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Surface Macromolecules and Virulence in Intracellular Parasitism: Comparison of Cell Envelope Components of Smooth and Rough Strains of *Brucella abortus*

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The surface topography of whole cells and the chemical composition of cell envelopes of a smooth-intermediate strain (45/0) and a rough strain (45/20) of *Brucella abortus* was examined. Electron microscopy of whole cells and thin sections did not reveal any gross surface difference(s). Only minor quantitative differences were observed in total lipids, proteins, and the murein layer. However, the lipopolysaccharide composition of the two strains was quite different. Both phenol- and water-soluble lipopolysaccharide fractions were obtained from the strain of higher virulence (45/0), whereas only aqueous lipopolysaccharide could be isolated from the rough strain. In addition to being toxic, the phenol-soluble lipopolysaccharide may be a key virulence factor in intracellular survival of *B. abortus* within phagocytic cells.

The role of surface macromolecules in determining the virulence of extracellular parasites has been well established (46). The polysaccharide capsule of *Streptococcus pneumoniae* (44) and some gram-negative bacteria (e.g., *Klebsiella pneumoniae*), as well as protein antigens such as the M-protein of *Streptococcus pyogenes* (44) and the V/W antigen of *Yersinia pestis* (45), are a few examples of virulence factors that enable bacteria to resist the action of phagocytic cells.

It is not clear what role(s) surface components play in the virulence of facultative intracellular parasites such as *Brucella*, *Listeria monocytogenes*, and the mycobacteria. Since these bacteria are readily ingested, but not killed, by phagocytes (32), it is obvious that surface components of intracellular parasites play some role other than blocking phagocytosis. For example, they may promote adhesion and facilitate ingestion so that the bacteria are protected from bactericidal factors and antibiotics present in serum. It is also possible that these surface macromolecules protect the bacteria from the hostile environment within the phagolysosome (32) or somehow inhibit the degranulation usually observed in phagocytes after ingestion. The appropriate signal to initiate degranulation by the host phagocytic cell may never occur because of the unique surfaces of these bacilli.

If all aspects of microbial pathogenicity are considered, including the host response, then both the interaction with phagocytes and the subsequent intracellular survival should be considered in a model to explain the dynamic host-parasite relationship of intracellular parasites. The bactericidal activity of phagocytes must be increased to promote resistance and terminate active infections.

Since it is difficult to extrapolate from one genus of facultative intracellular parasites to another, it was our objective to develop a model system using two closely related strains of *Brucella abortus* that differ markedly in virulence. Attenuated rough brucellae are more cytotoxic to macrophages in culture than are smooth bacilli (7), and thus it would appear that smooth highly virulent bacilli enter into a more successful host-parasite relationship. Although physiological differences may explain the decreased virulence and enhanced cytotoxicity of the rough bacilli (52), it is more likely that the chemistry of surface macromolecules plays a role in virulence. Further, attempts to relate surface macromolecule components of *Brucella* with virulence have used strains from different species and unrelated strains within a species (20, 23, 38).

Our approach has been to examine whole cells and to determine the chemical properties of cell walls and the constituents of the cell envelope of a smooth parent strain (45/0) and a rough variant strain (45/20) of *B. abortus* (28), with the objective of relating chemical structures with differences in biological activity. In an earlier

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report, we observed that cell walls of both strains were potent mitogens for lymphocytes, whereas isolated constituents of cell wall preparations had little or no mitogenic activity (19).

In this report, we describe the results of a comparative study of the proteins, lipoproteins, total lipids, murein layer, and lipopolysaccharide (LPS) composition of *B. abortus* strains 45/0 and 45/20. The only significant difference was that LPS was found in both the aqueous and phenolic phases after Westphal extraction of the smooth parent strain (45/0) but only in the aqueous phase of the rough variant strain (45/20). Possible implications of the LPS structure in the virulence of *B. abortus* are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. abortus* strain 45/0 was supplied by M. E. Meyer, University of California, Davis, and strain 45/20 was obtained from B. L. Deyoe, National Animal Disease Center, Ames, Iowa.

The smooth strain (45/0) of moderate virulence was first isolated from material of bovine origin by McEwen and Roberts (29). The reduced virulence of strain 45/0 in comparison with most field strains prompted interest in it for use as a vaccine. In attempts to determine whether its virulence could be increased by animal passage, the culture was serially passaged through guinea pigs at least 40 times. The culture remained smooth upon serial passage except for the 20th passage, which proved to be a rough colonial type. The organism reverted to a smooth form upon subsequent passages. The rough culture, designated as 45/20, has been used as a killed adjuvant vaccine (9, 10, 41). These cultures were selected because of their ease of handling, because strain 45/20 was directly derived from the parent strain 45/0, and because they exhibited surface properties typical of smooth and rough brucellae (1).

Both strains were grown in a medium containing tryptose, yeast extract, glucose, vitamins, and salts as described previously (39). Cells were harvested in mid-log phase, which corresponded to an optical density at 620 nm of 5 to 10 obtained with a 1:20 dilution in phosphate-buffered saline by use of a Spectronic 20 spectrophotometer (Bausch & Lomb). The bacteria were pelleted by centrifugation at 6,000 × *g* for 20 min in a Sorvall RC-2B centrifuge (4°C), washed once with phosphate-buffered saline, and resuspended in either distilled water or buffer.

