INFECTION AND IMMUNITY, Sept. 1970, p. 237-243

Vol. 2, No. 3
Printed in U.S.A.

Comparison of Chemical Components of Cell Walls of *Brucella abortus* Strains of Low and High Virulence[1]

GEORGE D. KELLERMAN,[2] J. W. FOSTER, AND F. F. BADAKHSH[3]
Department of Medical Microbiology, School of Veterinary Medicine, University of Georgia, Athens, Georgia 30601

Received for publication 13 April 1970

Amino acid, carbohydrate, and lipid components of cell walls of *Brucella abortus* strain 19A (low virulence) and strain 2308 (high virulence) were compared by thin layer chromatography (TLC) and by use of an amino acid analyzer. A total of 15 amino acids were detected by both chromatographic methods. Each amino acid was present in greater amounts in strain 2308 than in strain 19A when equal amounts of hydrolysates of cell wall and endotoxin-containing preparations were analyzed. A component with the same R_F value as ethanolamine was present in strain 2308 cell wall hydrolysates but was not revealed by TLC of strain 19A cell wall hydrolysates. This component was not detected with the amino acid analyzer. TLC of cell walls tagged with 2,4-dinitrofluorobenzene prior to hydrolysis showed that phenylalanine was a terminal amino acid in cell walls of *B. abortus* strains 19A and 2308, *B. suis* strain 1776, and *B. melitensis* strain 2500. Carbohydrates detected in cell walls of strains 19A and 2308 by TLC were tentatively identified as glucose, mannose, rhamnose, and galactose. Colorimetric tests were also positive for 2-keto-3-deoxy-octulosonic acid, heptose, and dideoxyhexose. At least seven lipid components were detected by TLC of ether extracts of cell walls of strains 19A and 2308. It is suggested that one or more lipids is important in maintaining cell wall structure, because isolated cell walls rapidly became fragmented after exposure to ether.

Many investigators have studied the chemistry of *Brucella* cells and their fractions in an effort to understand existing strain differences in antigenicity and virulence. The results of most of these studies are included in various reviews and monographs (8, 11, 15, 24). Detailed analyses of the amino acids, carbohydrates, and lipids which make up the antigenic and virulent components of *Brucella* cells have been less common. Bobo and Eagon (3) and W. J. Beasley (M.S. Thesis, Northwestern State College of Louisiana, Natchitoches, 1968) found fatty acids, phosopholipids, and seven or more amino acids in the cell walls of *B. abortus*. Ellwood, Keppie, and Smith (6) extracted an immunogen from *B. abortus* cell walls which contained eight amino acids, glucose, and glucosamine. Badakhsh and Foster (2) found that the same 15 amino acids occurred in *B. abortus* strain 19A (low virulence) and strain 2308 (high virulence) endotoxin-containing preparations (ECP), but that the lysine content was higher in strain 2308. In a preliminary report (Kellerman, Foster, and Badakhsh, Bacteriol. Proc., p. 124, 1967), we noted that 15 amino acids were present in the cell wall as well as in ECP. In both of the latter studies, thin-layer chromatography (TLC) of acid hydrolysates revealed an unidentified component which reacted strongly with ninhydrin. The present paper extends our previous observations.

[1] Taken in part from a Ph.D. dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree from the University of Georgia. A portion of this paper was presented at the 67th Annual Meeting of the American Society for Microbiology, New York, N.Y., May 1967. Published as paper number 776, Institute of Comparative Medicine, School of Veterinary Medicine, University of Georgia, Athens.

[2] Present address: Spalding College, Louisville, Ky.

[3] Present address: Wallace Laboratories, Cranbury, N.J.

MATERIALS AND METHODS

Production of preparations. *B. abortus* strains 19A and 2308 were grown in the chemically defined medium developed by Rode, Oglesby, and Schuhardt (18). After inoculation, 20 flasks of medium were incubated on a shaker oscillating at 206 cycles per min at 35 C for 36 hr. Three lots of each strain were produced. Cell walls and protoplasm (or cytoplasm) were prepared from each cell suspension by methods described by Foster and Ribi (10), or Foster, Cowan, and Maag (9). ECP was prepared by aqueous ether extraction and ethyl alcohol precipitation (10).

