Increased Host Resistance to Infection Elicited by Lipopolysaccharides from *Brucella abortus* (34111)

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Lipopolysaccharides (LPS) obtained from the cell walls of many different enterobacteriaceae are known to increase the resistance of the host to a variety of infections (for review, 1). Such materials, often called "endotoxin," have been isolated from strains of the genus *Eschericia, Serratia,* and *Salmonella.* Although the immunogenic effect of Brucella endotoxin on Brucella infections has been well established, there has been no previously published work on the ability of Brucella lipopolysaccharide to increase resistance to infections by other microorganisms. The present paper describes the effect of Brucella lipopolysaccharide on the host resistance to infections and draws attention to the differences between the biological properties and chemical composition of Brucella lipopolysaccharide and those isolated from other gram-negative microgorganisms.

Materials and Methods. Microorganisms. Brucella lipopolysaccharides were extracted from *Brucella abortus* strain 19-9R a rough, nonpathogenic strain derived from the *Brucella abortus* 19S vaccine strain. The bacteria were grown in a casein hydroyzate, yeast extract, glucose medium at 37° with continuous aeration for 48 hr, harvested by centrifugation, and stored in the frozen state. *Escherichia coli* (rough H31) or *Serratia marcescens* (*Bizio*) were grown on a semisynthetic medium at 30° for 20 hr, harvested by centrifugation, and frozen.

Extraction of lipopolysaccharides. Cells from each of the bacteria were thawed and suspended in saline at 4°. The cell suspensions were processed through a "Ribi" cell fractionator (I. Sorvall, Inc., Norwalk, Connecticut) at 30,000 psi at 6–9°. The cell walls were separated from the cytoplasm by centrifugation and subjected to an aqueous phenol extraction using a modification of the Westphal procedure (2). The extracted cell wall material was resuspended in saline, heated to 68°, and stirred with an equal volume of 88% phenol at 68° for 1 min. The mixture was cooled to 5°, centrifuged, and the phases separated by centrifugation. The phenol phase was re-extracted three times at 68° with equal volumes of saline. The phenol phases were discarded. The aqueous phases were combined, dialyzed against distilled water at 4°, and lyophilized.

Chemical analysis. Total carbohydrate was obtained by the *l*-tryptophan-sulfuric acid procedure (3). Protein was determined by the biuret assay (4). The LPS backbone sugar, 2-keto-3-deoxyoctonate (KDO), was determined by the thiobarbituric acid method (5) after a 45-min hydrolysis in 0.1 N H_2SO_4 at 100°. The KDO used as a standard was synthesized according to the procedure of Ghalambor *et al.* (6). Lipid and β-hydroxymyristic acid contents were determined by gas chromatography using the procedure of Metcalfe and Schmitz (7) on a 4-ft column packed with 3.8% W-382 on 80/100 mesh Diatoport S (Hewlett Packard) at 185°. Absence of β-hydroxymyristic acid was further established by acetylating the fatty acid mixture and subjecting the product to the same chromatographic procedure.

Pharmacological tests. Pyrogenicity was evaluated in male New Zealand albino rabbits according to the procedures of Landy and Shear (8) and Keen *et al.* (9). The Shwartzman reaction was carried out as described by Ribi *et al.* (10). The epinephrine-induced dermal necrosis test was carried out as described by Falk and Spink (11). The standard errors of the means were calculated

TABLE I. Increased Resistance to Infection with *Salmonella typhosa* (Ty2) in Mice Pretreated with *Brucella abortus* 19-9R Lipopolysaccharide.

Treatment	Dose (mg/kg)	No. surviving/no. treated at: 24 hr	48 hr	72 hr	PD_{50}[a] (mg/kg)
Control (none)	—	4/20	3/20	2/20	—
B. abortus (LPS)	0.01	6/20	6/20	6/20	0.12
	0.10	10/20	9/20	7/20	(0.055–0.26)
	1.00	17/20	16/20	16/20	
	10.0	20/20	20/20	20/20	

[a] PD_{50} based on survivors at 72 hr; challenge dose: 4.2×10^8 viable cells.

according to the method of Miller and Tainter (12).