Cultures were transferred monthly by plating on Trypticase soy agar. After 5 days of incubation at 37°C, colonies typical of either the smooth phase (1) of strain 45/0 or the rough phase of strain 45/20 were transferred to potato infusion agar slants. The slants were incubated for 24 h at 37°C and then stored at 4°C. The surface properties of each strain were also periodically monitored by acriflavine agglutination, spontaneous agglutination in saline, and *Brucella* phage sensitivity (1).

Electron microscopy. Negative staining of whole cells was performed as described by Parsons (35). A drop of sample was suspended on precoated Parlodion grids (0.5%) for 30 s and then removed. A drop of 2% phosphotungstic acid (supplemented with 0.1 mg of bovine serum albumin per ml) was then placed on the grid for 12 s, and the stained samples were viewed with an RCA EMU F2 electon microscope. Preparations for thin sectioning were prepared by standard procedures (35) and viewed with a Philips 300 electron microscope.

Preparation and enzymatic digestion of cell walls. Cell walls of both strains of *B. abortus* were isolated as described previously (22). Enzymatic digestion of isolated cell walls was carried out by the methods of Braun and Rehn (8). Cell wall preparations were suspended at an approximate absorbance of 1.0 at 578 nm, and individual enzymes were added until a 1:50 enzyme to substrate ratio was achieved. Cell wall sensitivity to papain, trypsin, Pronase, or lysozyme was determined by measuring the decrease in absorbance with time. The drop in absorbance at 578 nm was taken as evidence for cell wall disaggregation.

Isolation of LPS. Both the smooth-intermediate strain 45/0 and the rough strain 45/20 were extracted by the phenol-water procedure of Westphal as modified by Raff and Wheat (37). Cells were suspended in 44% hot aqueous phenol (5 g [dry weight] of cells per 100 ml), heated at 68°C for 30 min, and centrifuged after cooling to 4°C. The aqueous phase was removed, an equal volume of deionized water was added to the cell residue-phenol layer, and the mixture was extracted again. The aqueous phases were pooled, and the phenol phase was removed. The remaining cell residue was then extracted with hot 88% phenol (68°C for 30 min). The pooled aqueous and phenolic phases were each dialyzed against tap water for 3 to 5 days and fractionated by differential centrifugation at 27,000 and 100,000 × *g*.

Isolation of murein-lipoprotein complexes, lipoprotein, and mucopeptide. Murein-lipoprotein complexes were isolated from cell walls of both strains of *Brucella* by the procedure of Braun as previously described (22). Proteins solubilized from cell walls by sodium dodecyl sulfate were designated as "free protein." Proteins that remained with the mucopeptide-lipoprotein complex were designated as "bound proteins." Lipoprotein(s) and mucopeptide moieties were isolated from the mucopeptide-protein complex as described by Braun and Rehn (8) by suspending the complex in 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 8.2) and digesting it for 4 h with trypsin (enzyme-substrate ratio, 1:25). The digestion was stopped by the addition of hot 10% sodium dodecyl sulfate to a final concentration of 1%. After centrifugation, both the supernatant fluid and pellet were lyophilized and stored at −20°C. Protein was determined by the Lowry procedure (3). The assay for muramic acid was performed according to Hadzija (14), and that for total carbohydrate was determined by the phenol-sulfuric acid method (11) by using glucose as a standard.

Lipid and fatty acid analysis of *Brucella* cell walls and cell wall components. Total phospholipids and fatty acids were extracted by the procedure of Nelson and Buller (33). Aqueous suspensions of cells (10 mg/ml) were added to screw-cap tubes (16 by 125

mm) containing 6 ml of chloroform-methanol (1:2, vol/vol). The suspension was blended in a Vortex mixer for 2 min and extracted at room temperature for at least 1 h. After centrifugation for 20 min at 15,000 × *g*, the supernatant fluid was removed, and the residue was extracted overnight with 3 ml of chloroform-methanol (2:1, vol/vol) and centrifuged. The supernatant fluids from the two centrifugations were pooled and washed twice with 4 ml of water. The lipid extract was evaporated to dryness under a stream of N_2, resuspended in a known volume of chloroform, and designated as the free lipid-fatty acid extract.

Thin-layer chromatographic procedures of Skipski and Barclay (43) were utilized for analysis of lipids and fatty acids. Thin-layer chromatographic plates were prepared by use of a Brinkman spreading board, a Desagna adjustable spreader, and Camag silica gel (without binder). Thin-layer chromatographic plates 0.5 mm thick were poured, dried overnight, scored, activated for 1 h at 120°C, and maintained in a desiccator over anhydrous $CaSO_4$ until use. The lipid extracts were applied approximately 2 cm above the bottom of the plates and were developed with the appropriate solvent system. Phospholipids were separated with chloroform-methanol-water (65:25:4, vol/vol). Isopropyl ether-acetic acid (96:4, vol/vol) was used to separate nonhydroxylated fatty acids and hydroxylated fatty acids. Fatty acids were detected by spraying with a 0.2% solution of 2,7-dichlorofluorescein and visualization under ultraviolet light. Iodine vapor was utilized to detect phospholipid spots. Unknown fatty acids and lipids were identified by comparison with known standards, published R_f values, and the use of James plots (16).