Amino acid detection by TLC. Silica gel H was spread 0.2 mm thick on glass plates which were air-dried and activated at 110 C for 1 hr (16). Lyophilized cell walls or ECP were hydrolyzed in 5.0-

or 10-mg amounts in 6 N HCl for 24 hr in evacuated sealed tubes at 110 C. A preliminary experiment established this time to be optimal in respect to number of components resolved. After hydrolysis, samples were dried in vacuo and resuspended in sterile distilled water. This process was repeated five times or until *p*H indicator paper showed that acid had been removed. Samples of 50 nl each were applied about 1 cm from each edge in one corner of a plate. The plate was then equilibrated in the developing tank over the first solvent for 1 to 2 hr, lowered into the solvent, developed with chloroform, methanol, and 17% ammonium hydroxide (2:2:1, v/v) for 70 min, and air-dried for 20 min. The plate was next developed in phenol and water (75:25, v/v) for 120 min in a plane at right angles to that of the first developer. After this, the plate was air-dried, sprayed with ninhydrin, and heated at 110 C for 10 min; color development of spots was then recorded. Mixtures of known amino acids were chromatographed under the same conditions, and R_F values were compared with those of spots detected on the hydrolysates from preparations of strains 19A and 2308. Tracings were made of spots appearing on each plate for comparison of patterns, and color intensity was observed visually. Replicate hydrolysates were prepared from three lots of cell walls of each strain, and paired samples were chromatographed under identical conditions.

Detection of amino acids with an amino acid analyzer. Cell wall hydrolysates of one lot of *B. abortus* strain 19A and one lot of *B. abortus* strain 2308 were run in duplicate in 5- and 58-cm columns on an amino acid analyzer (Beckman model 120B). Samples were applied in 0.5-ml amounts on 8.0% cross-linked sulfonated polystyrene resin. Sodium citrate buffer was used as eluant. Buffer of *p*H 5.28 was used for the short column, and buffer of *p*H 3.28 was followed by buffer of *p*H 4.25 on the long column (23). Identification was made by comparison of chromatographs with known amino acids.

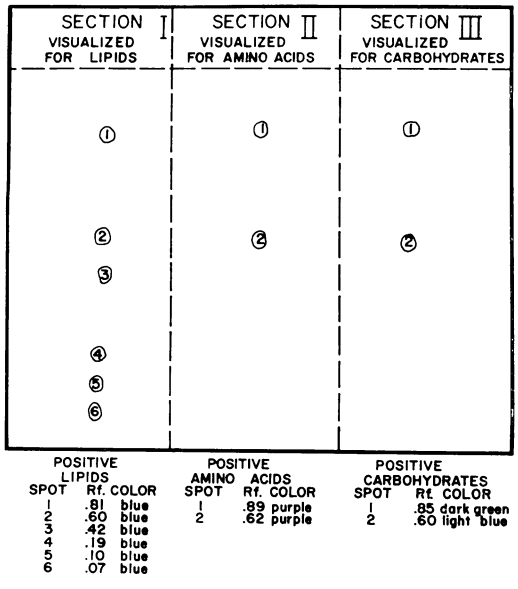

FIG. 1. *Tracings of thin-layer chromatograms of ether extracts of cell walls.*

Determination of N-terminal amino acid. Cell wall samples were tagged with 2,4-dinitrofluorobenzene (19). They were then hydrolyzed and analyzed by TLC as described above. Cell walls of *B. suis* strain 1776, *B. melitensis* strain 2500, and *B. abortus* strains 2308 and 19A were studied.

