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Detoxification and Immunogenic Properties of Endotoxin-Containing Precipitate of *Brucella abortus*[1]

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ABSTRACT

BADAKHSH, FRED F. (University of Georgia, Athens), AND JOHN W. FOSTER. Detoxification and immunogenic properties of endotoxin-containing precipitate of *Brucella abortus*. J. Bacteriol. **91**:494–498. 1966.—Endotoxin-containing precipitates (ECP) were prepared from *Brucella abortus* strain 19A by aqueous ether extraction followed by ethyl alcohol precipitation. Lysozyme was the most effective of several enzymes tried for detoxification of endotoxin present in the precipitate. Trypsin was shown to reduce mouse lethal toxicity but not rabbit dermal toxicity. Immunological studies of ECP and enzyme-treated ECP demonstrated that lysozyme did not harm the immunogenic property of ECP, whereas heat, ribonuclease, lipase, and proteolytic enzymes had an adverse effect. Serological reactivity of ECP was increased after lysozyme treatment, whereas ribonuclease reduced serological activity.

Endotoxin-containing preparations of *Brucella* have been shown to be immunogenic in mice (4), guinea pigs (Foster, Proc. Ann. Conf. Brucellosis Epidemiologists, 4th, *in press*), and swine (Edens and Foster, Am. J. Vet. Res., *in press*), but the use of these preparations as immunizing agents could cause problems because of their toxicity. One solution would be to reduce the amount of endotoxin administered to tolerable limits. However, greatly reduced amounts of antigen would produce reduced protection. A second solution would be to detoxify endotoxin-containing organisms or the antigenic fractions thereof. In spite of a variety of treatments, the loss of toxicity has been accompanied by a corresponding decrease in antigenicity (10, 13). The purpose of this study was to detoxify brucella endotoxin without adversely affecting immunogenicity of the endotoxin-containing preparations. It was also desired to see whether any such treatment might enhance immunogenicity.

MATERIALS AND METHODS

Isolation of organisms. B. abortus strain 19A was obtained from the stock culture collection of the School of Veterinary Medicine, University of Georgia. Smooth colonies were isolated as described by White and Wilson (14) with the use of a 1:4,000 crystal violet solution for about 30 sec to stain the colonies. They were also checked for failure to clump with 1:1,000 aqueous acriflavine (3).

Mass culture growth. Smooth colonies isolated as above were inoculated on Tryptose Agar (Difco) slants and incubated for 36 hr at 35 C. A 36-hr slant was used to inoculate a flask of the chemically defined medium (ROS) of Rode, Oglesby, and Schuhardt (11). This flask was incubated at 35 C on a shaker at 206 oscillations per min for 36 hr. Amounts of 5 to 10 ml of this culture were used to inoculate each of several 1-liter flasks containing 300 ml of ROS medium.

After 36 hr on the shaker at 35 C, the cultures were checked for purity by Gram stain, and cells were concentrated by centrifugation at 6,000 × *g* for 1 hr at 0 C.

Aqueous ether extraction. The method employed by Foster and Ribi (4) was applied except that cell sediment was resuspended in ice water, instead of saline, for ether treatment. The ethyl alcohol precipitate, herein designated as endotoxin-containing precipitate (ECP), was lyophilized and stored in a desiccator at room temperature until use.

Treatment of ECP with enzymes. Enzyme treatment consisted of incubating 60 mg of ECP with 30 mg of respective enzymes for 3 hr at 37 C in a water bath as follows: ribonuclease (five times crystalline; Nutri-

[1] Part of this work is taken from a thesis presented by the senior author in partial fulfillment of the requirements for the M.S. degree, University of Georgia, 1965.

tional Biochemicals Corp., Cleveland, Ohio), *p*H 7.6; pepsin (three times crystalline; Nutritional Biochemicals Corp.), *p*H 2.5; egg white lysozyme (three times crystalline; Nutritional Biochemicals Corp.), *p*H 7.2; lipase (Nutritional Biochemicals Corp.), *p*H 7.0; pancreatic trypsin (1:250; Difco), *p*H 8.2; and pronase (*Streptomyces griseus* protease; Calbiochem), *p*H 3.6. All reactions except that for pepsin were run in 0.15 M phosphate buffer. The *p*H was adjusted to 2.5 with HCl for the pepsin treatment.

Two methods were used for the treatment of ECP with enzymes. The differences in methods one and two had little, if any, effect on results obtained in this study.

Method one: ECP was treated with enzymes in the manner described for cell walls by Bobo and Foster (2). The ECP suspensions were stored at −20 C until use.

