JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1987, p. 2132–2135
0095-1137/87/112132-04$02.00/0

Vol. 25, No. 11

Isolation and Antigenic Reactivity of *Brucella ovis* Outer Membrane Proteins

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Received 5 June 1987/Accepted 3 August 1987

Brucella ovis **cell membranes were isolated from fractured and lysozyme-treated cells by ultracentrifugation. These preparations appeared to consist largely of outer membranes, as judged from the results of ultracentrifugation experiments in sucrose density gradients under conditions that are widely used to separate inner and outer membranes of gram-negative bacteria. The sequential detergent extraction of cell membranes yielded mainly lipopolysaccharide and three groups of outer membrane proteins. In immunoblotting, lipopolysaccharide had good antigenic reactivity with all sera from rams exposed to *B. ovis* (vaccination or natural infection), but some outer membrane proteins reacted strongly only with sera from immune (vaccinated) rams, not from infected rams, suggesting a possible diagnostic role for such proteins in predicting immunity or infection.**

The major antigens of *Brucella* spp. are the lipopolysaccharide (LPS) and outer membrane proteins (OMP) (8, 16, 25). Crude antigen preparations used in the diagnosis of Brucella infection are known to contain LPS (4), and we have successfully used purified *Brucella ovis* LPS in the serodiagnosis of ram epididymitis (3). A hot saline extract of *B. ovis* cells containing LPS and OMP, when used as the antigen, detected more infected rams in an enzyme-linked immunosorbent assay than LPS alone (18). A sodium deoxycholate extract of *B. ovis* membrane vesicles has also been successfully used for the serodiagnosis of *B. ovis* infection in rams (5, 6). Recently, some studies have been reported on the OMP of *Brucella* (17–19, 22, 23) and its usefulness in serodiagnosis of infections.

Antibodies against OMP are present in infections with most gram-negative bacteria (10, 13, 15), and OMP have also been reported to confer protective immunity (1, 8, 11). The purpose of this study was to isolate and characterize membrane proteins from *B. ovis* by sequential detergent extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and to study their antigenic reactivity by immunoblotting.

MATERIALS AND METHODS

Preparation of cell membranes. *B. ovis* was isolated from the semen of a ram with clinical epididymitis and grown on tryptose agar supplemented with 7.5% bovine calf serum for 3 to 5 days at 37°C in 10% carbon dioxide in air. Cells were harvested in phosphate-buffered saline (PBS, pH 7.2) with sterile glass beads, strained through several layers of sterile cheesecloth, and centrifuged at 5,000 × *g* for 20 min at 4°C. The cell pellet (4.2 g, wet weight) was suspended in 60 ml of PBS containing 30% sucrose and 10 mM Mg^{2+} and stored frozen at −20°C until use.

For cell membrane preparation, cells were thawed at room temperature, and 1 mg of DNase and 1 mg of RNase (Sigma Chemical Co., St. Louis, Mo.) were added per 4.2 g of cells (wet weight). Cells were broken by two passages through a French pressure cell at 20,000 to 24,000 lb/in^2. The broken-cell preparation was incubated at 37°C for 40 min and then centrifuged at 5,000 × *g* for 20 min at 4°C. The pellet was discarded, and the supernatant fluid centrifuged at 82,500 × *g* for 20 h at 4°C. The membrane pellet (0.63 g, wet weight) was suspended in 50 ml of PBS, and 5 mg of lysozyme (Sigma) was added. The suspension was incubated for 24 h at 37°C and then pelleted and washed by centrifuging at 154,400 × *g*, each time for 4 h at 4°C.

Separation of outer from inner membranes. We attempted to separate outer from inner membranes by sucrose density gradient centrifugation of the above membrane preparation. Part of the membrane pellet (about 0.1 g) was suspended in 12.5 ml of PBS, and 6 ml of this suspension was layered onto a sucrose density gradient consisting of 3 ml of 55% sucrose and 6 ml each of 50, 45, 35, and 30% sucrose in PBS. After centrifugation at 82,500 × *g* for 18 h, the centrifuge tube was pierced at the bottom and fractions were collected in tubes. The absorbance of each fraction was measured at 280 nm, and its density was calculated from the refractive index.

Detergent extraction of cell membranes. Membrane proteins were extracted from the lysozyme-treated cell membrane preparation by the method of Squire et al. (20). A sequential extraction scheme with different detergents was used (Fig. 1). In all extractions, the volume of detergent solution was at least eight times the volume of membranes. All centrifugations were done at 154,400 × *g* for 4 h at 4°C.

The detergents were removed by ultrafiltration through a YM-10 membrane (Amicon Corp., Danvers, Mass.). Each fraction was diluted with 4 volumes of PBS and filtered until less than 7 ml was left in the ultrafiltration cell (model 52, Amicon Corp.). The cell was filled with PBS and filtered again. The process was repeated at least three times.