Detection of carbohydrates by TLC. Silica gel H was suspended in 0.1 M boric acid, spread 0.2 mm thick on glass plates, air-dried, and activated at 110 C for 1 hr (16). Duplicate 3.5-mg samples of one lot of cell walls and cytoplasm were hydrolyzed in 1 N HCl for 4 hr in evacuated sealed tubes. A preliminary experiment indicated the optimal time and acid strength in terms of numbers of components

TABLE 1. *R_F values of components of cell walls and cytoplasm of B. abortus strain 19A and strain 2308 compared with known amino acids*

Amino acid	Known sample		Cell walls				Cytoplasm			
			Strain 19A		Strain 2308		Strain 19A		Strain 2308	
	1[a]	2	1	2	1	2	1	2	1	2
Lysine	.10	.05	.14	.08	.14	.08	.09	.10	.09	.10
Arginine	.13	.18	.16	.18	.16	.18	.19	.20	.19	.20
Glutamic acid	.38	.08	.38	.06	.38	.09	—	—	—	—
Serine	.46	.18	.50	.10	.50	.08	.48	.12	.48	.12
Glycine	.40	.14	.44	.15	.45	.15	.42	.15	.42	.15
Aspartic acid	.50	.16	.52	.16	.46	.22	.52	.16	.52	.16
Alanine	.56	.20	.58	.21	.58	.21	—	—	—	—
Threonine	.65	.14	.66	.18	.66	.18	—	—	.65	.20
Histidine	.59	.33	.56	.31	.56	.31	—	—	—	—
Valine	.72	.53	.70	.40	.70	.40	.72	.45	.72	.45
Leucine or isoleucine	.75	.60	.73	.56	.72	.60	.75	.56	.75	.56
Phenylalanine	.96	.75	.90	.75	.90	.75	.95	.75	.95	.75
Tryosine	.56	.61	.55	.60	.56	.47	.55	.60	.55	.60
Proline	.34	.43	.34	.43	.34	.43	.35	.40	.35	.40
Glucosamine	.44	.15	—	—	—	—	—	—	—	—
Galactosamine	.51	.12	—	—	—	—	—	—	—	—
Diaminopimelic acid	.17	.03	—	—	—	—	—	—	—	—
o-Phosphoryl ethanolamine	.31	.05	—	—	.33	.08	—	—	—	—
Ornithine	.19	.09	—	—	—	—	—	—	—	—

[a] All tests were done with two solvents. Solvent 1 was chloroform, methyl alcohol, and 17% NH_4OH (2:2:1, v/v). Solvent 2 was phenol and water (75:25, w/w). Indicator was ninhydrin.

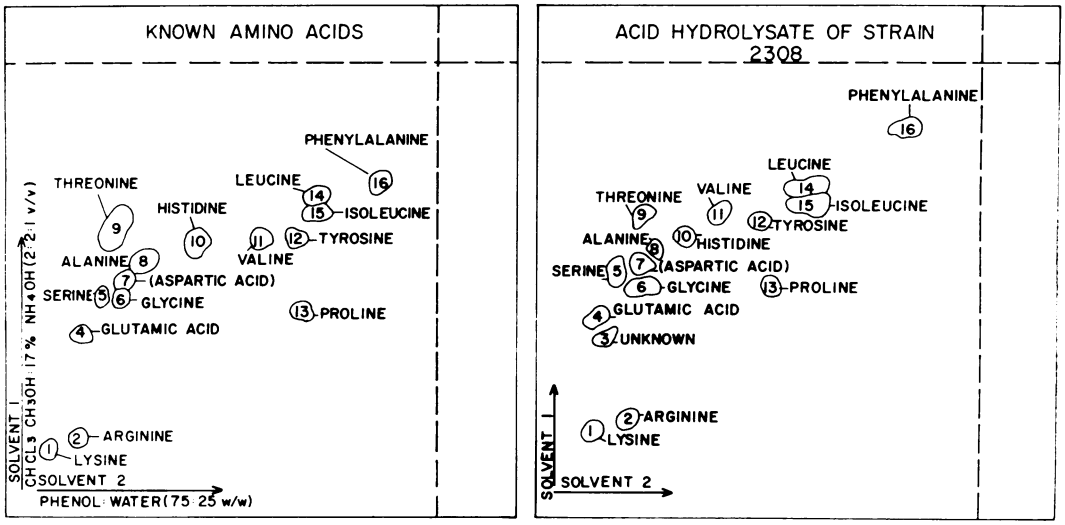

FIG. 2. *Comparison of tracings of thin-layer chromatograms of known amino acids and hydrolysates of B. abortus strain 2308. The "unknown" spot has an R_F value similar to that of o-phosphoryl ethanolamine.*

