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Comparative Analyses of Proteins Extracted by Hot Saline or Released Spontaneously into Outer Membrane Blebs from Field Strains of *Brucella ovis* and *Brucella melitensis*

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Sheep infected with *Brucella ovis* produce antibody responses to the rough lipopolysaccharide and to proteins present in hot saline (HS) extracts of *B. ovis* (J. I. Riezu-Boj, I. Moriyón, J. M. Blasco, C. M. Marín, and R. Díaz, J. Clin. Microbiol. 23:938–942, 1986). The distribution and antigenic relatedness of proteins in HS extracts and in outer membrane blebs were established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting for 41 strains of *B. ovis* and 26 strains of *Brucella melitensis* of diverse geographic origin. Five major groups of proteins were identified in HS extracts of *B. ovis* that had been freed of rough lipopolysaccharide: proteins of 43 kilodaltons (kDa), group A (25.5 to 32.0 kDa), group B (21.5 to 22.5 kDa); group C (18.0 to 19.5 kDa), and group D (13.0 to 15.5 kDa). Group A, B, C, and D proteins were also present in blebs. The profiles of proteins in HS extracts or blebs from strains of both *Brucella* species were very similar. Cross-reactions were demonstrated among HS extracts and blebs of all strains tested in immunoblots performed with an antiserum against the HS extract of a reference strain of *B. ovis*. Evidence was also provided of an antigenic relationship between group 3 proteins of the outer membrane and some of the proteins in groups A, B, and C. The conservation of these antigens and their immunogenicity in infected animals provide promise that they may serve as components of an effective subcellular vaccine for ovine brucellosis.

Ovine brucellosis is caused by *Brucella ovis* and *B. melitensis* and rarely by *B. abortus* (13, 20, 24). Reduced fertility of rams is the most serious consequence of this disease (29), although abortion also occurs (19, 27, 47). The principal vaccine used against ovine brucellosis has been REV 1, a live attenuated strain of *B. melitensis* (5, 6, 8, 15, 28). Although REV 1 is effective when administered to lambs (5), it is less effective in vaccination of adult animals (53). Moreover, this vaccine is virulent for humans (15), and its use is prohibited in the United States due to the possibility of transmitting *B. melitensis*. Bacterins of *B. ovis* in several adjuvants have been tested in rams and have provided protection against infection and disease to 50% or more of the vaccinated animals (1, 6, 8, 26, 28, 49, 50). However, bacterins were subject to several disadvantages including the need for multiple vaccinations, the development of lesions at the site of injection, and the induction of immune responses that interfered with the interpretation of diagnostic tests (30, 50, 58).

In the course of our studies we noted that rams and ewes infected with *B. ovis* produced a strong antibody response to proteins in hot saline (HS) extracts of *B. ovis*, and these reactions have already formed the basis of an improved diagnostic test (45). HS extracts contain a variety of proteins, and preliminary evidence had suggested that these included group 3 outer membrane (OM) proteins (OMPs) (45). We wished to test the possibility that these proteins, which were highly immunogenic in the course of natural infection, might also serve as effective components of an improved nonliving vaccine. The OMPs of *B. ovis*, a naturally occurring rough form lacking O side chains, would be exposed on the cell surface and could therefore potentially induce not only cell-mediated immune responses but also antibodies capable of mediating protective immunity. For purposes of vaccine development, it was necessary first to determine whether the proteins were conserved within the species and were antigenically related among field strains of diverse origin. It was also considered important to determine whether related proteins occurred in *B. melitensis*, the other major cause of ovine brucellosis. The present study was performed to accomplish these objectives. Similar comparisons were made with proteins derived from OM blebs (free membranous material [17]). Finally, an effort was made to establish the relationships of the principal proteins in HS extracts and blebs to each other and to detergent-extracted OMPs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 41 strains of *B. ovis* and 26 strains of *B. melitensis* were tested. Forty strains of *B. ovis* were isolated from infected sheep, of which 18 were obtained from the United States, 15 were from France, and 7 were from Spain (Table 1). The avirulent CO_2-independent *B. ovis* strain REO 198 was also included in this study. *B. melitensis* strains were isolated from infected animals or humans. Twelve strains were obtained from Spain, and 12 were obtained from other European countries (Table 2). Two strains of *B. melitensis* REV 1 which differed in pathogenicity were also analyzed (Table 2). Each field strain of *B. ovis* and *B. melitensis* was isolated from a different flock. The identity of each field strain had been established by standard biochemical reactions (2).

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TABLE 1. Derivation of *B. ovis* strains[a]

Origin	Strain
ATCC[b]	ATCC 25840
NADC[c]	REO 198
United States	
Montana	6-1107, 6-1192
Utah	L81-624-46, L81-624-47, L81-624-48, L81-624-50, L81-624-53, L81-624-56, 414646, Utah no. 4, 3-1640
Idaho	Arrata Blue, Arrata Y126, 4-0084, B0338
Wyoming	A2333
Spain	
Huesca	21, 22, 23, 24, 25, 26
Zaragoza	C1
France	
Alpes maritimes	NB2[d]
Pyrenées Atlantiques	PA21[d], 2895, 2892, 2953, 2950, 2933, 2942, 2919, 2987, 2920, 2889, 2976, 82-90

[a] All strains were isolated from sheep except strain C1, which was isolated from the epididymis of a rabbit 1 month after intraperitoneal inoculation of 5×10^8 organisms of a field isolate. Strains ATCC 25840 and REO 198 are reference strains. All others are field isolates.
[b] ATCC, American Type Culture Collection, Rockville, Md.
[c] NADC, National Animal Disease Center, Ames, Iowa.
[d] CO_2-independent strains.