Quantitation of *Brucella* lipids and free fatty acids by [^{14}C]acetate incorporation. The free fatty acids and bound fatty acids (lipids/lipoprotein) of *B. abortus* 45/0 and 45/20 were radioactively labeled by addition of 5 to 10 μCi of [^{14}C]acetate per ml of tryptose-yeast extract growth medium (minus glucose). After one or two generations of growth in the presence of the radioactive label, the bacteria were harvested by centrifugation, and free lipids and fatty acids were extracted as described above. Nonradioactive cells were added when necessary as a carrier to improve quantitative extraction of lipids and fatty acids.

Nonradioactive and [^{14}C]acetate-labeled lipids and fatty acids were chromatographed on thin-layer chromatographic plates and visualized with iodine vapor as described above. Spots were scraped into counting vials containing 4 ml of a counting solution of 5 g of 2,5-diphenyloxazole and 0.1 g of dimethyl 1,4-bis-2(5-phenyloxazole)-benzene per liter of toluene. The samples were counted in a Packard model 3375 liquid scintillation spectrometer, and counting efficiency was determined with the use of a nitromethane quench curve.

Hydrolytic release of bound fatty acids was accomplished by the procedure of Langworthy and Buller (25). Normally, nitrogen-dried samples were suspended in 3 ml of 2 N HCl in methanol and heated for 16 h at 80°C (Kallikainen model 100 block heater). After hydrolysis and extraction with either petroleum ether-ethyl ether (1:1, vol/vol) or petroleum ether, fatty acids were analyzed by thin-layer and gas-liquid chromatography (30).

Polyacrylamide gel electrophoresis of *Brucella* proteins. Samples containing bacterial protein (whole cells, cell walls, mucopeptide-lipoprotein complex) were extracted from both strains of *Brucella* with a phenol-acetic acid-water mixture (4:2:1, vol/vol) at a ratio of 100 μg of sample per 100 μl of extraction solution and were centrifuged at 40,000 × *g* for 30 min (31). The resulting supernatant fluid was analyzed by polyacrylamide slab gel electrophoresis (model 50 Hoefer Science slab gel electrophoresis apparatus). Slab gels were prepared by addition of 10 mg of ammonium persulfate to 10 ml of solution A (acrylamide, 7.5 g; urea, 30 g; *N,N*-methylene bisacrylamide, 0.2 g; acetic acid, 35 ml; and water to 100 ml), followed by addition of 0.1 ml of *N,N,N',N'*-tetramethylethylenediamine. The running buffer was 10% (vol/vol) aqueous acetic acid. The anode was set as the upper electrode. Samples were loaded and subjected to electrophoresis for 1 h at 30 mA and then for 5 to 6 h at 50 mA. Proteins were visualized by staining for 1 h in 0.025% Coomassie brilliant blue stain prepared by adding 11 ml of 0.25% Coomassie brilliant blue to 110 ml of methanol-acetic acid-water (5:1:5, vol/vol). Destaining of gels was performed by soaking the stained gel in an aqueous solution of 7.5% acetic acid and 5% methanol.

Limulus lysate test. Endotoxin and endotoxin-like substances in the cell walls of *B. abortus* strains 45/0 and 45/20 were detected by the procedure outlined by Sigma Chemical Co., St. Louis, Mo.

Prior to limulus assays, all glassware was soaked overnight in a 1% E-Toxa-Clean solution and rinsed 10 times in tap water, 5 times in distilled water, and finally once in pyrogen-free water. The glassware was then autoclaved at 121°C, followed by oven heating (175°C for 3 h). All assays were incubated in 10-ml conical tubes with pyrogen-free aluminum-foil covers. Sample tubes contained 0.1 ml of test solutions, 0.1 ml of pyrogen-free water, and 0.1 ml of E-Toxate. Positive control tubes contained 0.1 ml of *Salmonella typhimurium* LPS (1 or 10 μg) and 0.1 ml of E-Toxate solution. All assay tubes were incubated at 37°C and examined after 30 and 60 min for gel formation.

Amino sugar analysis. Total hexosamine content of the mucopeptide-protein complex was determined by the procedure of Strominger (47) after hydrolysis in 8 N HCl for 12 h at 100°C. Qualitative identification of amino sugars was achieved by descending paper chromatography with the use of Whatman 1MM paper developed with ethyl acetate-pyridine-acetic acid-water (5:5:1:3, vol/vol).

Duplicate chromatograms were developed, and amino sugars were detected by ninhydrin spray and alkaline silver nitrate (51). Identification of amino sugars was based on co-chromatography with known standards.

Toxicity of cell wall preparations. Mouse lethality was used to determine the biological toxicity of *Brucella* cell walls and cell wall components. Whole cells or cell walls were suspended in saline, and 0.1-ml amounts of serial dilutions were injected intraperitoneally into female CF-1 mice (20 to 25 g). The mice received food and water ad libitum and were moni-

tored for signs of stress (e.g., weight loss, ruffled fur, or death).