resolved and intensity of colors developed with the indicator used. Samples were applied in 200-nl amounts about 1 cm from the edge of a plate and about 3 cm apart. After 1 to 2 hr of equilibration in the developing tank, plates were lowered and developed for 2 hr in *n*-propanol, 17% ammonium hydroxide, and water (6:2:1, v/v). After air-drying, plates were sprayed with anisaldehyde in sulfuric acid, heated at 100 C for 15 min, and observed for colored spots. Known carbohydrates were applied singly and in mixtures, and were chromatographed in a similar manner for comparison of colors and R_F values.

Other chemical determinations. Nitrogen was determined by the micro-Kjeldahl technique (12), total carbohydrate by the Dische-Shettles method (5), hexoses by the anthrone method (21), hexosamine by the Rimington (17) modification of the Elson-Morgan method, and heptose by the method of Slein and Schnell (22). Dideoxyhexose and 2-keto-3-deoxyoctulosonic acid were detected by the methods described by Anacker et al. (1).

Detection of lipids by TLC. Cell walls from *B. abortus* strains 19A and 2308 were extracted in separate 10.0-mg amounts with anhydrous ethyl ether for 24 hr. Suspensions were shaken periodically in a separatory funnel. After extraction, the ether extract was filtered through Whatman no. 40 paper. Ether was evaporated by passing nitrogen gas through the filtrate, and the extract was reconstituted in 0.5 ml of ether for chromatography. Silica gel H was spread 0.2 mm thick on plates, activated at 110 C for 1 hr, and equilibrated for 1 hr over solvent (16). Samples of 75 nl were applied 1 cm from one edge of a plate and 3 cm apart, and were developed for 30 min in xylene or ether in one dimension only. Plates were then air-dried, sprayed with phosphomolybdic acid, and heated at 110 C for 10 min before observation of the results.

Detection of complexes. Plates prepared in a manner similar to that used for lipid analysis were also used to detect complexes which might be reactive to all indicators used for TLC in this study, except that triplicate 50-nl samples of ether extract of cell walls were placed in each of three spots on a plate (Fig. 1). After development in xylene for 30 min, the plates were air-dried. One section was sprayed with ninhydrin, a second with phosphomolybdic acid, and the third with anisaldehyde in sulfuric acid. Glass shielding was used to insure that the desired indicator would be sprayed only on that section for which it was intended. After all three sprays had been applied, the plates were heated at 110 C for 10 min and the spots were recorded. Unsprayed plates were also viewed with ultraviolet light of 350 nm.

Dermal toxicity of ether extracts of cell walls for rabbits. The ether extract of cell walls was mixed with sterile distilled water (1:1, v/v). Ether was removed by passing nitrogen gas through the mixture, and twofold dilutions were made of the remaining aqueous material in water. Intradermal injections were made of 0.1-ml amounts of each dilution in horizontal rows randomized on three rabbits (13). Injection sites were observed for evidence of edema, erythema, and necrosis every day for 1 week.

RESULTS

Amino acid analysis. A comparison of R_F values of known amino acids obtained by TLC with those obtained for spots in hydrolysates of strain 2308 and 19A cell walls is presented in Table 1. The patterns obtained with the known amino acids (Fig. 2) were very similar to those obtained by Fahmey et al. (7). A total of 15 amino acids were identified in the cell wall hydrolysates of both strains, and 14 of the amino acids detected

