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Endotoxic Activity of Rough Organisms of *Brucella* Species

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A rough-specific antigen extracted from the rough species *Brucella ovis* and lipopolysaccharide extracted from smooth *Brucella abortus* demonstrated equivalent levels of activity in tests for mouse lethality and limulus lysate clotting activity. Acetone-extracted whole cells of *B. ovis* and of *B. canis* and of a rough mutant of *B. abortus* had the same toxicity for mice, but it was not possible to extract endotoxin from *B. canis* by the methods used.

Lipopolysaccharides (LPS) extracted from smooth (S) *Brucellae* possess many structural and biological properties in common with the endotoxins of the *Enterobacteriaceae* (15). Although LPS (glycolipids) obtained from rough (R) mutants of the salmonellae are as toxic as LPS from smooth strains (13), there has been no evidence of endotoxin activity in whole cells or extracts from R strains of *Brucellae* (2, 11, 18), but the investigations have been limited in scope.

In the course of experiments with the R-specific antigen, which can be extracted in 0.15 M NaCl at 100 C from the R species *Brucella ovis* (10, 17), we found it to be as toxic for mice as the LPS of S *Brucella abortus*. Also, the R-specific antigen and the S-LPS were found to have equivalent potency in the limulus lysate clotting assay (19). This is the first time that endotoxic activity has been associated with an extract of R *Brucella*, and it opens up possibilities of more completely characterizing the *Brucella* endotoxin in terms of its similarities as well as its differences from endotoxin from the *Enterobacteriaceae*.

The work described here was designed to reexamine the toxicity of whole cells and of extracts of R variants of *B. abortus* and *Brucella melitensis*, as well as of the R species *Brucella ovis* and *Brucella canis*.

MATERIALS AND METHODS

Bacterial strains. *B. abortus* S strain 11-19 is used by the U.S. Department of Agriculture for producing agglutination test antigens (1). *B. abortus* R strain 45/20, *B. melitensis* R strain B115, and two strains of the rough species *B. ovis* REO 198 and *B. canis* RM666 have been used in our previous studies (7).

Cells were propagated in liquid medium in a fermenter as described (1) or in shake flasks in Trypticase soy broth (BBL) with the addition of 2% sterile fetal calf serum.

B. abortus 11-19, *B. melitensis* B115, and *B. canis* RM666 were grown in a fermenter at the Veterinary Services Laboratory, U.S. Department of Agriculture, Ames, Iowa, and were shipped to Madison in cold storage as a thick slurry in water. *B. ovis* REO 198 was propagated in a fermenter at the Station of Pathology of Reproduction, INRA, Nouzilly, France.

Bacterial preparations. For whole-cell preparations, the harvested cells were washed in saline three times, then extracted three times in cold acetone, and lyophilized.

The hot-saline extract of *B. ovis* REO 198 was prepared in France as described (10). Briefly, the method consisted of subjecting a suspension of cells in 0.15 M NaCl to flowing steam for 30 min, centrifuging at 10,000 × *g*, dialyzing and concentrating the supernatant fluid, and then centrifuging at 105,000 × *g* for 6 h. The pellet was reconstituted in water and lyophilized, and this contained the R-specific antigen. A second small preparation was made in Madison by the same method, except that the cells were grown in shake flasks. Sterility was checked at each stage of preparation.

B. canis cells grown in a fermenter were extracted by the hot-saline method described above. *B. abortus* 11-19 cells, grown in a fermenter, were extracted by the hot phenol-water method as modified for brucella by M. S. Redfearn (Ph.D. thesis, Univ. of Wisconsin, Madison, 1960) and Leong et al. (15). Material from both the aqueous and phenol phases was lyophilized and examined for endotoxic activity.