Reagents and chemicals. Deoxyribonuclease, lysozyme, trypsin, and muramic acid were obtained from Sigma Chemical Co. Papain and Pronase were purchased from Worthington Biochemical Corp., Freehold, N.J. Sodium [1,2-^{14}C]acetate (58.7 mCi/mmol) was from New England Nuclear Corp., Boston, Mass. Reference fatty acid methyl ester standards for gas-liquid chromatography were obtained from Sigma Chemical Co. and Applied Science Laboratories, State College, Pa. β-Hydroxymyristic acid was kindly provided by C. S. Buller of this department. All other general reagents and solvent were analytical reagent grade.

RESULTS

Surface topography. Both strains of *B. abortus*, 45/0 and 45/20, were examined by classical methods used to distinguish between smooth and rough strains of *Brucella* (1). Strain 45/0 did not absorb crystal violet and was not agglutinated by either saline or acriflavine, but was susceptible to *Brucella* phage infection. In contrast, strain 45/20 was agglutinated by saline and acriflavine and was resistant to phage infection, all of which are properties of rough strains of *Brucella*.

These differences prompted us to examine the surfaces and membrane structure of both strains by electron microscopy. The bacterial surfaces of both strains appeared extremely convoluted and somewhat granular (Fig. 1 and 2), and no significant differences were observed in their surface topography.

Thin sections of the two strains (Fig. 3 and 4) revealed an inner-outer membrane structure similar to that which has been observed in numerous other gram-negative bacteria. The inner and outer membranes were evident (Fig. 3 and 4), and no unique structural features were observed in either strain.

Chemical analysis of isolated cell walls. As described previously (22), cell wall preparations of both strains were examined for carbohydrate, 2-keto-3-deoxyoctonate, muramic acid, protein, and dideoxyaldoses. The only significant difference between the strains was in the level of dideoxyaldoses; strain 45/0 and a four-fold higher level than strain 45/20.

Digestion of cell walls with hydrolytic enzymes. Because *Brucella* may be able to withstand the hydrolytic enzymes within the phagolysosome (32), it was of interest to determine the relative resistance of each strain to various enzymes. A decrease in absorbance at 578 nm measures enzymatic cleavage and cell wall disaggregation. Both lysozyme and trypsin decreased the optical density of strain 45/20 cell walls, but had little or no effect on those of 45/0 (Table 1). Pronase decreased the absorbance slightly whereas papain had minimal activity on cell walls of strain 45/0.

Lysozyme was active against 45/20 cell walls, with increased disaggregation noted upon sub-