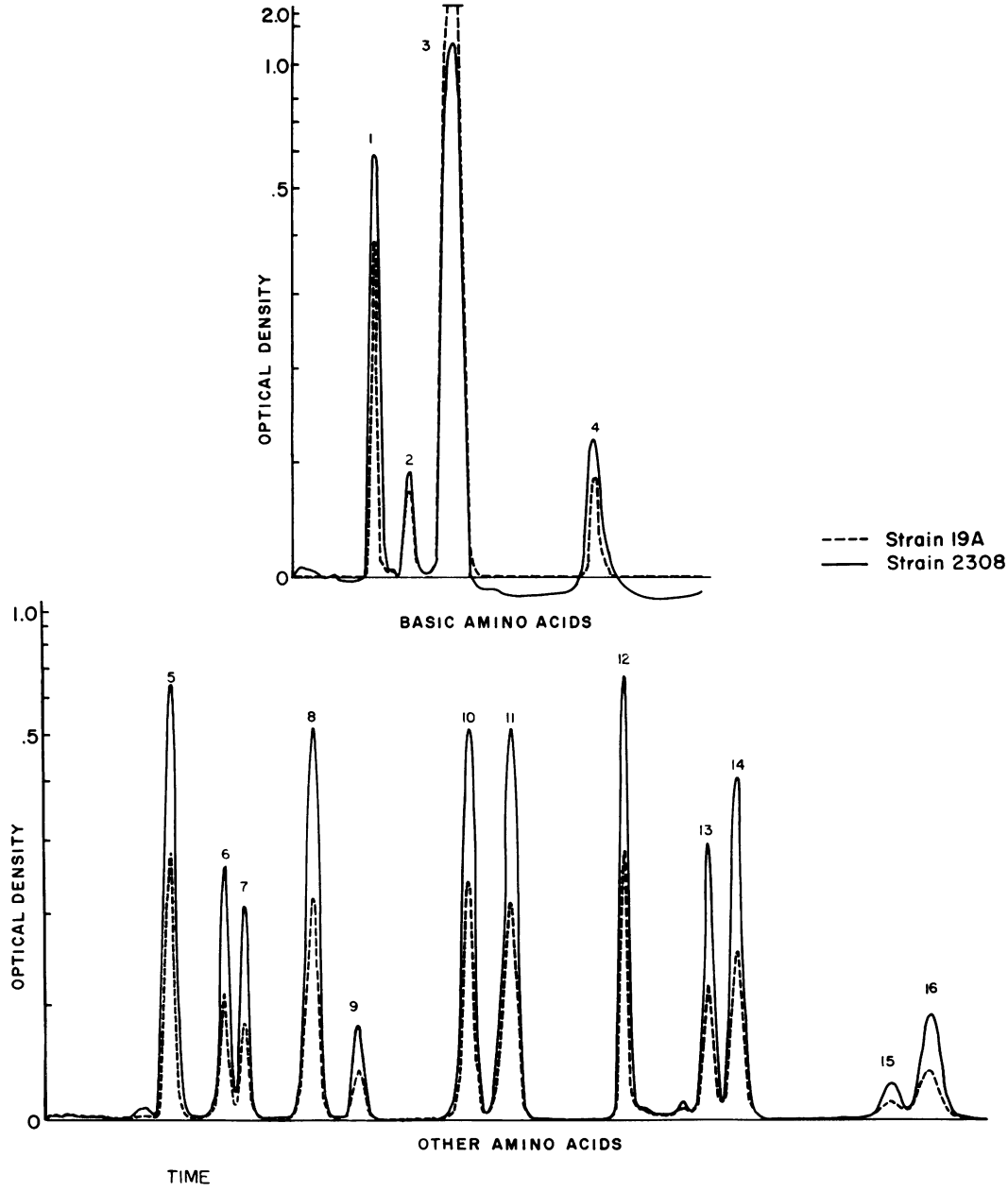

FIG. 3. *Comparison of the endotoxin of B. abortus strains 19A and 2308 on a Beckman model 120B amino acid analyzer. Analysis was carried out on a 150-cm column for the acid and the neutral amino acids and on a 15-cm column for the basic amino acids. For total analysis, 0.5 ml portions of the samples were pipetted onto the columns. Column packing was styrene, and chart speed was 0.1 inch/min (0.25 cm/min). Identification was made by a comparison with known amino acids. Short column: (1) lysine, (2) histidine, (3) NH_3, (4) arginine. Long column: (5) aspartic acid, (6) serine, (7) threonine, (8) glutamic acid, (9) proline, (10) glycine, (11) alanine, (12) valine, (13) isoleucine, (14) leucine, (15) tyrosine, (16) phenylalanine.*