Analysis of membrane proteins. (i) Chemical analysis. The protein content of the fractions was assayed by a modified Lowry procedure (14). 2-Keto-3-deoxyoctonate, a constituent of LPS, was analyzed by the thiobarbiturate reaction (24). Succinate dehydrogenase activity as a marker for inner membranes was assayed as described by Ellar et al. (9) and expressed as dichloroindophenol reduced.

(ii) SDS-PAGE. SDS-PAGE was carried out in 10% minigels by the method of Laemmli (12). Protein (10 μg) was applied to each well, and after electrophoresis the gels were stained with Coomassie blue. The molecular weights of the proteins were estimated from their positions in the gel

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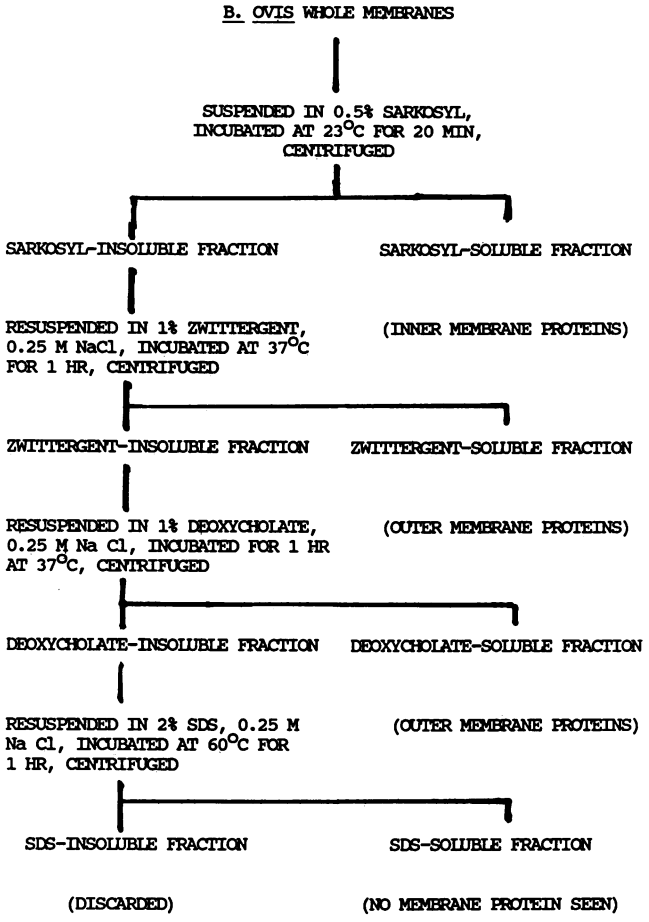

FIG. 1. Sequential extraction of *B. ovis* membrane proteins.

relative to those of known protein standards run on the same gel.

(iii) Immunoblotting. Following SDS-PAGE, the protein bands were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.) by electrophoresis at 40 V for 3 h as described by Towbin et al. (21). The membrane was cut into vertical strips corresponding to the SDS-PAGE bands, incubated in 1% bovine serum albumin for 1 h at 37°C to block the nonspecific binding of serum proteins, washed five times in PBS, and incubated overnight at 4°C in ram sera diluted 1:1,000 in PBS-Tween 20. After this the strips were washed five times and incubated in rabbit peroxidase-conjugated immunoglobulin G antiserum fraction made against sheep immunoglobulin G (diluted 1:1,000 in PBS-Tween 20) for 2 h at 37°C, washed again five times, placed in a substrate solution of α-chloronaphthol (Bio-Rad) containing 0.01% hydrogen peroxide, and allowed to develop until the bands were visible. The strips were then washed with distilled water and stored in the dark.

Five sera from four different groups of rams were used in immunoblotting: (i) vaccinated immune rams which did not become infected on experimental challenge; (ii) vaccinated animals which became infected on experimental challenge; (iii) naturally infected rams; and (iv) noninfected rams. These sera were collected in a related study of vaccination against *B. ovis* (2). Sera from groups 1 and 2 were collected 1 day before challenge (79 days after vaccination). Sera from groups 3 and 4 were collected 1 week after tests for infectivity. Groups 1, 2, and 3 had complement fixation titers of ≥160; group 4 had an undetectable titer.

RESULTS

Characterization of membrane proteins. SDS-PAGE of the sequentially extracted membrane proteins is shown in Fig. 2. The first extraction with Sarkosyl, which supposedly extracts inner membrane proteins, yielded only a few weak bands. The Zwittergent extract had many bands, supposedly corresponding to LPS and OMP. The deoxycholate extract contained the same bands as the Zwittergent extract, but the band intensities were different, suggesting a different extraction selectivity for deoxycholate.