Cells of *B. ovis*, *B. canis*, *B. abortus* 45/20, and *B. melitensis* B115 grown in shake flasks were washed in water, treated successively with ethanol, acetone, and twice with ether, and dried in vacuo. They were then treated with an extraction mixture containing liquid phenol, chloroform, and petroleum ether according to the method of Galanos et al. (8). The antigens which were obtained were insoluble in water, but suspensions injected into rabbits stimulated production of antisera with R specificity (6). For toxicity studies in mice, the suspended particles were homogenized with an MSE 150-W sonic vibrator and injected intraperitoneally. These preparations are designated as R(G) antigens.

A commercial preparation of *Escherichia coli* O127:B8 LPS (Difco) served as a control for endotoxin tests.

Mouse lethality tests. The mice were CF1 females weighing 22 g obtained from ARS Sprague Dawley, Madison, Wis. They were injected with 0.2 ml of the preparation in pyrogen-free water. They were observed several times a day for characteristic signs of toxicity, and the total number of dead 48 h after injection was recorded. The intraperitoneal route of injection was generally used. Comparative studies with the S- and R-soluble antigens and the whole cells of *Brucella* had shown that there were no significant differences in the results between the intravenous and intraperitoneal routes.

Limulus lysate clotting activity. Limulus lysate was obtained from Associates of Cape Cod, Inc., Woods Hole, Mass. All dilutions were made in pyrogen-free water (Travenol) with oven-heated glassware or pyrogen-free disposable tubes and pipettes (Falcon). The assay was performed as described by Sullivan and Watson (19).

RESULTS

Mouse lethality of whole cells of *Brucella*. There were significant differences in mouse lethality among the various preparations of R *Brucella* cells (Table 1). *B. ovis* and *B. canis* cells were of approximately equal toxicity, with 90 to 100% of mice being killed by 20 mg. *B. abortus* 45/20 was less toxic, and *B. melitensis* B115 cells did not kill mice at the 20-mg level. On a weight basis, the smooth *B. abortus* cells were approximately twice as toxic as the rough.

Endotoxic activity of soluble antigens of *Brucella*. Table 2 shows that the R-specific antigen from R *B. ovis* had the same level of activity in mouse toxicity and limulus lysate tests as the S-LPS in the phenol phase of the phenol-water extract of S *B. abortus*. In contrast, the material in the aqueous phase extracts of S *B. abortus* was not toxic, and 100 ng was required to produce limulus lysate gelation. Results with the control preparation of LPS from *E. coli* are included to show the sensitivity of the tests.

The R-specific antigen used in this experiment had been prepared from a large batch of *B. ovis* cells grown in a fermenter. A second smaller preparation with sterility checks performed at each stage was also toxic for mice, minimizing the possibility that contamination during large-scale production of the antigen could account for toxicity.

B. canis cells were extracted with hot saline in the same manner as used with the *B. ovis* cells. The yield after final centrifugation was one-tenth of that obtained with *B. ovis* cells, and the material was not toxic for mice at doses of 2 mg. R-specific antigen was not detected in immunodiffusion with anti-R serum. The supernatant fluid above the final pellet was also lyophilized and tested. This was not toxic at doses of 10 mg and did not contain R-specific antigen. Since the *B. canis* cells used in this extraction had been stored at 4 C in thick slurry for several months before shipment to Madison, it was thought that the R antigen might have leached out during storage. The first supernatant fluid from the slurry had been retained. This was dialyzed, lyophilized, and tested. It was not toxic at doses of 10 mg and did not contain R-specific antigen.

Mouse lethality of R(G) antigens. It was found that 4 mg of R(G) antigens of *B. ovis*, *B. canis*, or *B. abortus* 45/20 killed all mice injected, whereas this dose of the antigen from *B. melitensis* B115 was not lethal. None of the preparations killed mice at the 2-mg dose level.