Increase in host resistance. The ability of LPS to increase host resistance to infections was evaluated as previously described (13) in male mice of the Swiss Webster strain, weighing about 20 g. *Salmonella typhimurium* (3S), *Salmonella typhosa*(Ty2), *Diplococcus pneumoniae* (SV1), and *Streptococcus mastitidis* (SP1) were used as infective agents and were administered intraperitoneally. With all microorganisms, the number of viable cells given was adjusted in preliminary titrations to produce death in at least 90% of animals within 48 hr after infection. Lipopolysaccharide was administered once intraperitoneally at 4 or 5 different dose levels 24 hr prior to infection. Groups of 20 mice were used at each dose level. When 90% of the untreated control animals were dead, usually 24–48 hr after infection, the median protective dose, PD_{50}, that is, the dose at which 50% of the treated animals survived, was calculated (14). Each experiment was repeated 3–4 times with similar results.

Results. Increased resistance to infections. The results of an experiment carried out to determine the effect of lipopolysaccharide extracted from *Brucella abortus* 19-9R on the resistance to infections with *S. typhosa* are illustrated in Table I. Brucella LPS, given intraperitoneally 24 hr prior to challenge with the infectious microorganism, markedly increased the number of animals that survived the infection. Comparable results were obtained in repeated experiments when *Salmonella typhimurium, Diplococcus pneumoniae*, or *Streptococcus mastitidis* were used to produce infection.

The amount of Brucella LPS required to protect 50% of the animals against the various infections is shown in Table II. Brucella LPS increased the ability of animals to resist infection by four different microorganisms. Because the microorganisms used to produce the infections were immunologically unre-

TABLE II. Increased Resistance to Infection Produced in Mice by Lipopolysaccharides Extracted from *Brucella abortus* 19-9R.

Infectious agent	No. of viable organisms given	Lipopolysaccharide PD_{50}[a] (mg/kg)	Lipopolysaccharide Confidence limits[b]
Salmonella typhimurium (3S)	3.6×10^8	0.013	0.0081–0.021
Salmonella typhosa (Ty2)	4.2×10^8	0.096	0.055 –0.17
Diplococcus pneumoniae (SV1)	5.0×10^7	0.58	0.28 –1.22
Streptococcus mastitidis (SP_1)	2.4×10^7	0.96	0.60 –1.54

[a] Dose protecting 50% of mice from death by infection. Composite values obtained from four or five separate experiments using the same LPS.

[b] 95% confidence limits.

lated to Brucella from which the LPS was prepared, and because such LPS produced protection against all four of them, it appears that the protection that was achieved was nonspecific in character and independent of the presence of specific antibodies. The nonspecific character of the protection is further suggested by the fact that substantial protection was achieved as early as 24 hr after administration of LPS; *i.e.*, at a time before effective levels of antibodies could have been formed.

LPS extracted from *E. coli* and *Serratia marcescens* were prepared and their ability to increase resistance to infections compared with LPS extracted from *Brucella abortus*. In these experiments again *S. thyphimurium, S. typhosa, D. pneumoniae,* and *S. mastitidis* were used for challenge. The results, expressed in terms of the PD_{50} dose of representative preparations of the three different lipopolysaccharides, are given in Table III.

LPS prepared from *E. coli* or *S. marcescens* induced the well-known nonspecific increase in resistance to a variety of microorganisms in micrograms per kilogram amounts. The two lipopolysaccharides produced a similar degree of protective activity against all microorganisms against which they were evaluated. The LPS produced from *Brucella abortus*, on the other hand, was much less active. To produce protection with Brucella LPS, much larger amounts were needed than with LPS prepared from the other two gram-negative bacteria. Although all Brucella LPS were effective in increasing host resistance to infections, the amounts needed to produce this effect varied from batch to batch. Thus the mean protective dose of four different batches of Brucella LPS in animals challenged with *S. typhimurium* were 0.013, 0.058, 0.16, and 0.76 mg/kg. Brucella LPS also differed from the other two LPS's in that the amounts of Brucella LPS needed to produce protection varied to some extent depending on the infectious agent used.

Toxicity and other properties. LPS prepared from *Brucella abortus* was much less toxic than LPS prepared from other gram-negative microorganisms (Table IV). The mean lethal dose (LD_{50}) of LPS prepared from Brucella was 750 ± 158 mg/kg intraperitoneally. In comparison, the LD_{50} of LPS prepared from *Serratia marcescens* in a similar manner was 15.3 ± 4.4 mg/kg, and from *Escherichia coli* 20.2 ± 2.0 mg/kg. Pretreatment of mice with 10 mg/kg of Brucella LPS given alone or together with Freund's complete adjuvant did not change the toxicity of Brucella LPS administered to these animals 3 weeks later.