Method two: The aqueous phase was separated after aqueous ether extraction. Threefold concentration was accomplished by placing a dialysis bag containing Carbowax (polyethelene glycol; Union Carbide Corp., New York, N.Y.) in the aqueous phase overnight at 6 C. A 1-ml amount of concentrate was removed, and ECP was precipitated from it with ethyl alcohol; the precipitate was lyophilized. The weight of the precipitate obtained from 1.0 ml gave an indication of the number of milligrams of enzymes necessary for treatment of ECP to obtain material comparable to ECP prepared by method one. The *p*H of the solution was adjusted prior to adding enzyme and ECP, and the mixture was incubated for 3 hr in a water bath at 37 C. At the end of the incubation period, the mixture was precipitated with 70% ethyl alcohol (final volume), chilled to 6 C, and allowed to stand overnight. It was then centrifuged at 11,000 × *g* for 1 hr at 0 C, and the sediment was lyophilized and stored in a desiccator until use.

Toxicity tests were made in rabbits and mice with a portion of each of the preparations.

Rabbit dermal toxicity. Titrations were made in accordance with the technique of Larson et al. (7). Readings were made 24, 48, and 72 hr after inoculation. At 72 hr, the lesions appeared to be at their height, and the end points were consistent from animal to animal. The skin was dampened with alcohol to facilitate observation of lesions.

To show that the lesions in rabbit skin were not caused by the enzymes, separate skin tests were made with 70% ethyl alcohol-precipitated enzymes alone (Table 1).

Mouse LD_{50} test. The toxicity of the ECP and enzyme-treated ECP was determined by two separate experiments. In experiment one, 32 specific pathogen-free (SPF) white Swiss mice, 22 days old, were used for each treatment in four groups of eight. Each group received graded intraperitoneal doses as shown in Table 2. In experiment two, 32 conventional white Swiss mice, 22 days old, were used for each treatment in four groups of six. Each group received graded intraperitoneal doses as shown in Table 3. Some of the mice in experiments one and two died more than 5 days after inoculation. These were considered to have died from some cause other than endotoxin.

Study on immunity. The mice surviving the two LD_{50} experiments were challenged with 100,000 live *B. abortus* strain 2308 cells by the intraperiteoneal route. After 3 weeks, the mice were killed, and each spleen was removed aseptically and ground in 1.0 ml of diluent with a mortar and pestle. Plate counts were made of 0.01-g samples of spleen, or appropriate dilutions, on Difco Tryptose Agar (Tables 4 and 5) after 5 days of incubation at 35 C.

Serological studies. Serological reactivity of ECP and enzyme-treated ECP was measured by the capillary flocculation test (6).

Antisera were produced in rabbits by the intravenous injection of 1.0 mg each of *B. abortus* cells, untreated ECP, or enzyme-treated ECP. A second inoculation of 1.0 mg each was made in the hind footpad of each rabbit 14 days later.

RESULTS

Rabbit dermal toxicity test. The results, summarized in Table 1, show that 0.1 μg of untreated ECP provoked lesions in rabbit skins.

Comparison of the enzyme-treated ECP and untreated ECP show that there was little, if any, loss in dermal toxicity when ribonuclease, pronase, lipase, and trypsin were used. Detoxification of ECP was accomplished when lysozyme was used, since 400 μg was required to provoke a mild erythema.

Comparison of enzyme-treated ECP with enzymes alone (Table 1) shows that large quantities of ethyl alcohol-precipitated enzymes had to be inoculated to cause lesions. These results indicate that the lesions produced by enzyme-treated ECP were caused by the endotoxin present and not by the enzymes.

Mouse LD_{50} test. Results of LD_{50} tests are presented in Tables 2 and 3. These results again show that lysozyme was most effective in detoxi-

TABLE 1. *Dermotoxic effects of enzymes and enzyme-treated endotoxin-containing precipitate*

Enzymes	Lowest dose (μg) causing erythema*	
	Enzymes alone	Enzyme-treated ECP
Untreated	—	0.1
Ribonuclease	200	≦1.6
Pronase	25	≦1.6
Lysozyme	800	400.0
Lipase	200	≦1.6
Trypsin	400	≦1.6
Pepsin	200	≦1.6

* Amounts of 0.2 ml containing graded doses were given intradermally at each inoculation site in rabbits.

TABLE 2. *Mouse* lethality of endotoxin-containing precipitate (experiment 1)*

Treatment	Amt (mg)/ LD_{50}	Deaths per group of eight at dosage of			
		4.0 mg	2.0 mg	1.0 mg	0.5 mg
None	2.4†	6	3	1	0
Ribonuclease	2.8†	4	3	1	0
Pronase	1.9	8	4	1	0
Pepsin	4.0†	3	1	0	0
Lipase	4.0†	3	1	0	0
Lysozyme	>4.0	0	0	0	0
Trypsin	>4.0	1	0	0	0

* Specific pathogen-free white Swiss mice, 22 days old, inoculated intraperitoneally.