Three different groups of membrane proteins were distinguished by SDS-PAGE. Group 1 had only a single protein with an approximate molecular weight of 91,000 (91K protein). This protein was heat modifiable, since heating the Zwittergent extract for 3 min at 100°C resulted in the separation of this protein into several bands with increased mobilities, as seen on subsequent SDS-PAGE (data not shown). Group 2 proteins were less concentrated than group 3, as seen by the intensity of the bands. At least two distinct bands were visible, corresponding to approximate 54K and 36K proteins. Group 3 proteins consisted of at least three distinct proteins of approximately 25K, 21K, and 19K. Group 3 proteins were not heat modifiable. There were some variations in the distribution of these proteins among different isolates of *B. ovis* (data not shown).

Cell membranes prepared in this study seemed to contain only a small amount of inner membrane. On a sucrose density gradient, which separates outer from inner membranes, only one major peak was obtained with a buoyant density of 1.22 g/ml; equivalent to OMP (Fig. 3). This peak had a 2-keto-3-deoxyoctonate content of 8.62 μg/mg of protein and succinate dehydrogenase activity of 194.2 μmol/mg of protein per min, versus a 2-keto-3-deoxyoctonate content of 7.06 μg/mg of protein and succinate dehydrogenase activity of 328.8 μmol/mg of protein per min for the whole membrane, indicating some separation. A second smaller peak had a density corresponding to that of the inner membrane, but the amount of nondialyzable mate-

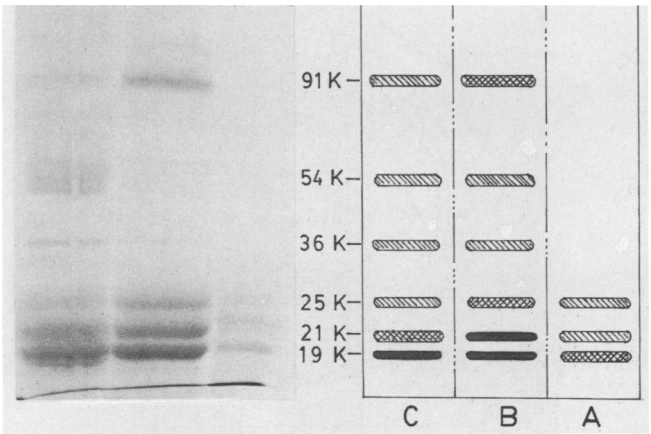

FIG. 2. SDS-PAGE of sequentially extracted *B. ovis* membrane proteins. Lanes: A, Sarkosyl-soluble membrane proteins; B, Zwittergent-soluble membrane proteins; C, deoxycholate-soluble membrane proteins. Molecular weights of OMP were calculated by comparison with six reference protein standards. Staining is indicated as strong (solid bands), medium (cross-hatched bars), or weak (hatched bars).

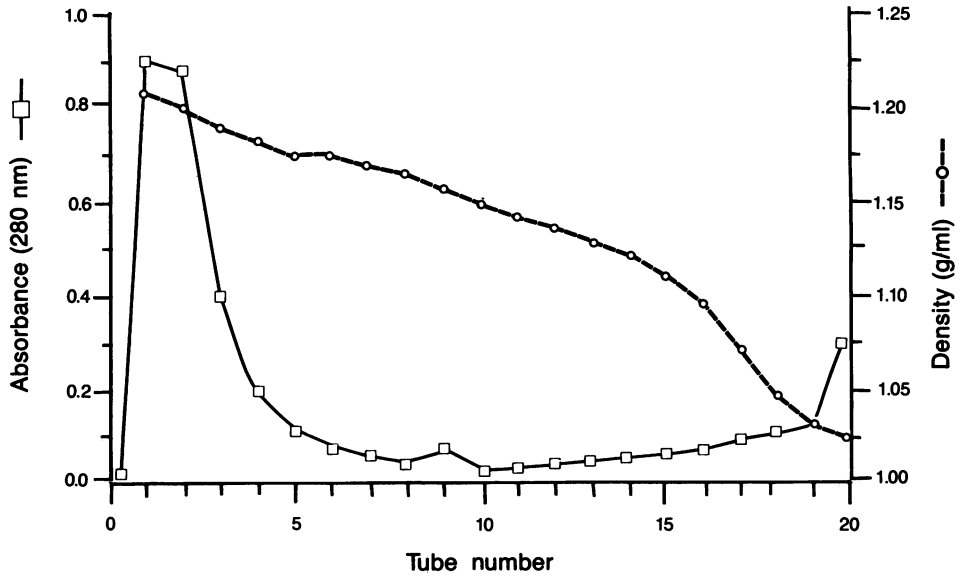

FIG. 3. Sucrose density gradient centrifugation of *B. ovis* whole membranes.

rial was small and insufficient for further analysis. The presence of a relatively small portion of inner membrane in *B. ovis* corresponds to the very small amount of supposed inner membrane proteins extractable with Sarkosyl.