in hydrolysates of ECP of both strains were the same as those found by Badakhsh and Foster (2). On the basis of color intensity of the spots, most amino acids were present in visibly higher concentrations in strain 2308 than in strain 19A. The same 15 amino acids were identified with the amino acid analyzer in hydrolysates of ECP, and those from strain 2308 were present in higher con-

centrations than those from strain 19A (Fig. 3). In addition, a component with the same R_F value as *o*-phosphoryl ethanolamine was detected by TLC of hydrolysates of cell walls and ECP of strain 2308 but was not detected in similar preparations from strain 19A. Strain 2308 also had a slightly higher nitrogen content (Table 2) than strain 19A. No extra component was detected in samples of strain 2308 run on the amino acid analyzer, although the extra component was present in replicate samples of three different lots of strain 2308 cell walls and absent in three different lots of strain 19A cell walls examined by TLC.

TABLE 2. *Comparison of chemical content of cell walls of B. abortus strains 19A and 2308*

Strain	Nitrogen	Protein	CHO	Hexoses (anthrone)	Hexosamine
	%	mg	%	%	%
19A	9	0.55	7.8	6.0	5.5
2308	10	0.62	10.0	8.0	3.8

TABLE 3. *Comparison of carbohydrates detected by thin-layer chromatography in B. abortus strains 19A and 2308 cell walls, B. abortus strain 19A aqueous ether extract, and B. suis ether extract*[a]

Sample tested	Sugars detected	Color	R_F
Cell walls, 19A	Glucose	Violet	.75
	Rhamnose	Blue-green	.41
	Galactose	Gray-green	.20
	Mannose	Gray-green	.15
Cell walls, 2308	Glucose	Violet	.75
	Rhamnose	Blue-green	.41
	Galactose	Gray-green	.20
	Mannose	Gray-green	.15
Aqueous ether extract, 19A endotoxin	Glucose	Violet	.65
	Unknown	Green	.51
	Rhamnose	Blue	.34
	Unknown	Violet	.27
	Galactose	Gray-green	.20
Ether extract, *B. suis* strain 3B cells	Glucose	Violet	.63
	Unknown	Green	.51
	Unknown	Blue	.48
	Rhamnose	Blue	.31
	Galactose	Gray-green	.24
	Mannose	Blue-green	.17
Known mixture	Glucose	Violet	.75
	Rhamnose	Blue-green	.41
	Galactose	Gray-green	.20
	Mannose	Gray-green	.15

[a] Solvent was *n*-propanol, ammonium hydroxide, and water (6:2:1, v/v). Indicator was anisaldehyde in sulfuric acid.

TABLE 4. *Lipid components detected in ether extracts of Brucella abortus cell walls by thin-layer chromatography*

R_F values after development in		
Ethyl ether		Xylene (strain 19A)
Strain 2308	Strain 19A	
.95	.95	.98
		.89
		.82
.64	.64	.61
.43	.43	.30
.28	.28	.27
.15	.15	.13

TLC of hydrolysates of cell walls which had been tagged prior to hydrolysis with 2,4-dinitrofluorobenzene indicated that phenylalanine was a terminal amino acid in *B. abortus* strains 19A and 2308, *B. suis* strain 1776, and *B. melitensis* strain 2500.

Carbohydrate analysis. On the basis of R_F values and color of spots detected by TLC of cell walls (Table 3), tentative identification was made of glucose, galactose, rhamnose, and mannose. Detectable differences in these components were not observed between strains 19A and 2308. Unidentified spots were also seen in ECP from *B. abortus* strain 19A and *B. suis* strain 3b. On the basis of colorimetric tests, 2-keto-3-deoxyoctulosonic acid, heptose, and dideoxyhexose are all present in cell walls and ECP of *B. abortus* strains 19A and 2308. Strain 2308 had more total carbohydrate and hexoses and less hexosamine than strain 19A.

Lipid analysis. As shown in Table 4, as many as seven components were resolved in ether extracts of strain 19A cell walls when xylene was employed as a developer, but only five were resolved with ether as a developer. The same five spots were detected in hydrolysates of strain 2308 cell walls, and color intensity of the spots appeared equal. This study was made on duplicate samples from a single lot of cell walls.