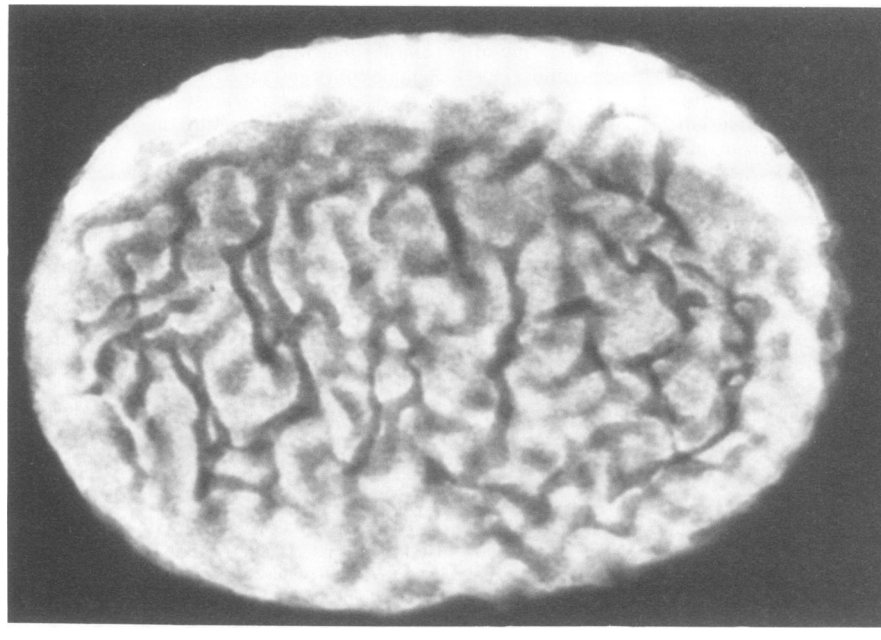


FIG. 1. *Negative-stained preparation of B. abortus 45/0. ×270,000.*

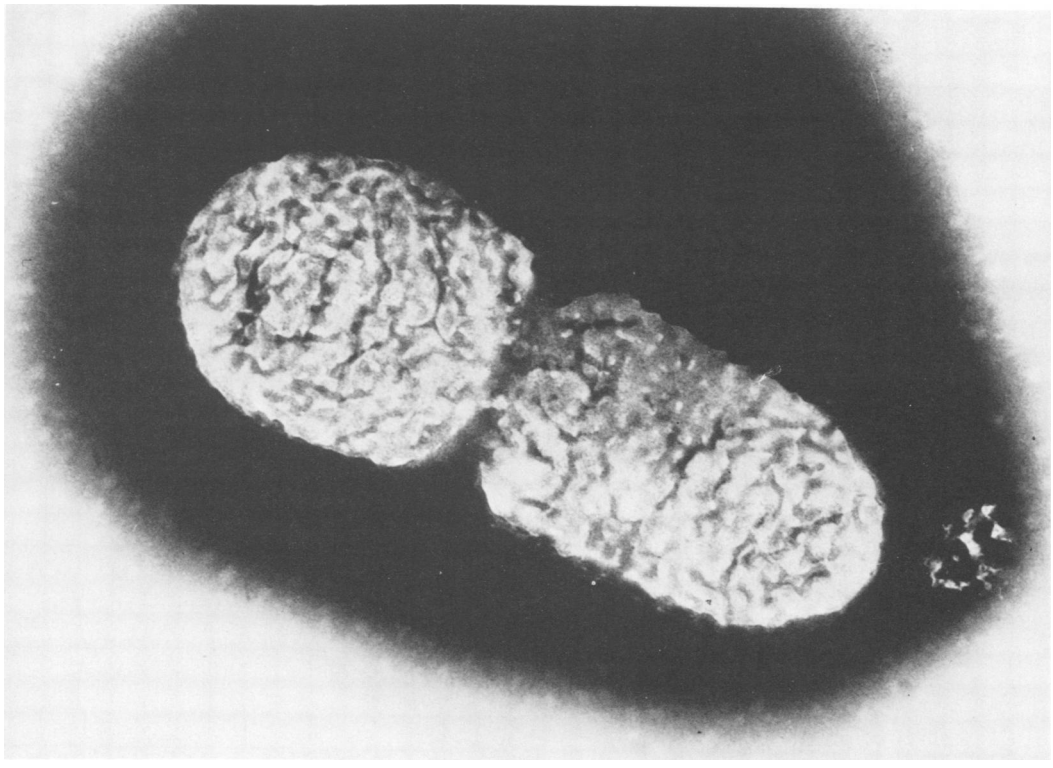


FIG. 2. *Negative-stained B. abortus 45/20. ×150,000.*

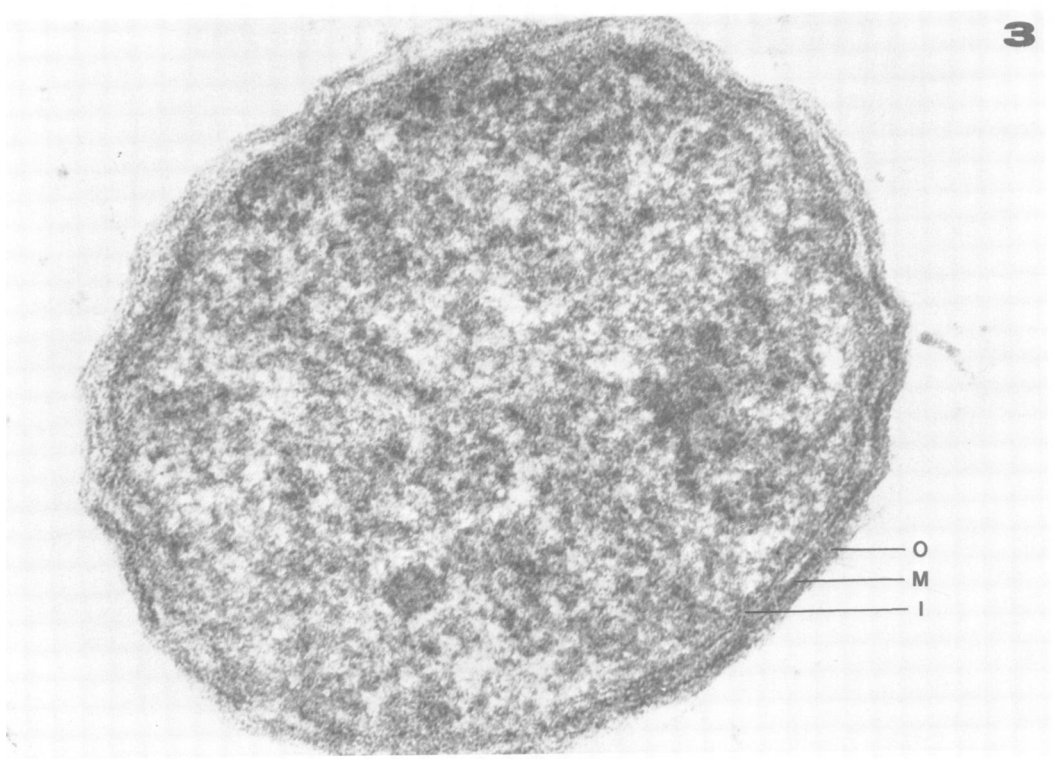
sequent addition of trypsin (Fig. 5). Strain 45/0 was not disaggregated by lysozyme and the subsequent addition of trypsin. In general, then, the cell walls of the rough strain were more susceptible to enzymatic hydrolysis than were those of the smooth intermediate strain. In support of these observations, only the rough strain, 45/20, was susceptible to the enzymatic procedure recently developed to isolate LPS with a higher degree of purity than possible with either phenol-water or trichloroacetic acid extraction (26).