TABLE 1. *Lethality for mice of acetone-extracted whole cells of Brucella*

Strain	Dose injected (mg)	No. dead at 48 h/no. injected	Mortality (%)
R *B. ovis* REO 198	20	12/12	100
	10	5/13	38
	5	0/12	0
R *B. canis* RM666	20	9/10	90
	10	7/10	70
	5	0/10	0
R *B. abortus* 45/20	20	7/12	58
	10	4/11	36
	5	0/6	0
R *B. melitensis* B115	20	0/12	0
	10	0/12	0
	5	0/6	0
S *B. abortus* 11-19	10	11/11	100
	5	8/11	72
	2.5	1/6	17

TABLE 2. *Lethality for mice and limulus lysate gelation activity of extracts from Brucella cells*

Prepn	Toxicity for mice			Limulus lysate gelation end point (ng)
	Dose injected (mg)	No. dead[a]/total	Mortality (%)	
B. ovis REO 198 R-specific antigen	2	4/4	100	0.1
	1	10/10	100	
	0.5	2/10	20	
	0.25	0/5	0	
B. abortus 11-19 S-LPS (phenol phase)	1	5/5	100	1
	0.5	1/10	10	
	0.25	0/5	0	
Aqueous phase	2	0/9	0	100
E. coli LPS	1	5/5	100	0.01
	0.5	2/5	40	

[a] 48 h after injection.

DISCUSSION

Despite repeated confirmations (2, 9, 15) of Redfearn's observation in this laboratory in 1960 that the toxic LPS of S *Brucellae* is found primarily in the phenol phase rather than in the aqueous phase of hot phenol-water extracts, various workers who have examined only the aqueous phase have erroneously reported that *Brucella* LPS is devoid of endotoxin activity (3, 12, 14). In fact, when the LPS from smooth *Brucella* is extracted in the appropriate fashion, it can be shown to have many structural and biological properties in common with endotoxins from the *Enterobacteriaceae* (15).

Earlier attempts to demonstrate endotoxin in heat-killed cells of non-S variants of *B. abortus* and the R species *B. canis*, or extracts from those cells, were unsuccessful (2, 11, 18, 20). These limited studies have led to the generally held belief that endotoxin is associated only with S *Brucellae* (16), whereas R mutants of the *Enterobacteriaceae* are known to have endotoxin (13).

R-specific antigens of *Brucella* may be extracted simply by heating saline suspensions of cells of *B. ovis*, *B. canis*, or R strains of *B. abortus* and *B. melitensis* (6, 17). These antigens are all cross-reactive regardless of the species from which they are extracted. The R-specific antigen is unrelated to the major determinants of S-LPS, and differs as well in chemical and physical characteristics (6, 10, 17).

We have shown here that the R-specific antigen from *B. ovis* has endotoxin activity, and that on a weight basis it is as toxic as the S-LPS from *B. abortus*. Whole killed cells of *B. canis* and R *B. abortus* strain 45/20 also have a significant content of endotoxin. These data suggest that a toxic, probably lipid A-like component is associated with different macromolecules in the S and R *Brucellae*. It remains to be determined whether variants which are devoid of toxicity, such as the *B. melitensis* strain B115 used here, are deficient in the toxic moiety. This strain has been shown to contain R-specific antigen indistinguishable by immunoelectrophoresis from that of *B. ovis* (6).

Although the *B. canis* cells were toxic for mice, we were not able to recover the toxic fraction in any of the soluble extracts. Direct extraction of *B. canis* cells with phenol-chloroform-petroleum ether produced a water-insoluble preparation which was toxic. It was recognized early that in cultures of *B. canis* the organisms form mucoid colonies on agar or "ropey" cultures in broth (4). Even though R-specific antigen can be extracted from these cells with hot saline (6), the toxic lipid may be associated with other parts of the cell wall. Obviously, small differences in conditions of growth, as well as in procedures of extraction, may also influence the distribution of the toxic material, as is true with even a single strain of *Salmonella* (5).

The demonstration that R *Brucellae* contain endotoxin makes possible a systematic investigation of the similarities as well as the differences between these endotoxins and those of the other gram-negative bacteria. This could include a search for a biochemical basis for such physiological characteristics as the relatively weak pyrogenicity and chicken embryo lethality of *Brucella* endotoxins (15).

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