In a separate study on ether extracts of another lot of cell walls, extracts were developed with xylene as shown in Fig. 1. Only six components were detected on the section of the plate developed with phosphomolybdic acid. In the next section, developed with ninhydrin, two spots were detected which had R_F values corresponding to those of the most rapidly moving components detected with phosphomolybdate. In the third sec-

tion, developed with anisaldehyde in sulfuric acid, there were also two components with R_F values corresponding to the most rapidly moving components. Comparison of these data with those obtained in the study by Bobo and Eagon (3) suggests that these rapidly moving components might be phosphatidylethanolamine and lysophosphatidylethanolamine.

DISCUSSION

Although the endotoxic activity of *B. abortus* strain 19A is about as great as that of strain 2308 (10), strain 19A is less virulent than strain 2308 and is commonly used for vaccination of cattle. That is, in cattle and guinea pigs, strain 19A does not cause as severe an infection as strain 2308, and, if given to heifers 6 to 8 months old, strain 19A is an effective vaccine (4). Mice tolerate considerably higher doses of live strain 19A organisms than of strain 2308 organisms (10). Considering these points, one may expect that one or more "virulence factors" present in strain 2308 may not be present in strain 19A. There is a possibility that a metabolic difference is involved (25), but the question raised in this study regarded chemical differences in antigens or virulence factors. There were no obvious qualitative differences between lipid or carbohydrate components of strain 19A and 2308 cell walls or ECP as shown by TLC. There were possibly larger quantities of total carbohydrate and hexose and smaller quantities of hexosamine in strain 19A cell walls than in strain 2308 cell walls. However, the differences may not be significant as only one lot of cell walls was tested.

A qualitative difference in the cell walls and ECP of strain 19A and 2308 may exist, as an extra ninhydrin-reactive component was present in triplicate preparations in TLC of strain 2308 preparations but was not detected in comparable strain 19A preparations. These differences had been noted previously (2; Kellerman et al., Bacteriol. Proc., p. 124, 1967). To rule out the possibility that the extra component was an artifact with the mobility of ethanolamine, hydrolysates of autoclaved uninoculated culture medium were analyzed by TLC. Only the amino acids originally put into the medium were detected. There still might be some artifact of hydrolysis, but the reproducibility of the unknown spot suggests that something about strain 2308 was different from strain 19A preparations. Comparison of R_F values of the extra component with those from TLC of several known amino acids and other ninhydrin-reactive compounds indicated that the unknown compound was probably ethanolamine. None of the R_F values of components in the hydrolysates corresponded to diaminopimelic acid. This is in disagreement with Markenson et al. (14) but in agreement with Ellwood et al. (6). There was no evidence of an extra component in hydrolysates from strain 2308 ECP when it was compared with 19A in the amino acid analyzer. Since a different substrate was used for TLC and since solvents for the amino acid analyzer differed, one might expect some differences in components. However, there was excellent agreement regarding the 15 amino acids detected.

Phenylalanine was shown to be a terminal amino acid in cell walls of the *B. abortus*, *B. suis*, and *B. melitensis* strains studied. If phenylalanine has an important serological role, this might help explain the close serological relationships seen among these *Brucella* species.

Ether extraction removed some lipids from the cell walls of strains 2308 and 19A, as might be expected. It has been observed in previous studies (J. W. Foster and R. Brown, Bacteriol. Proc., p. 95, 1967) that ether treatment of *Brucella* cell walls causes extensive breakup. It is speculated that one or more of the lipids extracted is essential to maintaining the morphological integrity of *Brucella* cell walls. The lipids extracted also may be partly responsible for toxic reactions observed in rabbit skin and ascribed to *Brucella* endotoxin, but it is not certain that some small amount of endotoxin might not be contaminating the ether extract. As little as 0.01 μg of endotoxin caused a rabbit skin lesion in some experiments.

ACKNOWLEDGMENTS

We express our appreciation for technical assistance to Elizabeth Ward and for the figures to Ethel Foster. Results from the amino acid analyzer were obtained through the kind assistance of Sarah S. Cliett and Harry E. Peck of the Biochemistry Department.

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