Isolation of LPS. Several extraction procedures were used in an effort to isolate LPS from *B. abortus* 45/0 and 45/20. The phenol-water procedure of Westphal, as modified by Raff and Wheat (37), yielded LPS from both strains of *Brucella*. No LPS could be isolated from either organism by the chloroform-phenol-petroleum ether procedure of Galanos et al. (12) designed to extract R-form LPS.

Differential centrifugation was employed for primary fractionation of LPS isolated by the phenol-water procedure. Heavy LPS pelleted at 27,000 × *g*, and light LPS sedimented at 100,000 × *g*. LPS was present in both aqueous and phenolic extraction phases of the smooth strain, *B. abortus* 45/0, but only in the aqueous extraction phase of the rough strain, *B. abortus* 45/20 (Table 2). The centrifugation at 27,000 × *g* yielded a two-layered pellet with a lightly packed white upper layer which was water soluble and readily removed by rinsing. The remaining light-brown layer was removed by scraping with a spatula. These fractions have been noted as heavy white and heavy brown, respectively. Though the distribution of LPS in aqueous and phenolic phases varied between the two strains, it is important to note that the total yield of LPS from each organism was about 2%.

Limulus lysate activity of *Brucella* LPS. Purified cell walls prepared as described previously (22) and all LPS fractions exhibited a positive limulus lysate test at a concentration of 1 μg/ml. Jones et al. (18) observed that the phenolic-phase LPS of *B. abortus* was active in the limulus test at 1 ng and was 100-fold more active than an aqueous-phase preparation.

Whole-cell and cell wall proteins. Analysis of the whole-cell proteins revealed only minor qualitative and quantitative differences (Fig. 6), and no major differences were observed in the envelope proteins of either strain. The proteins released by trypsin digestion of the murein-lip-



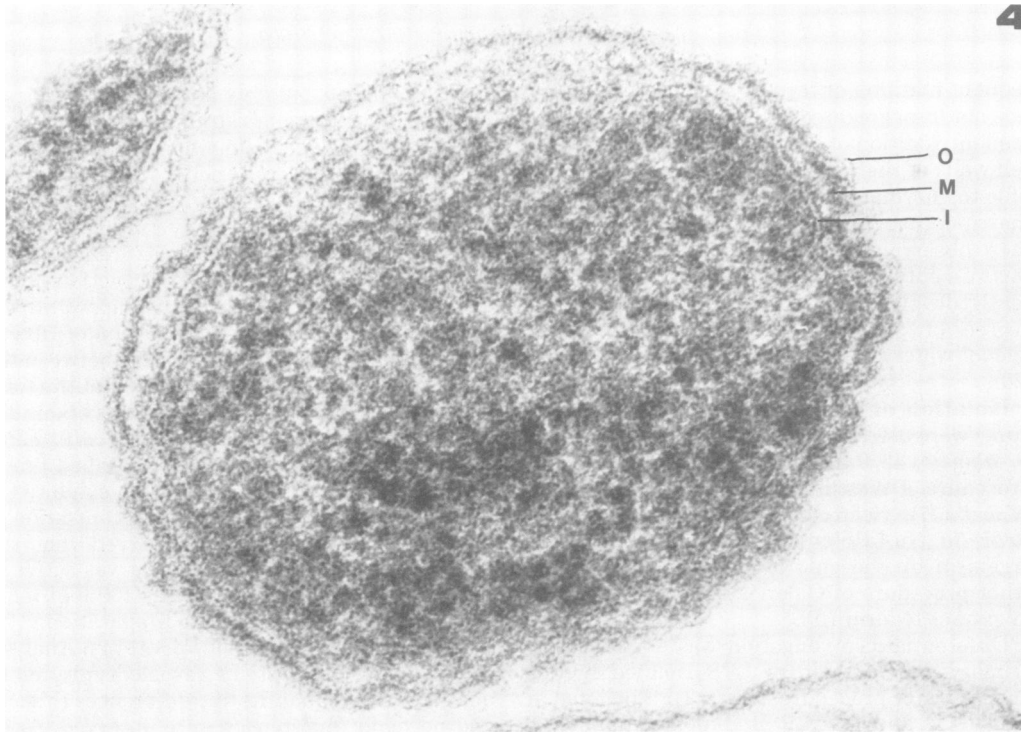


FIG. 3 and 4. *Thin sections of B. abortus 45/0 (Fig. 3) and 45/20 (Fig. 4). Envelope components are noted as inner membrane, I; mucopeptide, M; and outer membrane, O. ×270,000.*

oprotein complex are included for comparison. Again, no significant differences were detected.

Isolation and characterization of murein-lipoprotein complexes. The presence of a murein layer or mucopeptide in *B. abortus* and *B. melitensis* has been demonstrated (24), however, it was of interest to determine whether *Brucella* contains a lipoprotein covalently bound to murein as it is in *Escherichia coli* (8). This lipoprotein, first observed in *E. coli*, may constitute up to 40% of the outer membrane proteins. The residue remaining after extraction of cell walls of *B. abortus* with boiling sodium dodecyl sulfate was free from 2-keto-3-deoxyoctonate, a marker indicating LPS contamination. Preliminary experiments indicated that, although the mucopeptide-lipoprotein complex comprised a higher percentage of the cell wall of strain 45/20, there was no difference in muramic acid level and only a minimally higher level of hexosamine in the complex of strain 45/0 (Table 3).

