivin, A., and L. Mesrobeanu.** 1934. Remarques concernant la technique d'extraction du complexe polysaccharidique, antigènique refermé dans le bacille d'Aertrycke. C. R. Soc. Biol. **115**:304–306.
4. **Boyd, W. C.** 1966. Fundamentals of immunology, 4th ed. p. 703–733. Interscience Publishers, New York.
5. **Bradley, S. G.** 1979. Cellular and molecular mechanisms of action of bacterial endotoxins. Annu. Rev. Microbiol. **33**:67–94.
6. **Cooper, N. R., and D. C. Morrison.** 1978. Binding and activation of the first component of human complement by the lipid A region of lipopolysaccharides. J. Immunol. **120**:1862–1868.
7. **Diaz, R., P. Garatea, L. M. Jones, and I. Moriyon.** 1979. Radial immunodiffusion test with a *Brucella* polysaccharide antigen for differentiating infected from vaccinated cattle. J. Clin. Microbiol. **10**:37–41.
8. **Diaz, R., and M. A. Oyeledun.** 1977. Studies of some biological activities of *Brucella* endotoxin in normal and infected animals and the role of the hypersensitivity factor. Ann. Sclavo **19**:117–130.
9. **Galanos, C.** 1975. Physical state and biological activity of lipopolysaccharides. Toxicity and immunogenicity of the lipid A component. Z. Immunitaetsforsch. **149**:214–229.
10. **Galanos, C., and O. Lüderitz.** 1976. The role of the physical state of lipopolysaccharides in the interaction with complement. Eur. J. Biochem. **65**:403–408.
11. **Galanos, C., E. T. Rietschel, O. Lüderitz, and O. Westphal.** 1972. Biological activities of lipid A complexed with bovine serum albumin. Eur. J. Biochem. **31**:230–233.
12. **Galanos, C., J. Roppel, J. Weckesser, E. T. Rietschel, and E. H. Mayer.** 1977. Biological activities of lipopolysaccharides and lipid A from *Rhodospirillaceae*. Infect. Immun. **16**:407–412.
13. **Goodman, M. G., D. E. Parks, and W. O. Weigle.** 1978. Immunologic responsiveness of the C3H/HeJ mouse: differential ability of butanol-extracted lipopolysaccharide (LPS) to evoke LPS-mediated effects. J. Exp. Med. **147**:800–813.
14. **Izui, S., D. C. Morrison, B. Curry, and F. J. Dixon.** 1980. Effect of lipid A-associated protein on the expression of lipopolysaccharide activity. Immunology **49**: 473–482.
15. **Jacobs, D., and D. C. Morrison.** 1976. Lymphocyte triggering by bacterial LPS. Dissociation between immunogenicity of TNP-LPS and mitogenic and complement activating properties, p. 89–96. *In* V. P. Eysvoogel, D. Roos, and W. O. Zeijlemaker (ed.), Leukocyte membrane determinants regulating immune reactivity. Academic Press, Inc., New York.
16. **Jann, B., K. Reske, and K. Jann.** 1975. Heterogeneity of lipopolysaccharides. Analysis of polysaccharide chain lengths by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Biochemistry **60**:239–246.
17. **Jerne, N. K., C. Henry, A. A. Norden, H. Fuji, A. M. C. Koros, and I. Lefkovits.** 1974. Plaque forming cells: methodology and theory. Transplant. Rev. **18**:180–191.
18. **Jones, L. M., R. Diaz, and D. T. Berman.** 1976. Endotoxin activity of rough organisms of *Brucella* species. Infect. Immun. **6**:1638–1641.
19. **Leong, D., R. Diaz, K. Milner, J. Rudbach, and J. B. Wilson.** 1970. Some structural and biological properties of *Brucella* endotoxin. Infect. Immun. **1**:174–182.
20. **Lüderitz, O., C. Galanos, V. Lehmann, H. Mayer, E. T. Rietschel, and J. Weckesser.** 1978. Chemical structure and biological activities of lipid A's from various bacterial families. Naturwissenschaften **65**:578–585.
21. **Möller, G., A. Coutinho, E. Gronowicz, L. Hammerström, and E. Smith.** 1976. Role of mitogenic components of thymus-independent antigens, p. 291. *In* J. J. Oppenheim and D. L. Rosenstreich (ed.), Mitogens in immunobiology. Academic Press, New York.
22. **Moore, S.** 1972. The precision and sensitivity of amino acid analysis, p. 629. *In* J. Meienhofer (ed.), Chemistry and biology of peptides: Proceedings of the Third American Peptide Symposium. Ann Arbor Science Publishers, Inc., Ann Arbor, Mich.
23. **Moreno, E., and D. T. Berman.** 1979. *Brucella abortus* lipopolysaccharide is mitogenic for spleen cells of endotoxin-resistant C3H/HeJ mice. J. Immunol. **123**:2915–2919.
24. **Moreno, E., M. W. Pitt, L. M. Jones, G. G. Schurig, and D. T. Berman.** 1979. Purification and characterization of smooth and rough lipopolysaccharides from *Brucella abortus*. J. Bacteriol. **138**:361–369.
25. **Morrison, D. C., S. J. Betz, and D. M. Jacobs.** 1976. Isolation of a lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C3H/HeJ spleen cells. J. Exp. Med. **114**:840–846.
26. **Morrison, D. C., and D. M. Jacobs.** 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry **13**:813–818.
27. **Morrison, D. C., and L. F. Kline.** 1977. Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides (LPS). J. Immunol. **118**: 362–369.
28. **Morrison, D. C., M. E. Wilson, S. Raziuddin, S. J. Betz, B. J. Curry, Z. Oades, and P. Munkenbeck.** 1980. Influence of lipid A-associated protein on endotoxin stimulation of nonlymphoid cells, p. 30–35. *In* D. Schlessinger (ed.), Microbiology—1980. American So-

ciety for Microbiology, Washington, D.C.
29. **Paul, J.** 1975. Media for culturing cells and tissues, p. 90–123. *In* Cell and tissue culture. Churchill Livingstone, Edinburgh.
30. **Ribi, E., R. L. Anacker, R. Brown, W. T. Haskins, B. Malmgren, K. C. Milner, and J. A. Rudbach.** 1966. Reaction of endotoxin and surfactants. J. Bacteriol. **92:** 1492–1509.
31. **Rudbach, J. A., F. I. Akiya, R. J. Elin, H. D. Hochstein, M. K. Luoma, E. C. B. Milner, K. C. Milner, and K. R. Thomas.** 1976. Preparation and properties of a national reference endotoxin. J. Clin. Microbiol. **3:** 21–25.
32. **Sullivan, J. D., Jr., and S. W. Watson.** 1974. Factors affecting the sensitivity of *Limulus* lysate. Appl. Microbiol. **28:**1023–1026.
33. **Sultzer, B. M.** 1972. Genetic control of host responses to endotoxin. Infect. Immun. **5:**107–113.
34. **Urbaschek, B.** 1961. Versuche mit der abortauslösenden Lipopolysaccharid-Fraktion aus *Brucella abortus*. Zentralbl. Veterinaermed. **8:**150–157.
35. **Urbaschek, B.** 1964. Motility-promoting effect of the *Brucella abortus* and *Brucella melitensis* endotoxin on the smooth uterine muscle. Nature (London) **202:**883–884.
36. **Weckesser, J., G. Drews, and H. Mayer.** 1979. Lipopolysaccharides of photosynthetic prokaryotes. Annu. Rev. Microbiol. **33:**215–239.
37. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.