† It was assumed that eight mice would have died if inoculated with an 8-mg dose.

TABLE 3. *Mouse[a] lethality of endotoxin-containing precipitate (experiment 2)*

Treatment	Amt (mg)/ LD_{50}	Deaths per group of six at dosage of				
		16.0 mg	8.0 mg	4.0 mg	2.0 mg	1.0 mg
None	2.0	—[b]	6	5	3	1[c]
Ribonuclease	3.6	—	6	4	0	0
Pronase	1.1	—	6	6	5	3[c]
Pronase[d]		—	6	6	6	6
Pepsin	3.2	—	6	3	1	1[c]
Lipase	2.8	—	4	3	3	2[c]
Lysozyme	>16.0	0	0	0	0	0
Lysozyme[d]		6	6	6	6	6
Trypsin	5.8	—	5	3	1	—
Heated, 60 C, 1 hr	4.5	—	5	2	1	—

[a] Conventional white Swiss mice, 22 days old, inoculated intraperitoneally.

[b] Not done.

[c] It was assumed no mice would have died had they been inoculated with 0.5 mg.

[d] Enzyme-ECP combination not treated with 70% ethyl alcohol.

fication of *Brucella* endotoxin. Trypsin also seemed to have some effect. The other enzymes had little or no effect in reducing the toxicity of ECP. Either pronase- or lysozyme-treated ECP was lethal for mice at doses as low as 1.0 mg (Table 3). Treatment with 70% ethyl alcohol (overnight) at 6 C markedly reduced the mouse lethality of both enzymes. The other enzymes were not lethal at the concentration used.

The results in Tables 2 and 3 show no difference in mouse lethality in conventional and SPF mice as far as endotoxin is concerned. It does appear that lysozyme and pronase which had not been treated with ethyl alcohol were more lethal for conventional than for SPF mice. This might also be due to strain differences in the mice.

Immunological studies. In the first experiment, the average plate counts from the spleens of infected SPF mice receiving lysozyme-treated ECP were lower for each dosage group than the average plate counts from infected SPF mice receiving untreated ECP (Table 4). Approximately the same proportion of mice were protected in the untreated and lysozyme-treated dosage groups studied. No mice which received ECP treated with the other enzymes were protected. In the second experiment, the absence of any colonies on the plates (Table 5) showed that all mice receiving 16.0, 8.0, and 4.0 mg of lysozyme-treated ECP were protected. However, it was not possible to relate immunizing potency of lysozyme-treated to untreated ECP in this experiment, since the comparable doses were lethal for all mice. No mice which received ECP treated with the other enzymes were protected. Comparison was also made with a group of mice kept as controls, which received the same challenge doses. Table 5 shows that all were infected.

DISCUSSION

SPF and conventional white Swiss mice apparently are equally susceptible to the *Brucella* endotoxin in ECP. Their susceptibility to enzyme-treated endotoxin also seems to be similar. The mouse-lethal material present in ECP is affected by lysozyme, as shown by the ability of the mice to tolerate great doses of lysozyme-treated ECP, in contrast to their susceptibility to untreated ECP. It is of interest that Smith et al. (12) rapidly detoxified bacterial endotoxin by use of splenic extracts of dogs, and that lysozyme and other enzymes have been isolated from dog spleen (5). The data in Table 1 show that a rabbit dermatoxic principle is also affected by lysozyme. Mouse lethal toxicity and rabbit dermal toxicity may be different, since trypsin treatment of ECP appeared to reduce mouse lethal toxicity but not rabbit dermal toxicity.

Lysozyme greatly increased the serological reactivity of ECP. Increases in serological reactivity of *Brucella* cell walls have previously been shown by Olitzki and Sulitzeanu (9) with trypsin and pepsin treatment and by Bobo and Foster (2), who also used lysozyme, pronase, and trypsin treatment. In the present study, pronase treatment decreased serological reactivity (Table 6). No simple explanation can be offered for the high titers demonstrated when ECP was treated with lysozyme, but several possibilities come to mind. For example, the lysozyme may complex with ECP particles until they become very large and

TABLE 4. *Protection of mice[a] immunized with enzyme-treated endotoxin-containing precipitate (experiment 1)[b]*

Treatment	4.0 mg		2.0 mg		1.0 mg		0.5 mg	
	P/I[c]	Plate count[d]	P/I	Plate count	P/I	Plate count	P/I	Plate count
Untreated	1/2	75	2/5	475	1/7	4133	0/8	TNTC[e]
Lysozyme	4/8	32	3/8	100	1/8	1150	0/8	TNTC
Pronase	—[f]	—	0/3	TNTC	0/7	TNTC	0/8	TNTC
Pepsin	0/3	3,800	0/6	TNTC	0/8	TNTC	0/8	TNTC
Trypsin	0/7	TNTC	0/8	TNTC	0/8	TNTC	0/8	TNTC
Lipase	0/4	TNTC	0/7	TNTC	0/8	TNTC	0/8	TNTC
Ribonuclease	0/4	TNTC	0/5	TNTC	0/7	TNTC	0/8	TNTC

[a] Specific pathogen-free white Swiss mice challenged with 100,000 (by plate count) *Brucella abortus* strain 2308 given intraperitoneally to survivors of lethality experiments 2 weeks after inoculation.