A lipoprotein and/or proteins was released from the complex by trypsin digestion which represented 5.3% of the complex of 45/0 and 29.2% of the complex of 45/20. The material released by trypsin digestion was partially lipid as indicated by the presence of palmitic acid, oleic acid, *cis*-vaccenic acid, and nonadecanoic acid.

TABLE 1. *Effect of hydrolytic enzymes on isolated cell walls of Brucella abortus 45/0 and 45/20*

Enzyme	Relative drop in optical density[a]	
	45/0	45/20
Trypsin	0.06	0.13
Lysozyme	0.10	0.20
Pronase	0.10	0.25
Papain	0.03	0.13

[a] Obtained by subtracting the final absorbance at 578 nm from the initial absorbance at 578 nm.

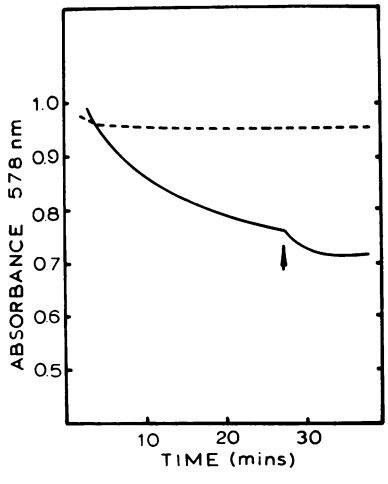


FIG. 5. *Effect of lysozyme and trypsin on isolated cell walls of B. abortus 45/0 (---) and 45/20 (——). Addition of trypsin noted by arrow.*

TABLE 2. *Yield of LPS from B. abortus 45/0 and 45/20*

Fraction	Yield (mg/g [dry wt] of cells)	
	45/0	45/20
Water light[a]	7.2	15.6
Water heavy white[b]	0.4	4.2
Water heavy brown[c]	0.4	1.9
Phenol light[a]	3.5	—
Phenol heavy white[b]	8.4	—
Phenol heavy brown[c]	0.6	—
Total	20.5	21.7

[a] LPS which pelleted at 100,000 × *g*.
[b] LPS which pelleted at 27,000 × *g*.
[c] A light brown layer (highly insoluble) which pelleted at 27,000 × *g* on the bottom of the tube.

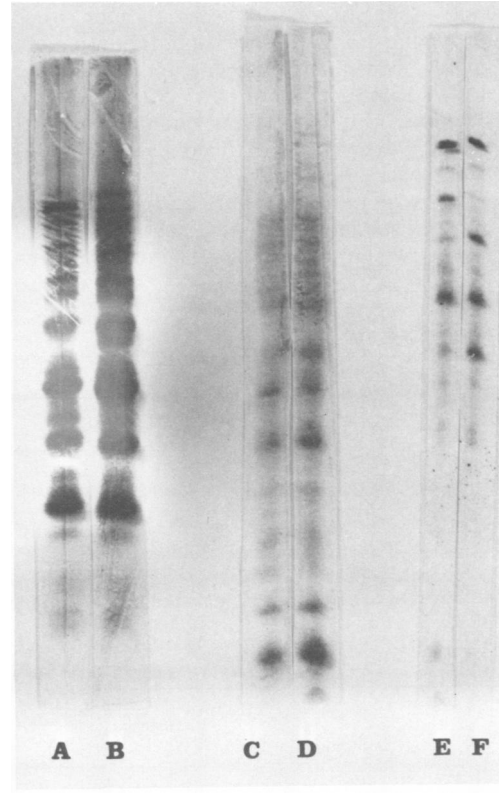


FIG. 6. *Protein profiles of smooth (gels A, C, and E) and rough strains (gels B, D, and F) of B. abortus. Whole-cell proteins, A, B; cell envelope proteins, C, D; and mucopeptide-lipoprotein complexes released by trypsin digestion, E, F.*

Interestingly, boiling sodium dodecyl sulfate solubilized 75% of the dry weight of strain 45/0 cell walls but only 54% of strain 45/20 cell walls. On the other hand, trypsin digestion released 21.2% and 63.5% of the protein of strains 45/0 and 45/20, respectively. Since significant differences in chemical composition were not observed, it appears that the greater resistance of strain 45/0 to enzymatic digestion may be due to higher numbers of cross-links in the mucopeptide layer and/or a different spatial configuration of the complex itself.

Characterization of free lipids. Thin-layer chromatographic analysis indicated the presence of the same five neutral lipids in each strain of *Brucella*, with three of the five lipids being ninhydrin positive, as is typical of *B. abortus* (5, 48–50). The lipids were identified as diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, lysophosphatidylethanolamine, an "ornithine-containing lipid."

Both strains incorporated equal amounts of [^{14}C]acetate into lipids. Thus, quantitative analyses of ^{14}C-labeled lipids from both strains of *Brucella* were performed (Table 4). Although there were quantitative shifts in the lipid distributions with increased [^{14}C]acetate labeling time, the two strains displayed similar patterns.