The Brucella LPS had only about 1/20 to 1/500 of the pyrogenicity of *E. coli* LPS and differed from endotoxins prepared from oth-

TABLE III. Increased Resistance to Infections Produced by Various Lipopolysaccharides in Mice.

Infectious agent	PD_{50}[a] (mg/kg) of lipopolysaccharides prepared from:		
	Brucella abortus 19-9R	*Escherichia coli*	*Serratia marcescens*
S. typhimurium	0.013 (0.0081–0.021)[b]	0.0028 (0.0013–0.0058)	0.0019 (0.0011–0.0033)
S. typhosa	0.096 (0.055–0.17)	0.0019 (0.0011–0.0032)	0.0058 (0.0029–0.0116)
D. pneumoniae	0.58 (0.28–1.22)	0.0038 (0.0023–0.0063)	0.014 (0.0093–0.021)
S. mastitidis	0.96 (0.60–1.54)	0.0055 (0.0031–0.0099)	0.0073 (0.0052–0.010)

[a] Dose protecting 50% of mice from death by infection. Composite values obtained from four or five separate experiments using the same LPS.

[b] 95% confidence limits.

TABLE IV. Properties of Lipopolysaccharides Isolated from Various Organisms.

	Serratia marcescens	*Escherichia coli*	*Brucella abortus*
LD_{50} mg/kg	15.3 ± 4.4	20.2 ± 2.0	750.0 ± 158.2
Shwartzman reaction	4/4[a]	8/8	0/6
Dermal necrosis	4/6[a]	7/7	0/6
Pyrogenicity[b]	5	1	0.043

[a] Number of rabbits showing a positive reaction divided by number of rabbits tested.
[b] Relative pyrogenicity where *E. coli* LPS = 1.

er gram-negative bacteria in not producing dermal necrosis or the Shwartzman reaction (Table IV).

Chemical composition. To establish the reason why there should be such a marked difference in the protective activity and other biological properties of LPS obtained from Brucella and LPS obtained from other gram-negative organisms, the chemical composition of these preparations was studied. Table V indicates that the various LPS do not differ markedly from each other in their content of protein, nitrogen, and carbohydrate. All preparations also contained 2-keto-3-deoxyoctonate (KDO), which is usually present in LPS derived from cell wall material of gram-negative bacteria. All preparations also contained appreciable amounts of glucosamine. The Brucella LPS, however, differs from the others in containing smaller amounts of lipid. Analysis of the fatty substances contained in the lipid fraction indicated that the LPS obtained from Brucella does not contain measurable amounts of β-hydroxymyristic acid. This constituent is known to be present in LPS obtained from other gram-negative bacteria (15).

Discussion. It has been known for more than 70 years that a variety of gram-negative organisms, such as *Escherichia coli, Proteus vulgaris, Salmonella typhosa,* and *Pseudomonas aeruginosa,* contain a "principle" which appears to be similar for all the species and which is capable of increasing the nonspecific resistance to infections by a variety of microorganisms (16). Rowley (17) and Landy and Pillemer (18) identified endotoxin, a lipopolysaccharide contained in the cell walls of these microorganisms, as the active principle responsible for increased host resistance.

Foster and Ribi (19) found that LPS extracted from *Brucella abortus* cell walls protected mice against infections with this organism. However, cell wall materials extracted from *Brucella abortus* have not been evaluated for their ability to increase host resistance to infections with other microorganisms. The findings reported here demonstrate that LPS obtained from *Brucella abortus* is capable of increasing host resistance to infections induced by several gram-negative and gram-positive microorganisms. In this respect, *Brucella abortus* LPS appears to be acting in a similar manner as endotoxin produced by other gram-negative microorganisms such as *E. coli, S. typhosa,* or *Serratia marcescens.*

Brucella abortus LPS, however, differs from the endotoxin of other gram-negative microorganisms in several respects. Considerably larger amounts of Brucella LPS are

TABLE V. Chemical Composition of *Escherichia coli, Serratia marcescens,* and *Brucella abortus* Lipopolysaccharides (in per cent).