Antigenic reactivity of membrane components. Immunoblotting was carried out on Zwittergent extract only, since this extract contained all detectable membrane fractions. Five sera from each of the four animal groups were tested. Since there were no qualitative differences among the sera within a group, only one representative serum from each of three groups is shown in Fig. 4. Sera from vaccinated rams that later became infected after an experimental challenge reacted only very weakly with the Zwittergent extract components, and therefore this group is not shown. Sera from *B. ovis*-vaccinated immune rats reacted strongly with LPS and all membrane proteins present in the Zwittergent extract. Sera from naturally infected rams reacted with LPS and the group 1 protein but not with group 2 proteins. Sera from noninfected rams did not react.

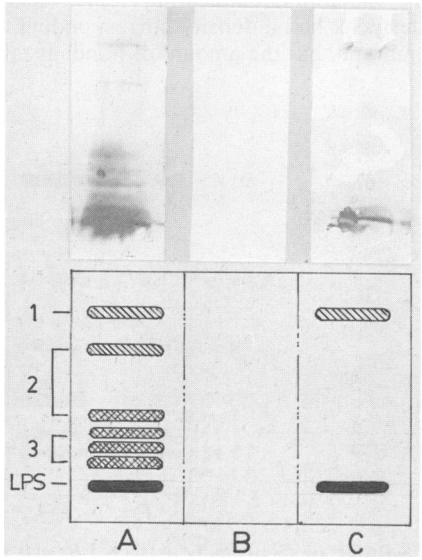

FIG. 4. Immunoblotting of Zwittergent-extracted OMP of *B. ovis* with immune, infected, and normal ram sera. Lanes: A, serum from an immune ram; B, uninfected normal ram serum; C, serum from a naturally infected ram. Numbers 1, 2, and 3 represent the positions of group 1, group 2, and group 3 proteins, respectively. LPS is also indicated. See Fig. 2 legend for symbols.

DISCUSSION

An attempt was made to separate inner and outer membranes of *B. ovis* and to isolate and characterize their membrane protein components. Sequential extraction with Sarkosyl, Zwittergent, and deoxycholate as described by Squire et al. (20) apparently extracted all membrane proteins, since further extraction with SDS did not yield any additional membrane proteins. The detergent extraction procedure did not separate LPS from proteins. Efforts to isolate inner from outer membranes by a sucrose density gradient, as used in studies with other gram-negative bacteria, resulted in one major peak corresponding to outer membranes. Analytical data on this fraction compared with that of total membranes suggest that some inner membrane material was removed by this procedure and that the small second peak may be real, but the amount of this material was inadequate for further analysis. It seems likely that the procedures used isolated only the bulkier and denser outer membrane. Another possibility is that the proportion of inner membranes in *B. ovis* is very small.

Three groups of membrane proteins were seen in *B. ovis* which corresponded to the OMP of other *Brucella* species (19, 22, 23). The role of group 1 proteins is not known. Group 2 proteins form the permeability channels of *Brucella* species (7) and induce both humoral and cell-mediated immune response in cattle (25). Proteins of molecular weights similar to those of groups 2 and 3 from *B. abortus* are important in protection against experimental *Brucella* infection in mice (8). An additional protein in group 2 corresponding to 54K was isolated in this study; this protein has not been reported earlier for *B. ovis*, but a 48K protein has been reported for *B. melitensis* (19). This is perhaps due to different isolation procedures, strain variation, or different culture conditions.

Immunoblotting indicates that rams exposed to *B. ovis* develop antibodies against LPS, irrespective of the fate of the infection (immunity or disease). This supports our choice of LPS as the diagnostic antigen for detecting *B. ovis* infection by an enzyme-linked immunosorbent assay (3). Chin et al. (8) isolated OMP from *B. ovis* by extracting membrane vesicles with sodium deoxycholate. This preparation, which would contain LPS and OMP, was successfully used in an enzyme-linked immunosorbent assay for the diagnosis of *B. ovis* infection in rams. Hot saline extract of *B. ovis* cells containing OMP and LPS reacted with serums of *B. ovis*-infected rams, but also cross-reacted with *B. melitensis* infection (18). Some of the OMP, especially the proteins in group 2, may have additional diagnostic value in predicting the outcome of vaccination or natural exposure to *B. ovis*. A strong antibody reaction to group 2 proteins seems to signal the state of protection, while a weak reaction or lack of reaction may indicate the state of disease. Whether or not these antibodies have a real protective role against epididymitis remains to be seen.

ACKNOWLEDGMENT

The work was supported by a grant from Hoffmann-La Roche Inc.

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