DISCUSSION

The objective of this work was to compare the surface topography of whole cells and the chemical composition of the cell envelopes of two closely related strains of *B. abortus* and to related any observable differences with virulence and intracellular survival within professional phagocytic cells. Electron microscopy of whole cells and thin sections did not reveal any gross differences, and chemical analysis of cell envelopes of both strains showed only minor quantitative differences in total lipids, proteins, and the properties of the mucopeptide-lipoprotein complex.

The major chemical difference in the cell preparations of the two strains was in the amount of dideoxyaldoses (22), a constituent of the polysaccharide component of LPS. Only the strain of higher virulence (45/0) contained a phenol-soluble LPS; the yield of the combined water-soluble LPS fractions from strain 45/20 was similar to the combined water-soluble and phenol-soluble LPS of 45/0. LPS has been detected in the phenolic-phase extract of other gram-negative bacteria (17, 37). Further, phenolic LPS has been extracted from virulent strains of *B. abortus* and *B. melitensis* (4, 17, 23,

TABLE 3. *Chemical properties of mucopeptide-lipoprotein complexes isolated from B. abortus 45/0 and 45/20*[a]

Strain	Complex[b]	Lipoprotein[c]	Muramic acid[d]	Hexosamine[d]	Carbohydrate (hexose)[d]
45/0	25.0	5.3	20.2	111.7	44
45/20	46.0	29.2	16.0	80.0	10

[a] The complex isolated from both strains contained no 2-keto-3-deoxyoctonate.
[b] Expressed as milligrams per 100 mg of cell walls.
[c] Lipoprotein-protein released by trypsin digestion, expressed as milligrams per 100 mg of complex.
[d] Expressed as micrograms per milligram of complex.

TABLE 4. *Distribution of the free lipids extracted from B. abortus 45/0 and 45/20*

Compound	Percentage of total					
	3 h[a]		6 h		9 h	
	45/0	45/20	45/0	45/20	45/0	45/20
Fatty acids (front)	1.5	1.2	1.3	1.3	2.1	2.3
Unknown	5.7	5.5	5.8	8.0	0.9	0.4
Diphosphatidyl-glycerol	23.9	27.5	30.6	19.1	8.2	8.4
Phosphatidyl-ethanolamine	26.4	18.1	22.8	25.7	39.1	32.9
"Ornithine" lipid	17.6	19.1	16.9	18.3	25.7	32.5
Phosphatidyl-glycerol	22.4	26.1	21.4	26.4	9.8	15.9
Lysophosphatidyl-ethanolamine	0.5	0.5	0.2	0.2	11.7	2.8
Unknown	0.2	0.2	0.1	0.2	0.5	1.6
Origin	1.0	1.0	0.6	0.6	1.5	2.8

[a] Time of incubation with [^{14}C]acetate.

27, 38); however, to the best of our knowledge, this report is the first account describing its absence in avirulent strains. Limited chemical characterizations of phenolic preparations from other laboratories make it difficult to establish either precursor-product or structure-function relationships. It may be that the phenolic LPS is an important factor in the intracellular survival of *Brucella*. Only phenolic LPS of *B. abortus* 45/0 exhibited mouse lethality; yet both aqueous and phenolic LPS induced carbohydrate depletion (21). Thus, the LPS of *Brucella* appears to be quite unique compared with enteric LPS and may be useful in studying structure-function relationships involved in endotoxin poisoning (42).

On the other hand, cell walls of strain 45/0 were remarkably more resistant to digestion and disaggregation by hydrolytic enzymes than cell walls from 45/20. The chemical basis of this enhanced resistance is not known. It is difficult to disrupt and prepare cell-free extracts of *Brucella* (39), and it may be that a high degree of cross-linking in the murein layer contributes to the refractory nature of *Brucella*, as is observed with *Staphylococcus aureus* (13).

Changes in colonial morphology which reflects the smooth to rough transition, common to both gram-negative and gram-positive bacteria, are subtle in the genus *Brucella* (1, 28). Nonsmooth variants can be observed only through oblique light, uptake of crystal violet, either acriflavine or saline agglutination, and phage susceptibility (2, 28). Further, the genus is unusually homogeneous in biochemical reaction and antigenic structure (28). Thus, it appears that minor surface changes, and the difficulties in detecting them (6, 15, 19, 20, 34, 36), are due to a minimum of chemical differences.

Other differences observed in comparison of virulent and attenuated strains of *Brucella* have included the ability of highly virulent strains of *B. abortus* to catabolize erythritol (44) and the high level of glutamate oxidation by avirulent strains as compared with highly virulent strains (52). It is likely that a successful intracellular parasite must adapt to the intracellular milieu as well as resist the killing systems of phagocytic cells.

Even though strain 45/0 described in this report is more resistant than 45/20 to the bactericidal activity of both human and guinea pig polymorphonuclear leukocytes (D. L. Kreutzer and D. C. Robertson, submitted for publication), it is not possible to say whether the phenol-soluble LPS contributes to intracellular survival. However, the potentially interesting chemical and biological activities of the aqueous-phase and phenolic-phase LPS seemed to warrant more intensive characterization, as described in the following paper (21).

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