Origin of LPS	Biuret protein	Kjeldahl nitrogen	Carbohydrate	Lipid	KDO[a]	β-OH MA[b]
E. coli	6.3	5.4	12.9	3.1	2.2	2.45
S. marcescens	14.7	2.6	34.5	4.4	6.2	2.9
B. abortus 19-9R	10.3	10.9	12.7	1.05	4.0	0

[a] 2-Keto-3-deoxyoctonate.
[b] β-hydroxymyristic acid.

needed to induce an increase in resistance of the host to infections than is required with endotoxins extracted from other gram-negative bacteria. With Brucella LPS, the amount needed to obtain protection varied appreciably with the infectious microorganism used. In this respect, Brucella LPS also differs from endotoxins obtained from other gram-negative bacteria investigated thus far which protect animals from infections evoked by most microorganisms in doses of a similar magnitude. Finally, Brucella endotoxin differs from other endotoxins in not containing measurable amounts of β-hydroxymyristic acid. This constituent is always present in appreciable amounts in lipopolysaccharides (endotoxins) from gram-negative bacteria that have been studied in this respect (15, 20). It is present even in LPS from *Bordetella pertussis* which otherwise differs quite significantly in chemical composition from LPS of *Escherichia, Serratia,* or *Salmonella* (21). It appears questionable whether a lipopolysaccharide that does not contain β-hydroxymyristic acid can properly be called endotoxin (22).

It is of interest to speculate whether the absence of β-hydroxymyristic acid in Brucella LPS is responsible for its low toxicity and its lack of ability to elicit the Schwartzman reaction or the epinephrine-induced dermal necrosis that is so characteristic of endotoxins extracted from other gram-negative bacteria.

In this connection the roughness of our Brucella strain may be of interest. Luderitz *et al.* (23), when studying the biological activities of S-(smooth) and R-(rough) LPS of *Salmonella minnesota,* found that LPS obtained from both strains had high pyrogenic activity of a similar order of magnitude. On the other hand, LPS derived from R-strains were much less toxic than LPS from S-strains.

It is well known that patients who have or have had brucellosis often exhibit a pronounced hypersensitivity for Brucella antigens (24). Several studies in animals indicate that animals do not respond in the usual manner to the first administration of Brucella LPS or of killed Brucella cells. Thus, Abernathy *et al.* (25) reported that infection with Brucella increased the lethality of Brucella endotoxin for mice. Using the sensitive mouse urinary nitrogen assay for endotoxin (26), Wilson *et al.* (27) observed that killed Brucella cells did not affect this test as did killed salmonellae or escherichiae. However when mice were passively sensitized with antibrucella serum, decreased urinary nitrogen excretion, similar to that produced by *E. coli* endotoxin, could then be obtained with Brucella cells. The authors explained these results by showing that mice have natural antibodies to escherichiae but not to brucellae. Kessel *et al.* (28) observed that endotoxin from *Brucella abortus* is not cytotoxic *in vitro* unless macrophage donors have first been sensitized by infection with live *Brucella abortus*. The importance of induced sensitization of normal laboratory animals was also studied by Freedman *et al.* (29) who found that Brucella endotoxin failed to depress water intake or to cause an increase in the number of hemolysin-producing spleen cells in mice unless the animals were pretreated with Brucella LPS. Thus, hypersensitivity to, or prior contact with, Brucella LPS or Brucella cells may be a prerequisite for the elicitation of certain characteristic effects with this agent.

It appears remarkable that Brucella LPS is capable of inducing good protective action in animals that did not have previous experience with Brucella and that do not have natural antibodies against these microorganisms. It indicates that the increase of resistance to infection produced by Brucella LPS does not depend on an induced state of hypersensitivity to this organism but depends on some other as yet unexplained mode of action.

Summary. Lipopolysaccharides isolated from cell walls of *Brucella abortus* 19-9R increase resistance in mice to infections with *Salmonella typhimurium, Salmonella typhosa, Diplococcus pneumoniae,* or *Streptococcus mastitidis*. In this respect, the action of Brucella LPS is similar to that of lipopolysaccharides obtained from other gram-negative bacteria. However, considerably larger amounts of Brucella LPS are required to produce protection than is the case with other

lipopolysaccharides. Brucella LPS also differs from other lipopolysaccharides in not eliciting the Shwartzman reaction or epinephrine-induced dermal necrosis and in having a low pyrogenicity. Brucella LPS does not contain measurable amounts of β-hydroxymyristic acid which is always present in appreciable amounts in lipopolysaccharides from other gram-negative bacteria. The ability of Brucella LPS to increase host resistance to infection appears to be independent of prior sensitization of the host to Brucella.

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