[b] Apparent discrepancies which occurred between Tables 2 and 4 are accounted for by the death of mice from causes other than ECP.

[c] P/I = no. protected/no. injected.

[d] Average number of colonies per 0.01 g of wet spleen.

[e] Colonies too numerous to count.

[f] Not done.

TABLE 5. *Protection[a] of mice immunized with enzyme-treated endotoxin-containing precipitate (experiment 2)*

Treatment	16.0 mg		8.0 mg		4.0 mg		2.0 mg		1.0 mg	
	P/I[b]	Plate count[c]	P/I	Plate count	P/I	Plate count	P/I	Plate count	P/I	Plate count
Untreated	—[d]	—	D[e]	—	D	—	1/3	233	1/5	TNTC[f]
Lysozyme	5/5	0	6/6	0	5/5	0	—	—	—	—
Pronase	—	—	D	—	D	—	0/1	2,950	0/1	TNTC
Pepsin	—	—	D	—	0/3	TNTC	0/4	TNTC	0/3	TNTC
Trypsin	—	—	0/1	3,000	0/3	TNTC	0/5	TNTC	—	—
Lipase	—	—	0/2	5,400	0/3	TNTC	0/3	TNTC	0/2	TNTC
Ribonuclease	—	—	D	—	0/2	TNTC	0/6	TNTC	0/6	TNTC
Heated, 60 C, 1 hr	—	—	D	—	D	—	0/4	TNTC	—	—

[a] Conventional white Swiss mice challenged with 100,000 (by plate count) *Brucella abortus* strain 2308 given intraperitoneally 2 weeks after inoculation. In controls (not immunized), none of 16 injected was protected. The plate count was 134,900.

[b] P/I = no. protected/no. injected.

[c] Average number of colonies per 0.01 g of wet spleen.

[d] Not done.

[e] D = mice died prior to challenge. Apparent discrepancies which occurred between Tables 3 and 5 are accounted for by the death of mice from causes other than ECP.

[f] Colonies too numerous to count.

TABLE 6. *Reciprocal flocculation titers of various Brucella abortus preparations**

ECP treatment	Antiserum to			
	Viable organisms	ECP	Ether-treated cell wall	Acetone-treated cell wall
None	1,280	1,280	40	1,280
Ribonuclease	320	40	20	320
Pronase	640	160	40	320
Lysozyme	≧10,240	≧10,240	80	≧10,240

* Antiserum produced by intravenous inoculation followed by footpad inoculation.

easy to precipitate with few antibody molecules. Another possibility may be that the lysozyme is breaking down the ECP particles in such a manner that more antibody-accepting sites become available for the antibodies to combine with. The loss in toxicity is compatible with either possibility. Complexing could "cover" or bind toxic groups, or lysozyme could destroy them.

The findings in this study relative to the immunization of mice are compatible with those of Markenson, Sulitzeanu, and Olitzki (8) and Foster and Ribi (4), which establish that *Brucella* cell walls will immunize mice and that the endotoxin-containing precipitate derived from cell

walls immunizes mice. Foster (*in press*) has shown that guinea pigs are immunized by *B. suis* ECP preparations similar to those prepared by Foster and Ribi. Edens and Foster (*in press*) have shown that swine also receive a significant degree of immunity from *B. suis* ECP. In swine, lysozyme treatment did not seem to affect immunogenic potency. It appears possible that lysozyme-treated ECP may be usefully employed as an immunogenic agent.

The immunogen may not be the endotoxin, because the lability of the immunogen in ECP to heat, pepsin, trypsin, and pronase (Tables 3 and 5) points to the possible protein or peptide nature of the agent. Allen (1) also presented evidence that there is a heat-labile *Brucella* immunogen. The lability of the immunogen to lipase and ribonuclease could indicate that a large antigenic complex which contains protein or peptide is being broken down. The resistance of the immunogen to lysozyme argues against the mucopeptide bonds being essential to immunity. Thus, we are left with peptide or protein, lipid, and ribonucleic acid as possible necessary members of the immunogenic complex. The lipase could conceivably have had some proteolytic enzymes as impurities, but the ribonuclease was highly purified and should have had little nonspecific activity. It is premature to speculate further on the nature of the immunogen.

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