Upon receipt, each strain of *B. ovis* was transferred to Schaedler agar plates (BBL Microbiology Systems, Cockeysville, Md.) containing 10% bovine blood and cultivated for 3 days at 37°C in an atmosphere of 10% CO_2. Strains of *B. melitensis* were grown in a like manner for 2 days in air. *B. melitensis* cells were suspended in sterile phosphate-buffered saline (pH 7.2, 0.1 M), and *B. ovis* cells were suspended in a cryoprotective mixture composed of 5% bovine serum albumin, 7.5% sucrose, and 1% sodium glutamate in distilled water. These stock suspensions were frozen in portions at −70°C.

In preliminary trials field strains of *B. ovis* had been found to grow poorly in broth media, even with a supplement of 0.5% yeast extract, but they grew well on blood agar plates.

TABLE 2. Derivation and properties of *B. melitensis* strains[a]

Origin	Biotype	Host	Strain
	1		Rev 1
France			
Corse	3	Goat	81-151
H. Vienne	3	Sheep	81-131
Paris	3	Sheep	76-303
H. Alpes	3	Sheep	83-27
Alpes maritimes	3	Sheep	81-35
Var	3	Sheep	78-172
Savoie	3	Sheep	82-17
Savoie	3	Cow	80-373, 83-5
Aveyron	3	Cow	81-142
Portugal	1	Human	85-7
Greece	3	Sheep	83-183
Spain			
Navarra	1	Human	6394/86, 2731/85, 1408/86, 9819/85, 4921/86, 1928/85, 5249/86, 1517/86, 1987/85, 11190/85, 4815/85, 5007/85

[a] Two REV 1 isolates were tested, one typical (received from G. G. Schurig), and one atypical (isolated from an aborted lamb by J. M. Blasco). *All other* strains are field strains.

Although *B. melitensis* field strains grew optimally in broth, all strains of both *Brucella* species were grown on blood agar plates to avoid possible variations in the expression of proteins due to differences in the culture medium. To prepare cells for extractions, a thawed vial of stock suspension was streaked onto blood agar plates and incubated as described above. The confluent growth was suspended in phosphate-buffered saline and spread over several blood agar plates (15-cm diameter), which were incubated at 37°C for 2 days in air to grow *B. melitensis* or for 3 days in 10% CO_2 for *B. ovis*.

Extraction procedures. HS extracts were obtained as described previously (9, 45) by suspending live cells in physiological saline (10 g of packed cells per 100 ml) and heating in flowing steam for 15 min. After centrifugation at 12,000 × *g* for 15 min, the supernatant was dialyzed for 2 days at 4°C against several changes of distilled, deionized water. The dialyzed material was centrifuged for 5 h at 100,000 × *g*, and the pellet (HS) was suspended in distilled, deionized H_2O, lyophilized, and stored at room temperature. HS proteins were purified by extraction of 2 mg of crude material with 7 ml of a mixture of petroleum ether, chloroform, and phenol (PCP) (16) for 1 min at 16,000 rpm in a homogenizer Sorvall Omnimixer, Du Pont Co., Wilmington, Del.). The sediment (HS-PCP fraction) after centrifugation at 12,000 × *g* was washed once in distilled, deionized H_2O, suspended, and lyophilized.

OM blebs were obtained by a modification of the published method (17). Growth from agar plates was suspended in sterile phosphate-buffered saline and collected by centrifugation at 10,000 × *g* for 20 min. The supernatant was passed through a 0.45-μm filter (Millipore Corp., Bedford, Mass.), and blebs were sedimented from the filtrate by centrifugation at 100,000 × *g* for 2 h.

Rough lipopolysaccharide (R-LPS) from *B. ovis* REO 198 was obtained from complete cells as described by Galanos et al. (16). R-LPS was also obtained from HS by elimination of the proteins with the PCP method (45) followed by precipitation of the R-LPS with water from the phenol phase (16). Smooth LPS (S-LPS) was obtained from HS extracts of *B. melitensis* after PCP extraction by precipitation of the S-LPS in the phenol phase with methanol (M. S. Redfearn, Ph.D. thesis, University of Wisconsin, Madison, 1960).

OMPs from *B. ovis* strains 6-1107 and REO 198 were prepared by sequential detergent extraction of cell envelopes (35, 54). For purposes of immunization, the OMPs of strain REO 198 were extracted by the PCP method to remove LPS. About 6 mg of the purified proteins were solubilized with 0.25% sodium dodecyl sulfate (SDS) and separated by SDS-polyacrylamide gel electrophoresis (PAGE) with a one-well comb. The area of acrylamide containing the group 3 proteins was cut out, washed in saline, homogenized in saline, and emulsified with incomplete Freund adjuvant.

Antisera. Antisera were raised in male Flemish giant chinchilla cross-bred rabbits, except for sera against R-LPS, which were obtained from outbred rabbits. Sera against HS or blebs were obtained by four weekly intramuscular injections of 0.6 mg of HS or blebs from *B. ovis* REO 198 in incomplete Freund adjuvant. Sera against R-LPS were produced by the same course of immunization with four doses of 0.5 mg of R-LPS from *B. melitensis* 115, which was suspended in saline by sonication before emulsification in incomplete Freund adjuvant. Rabbits were exsanguinated 1 week after the last immunization.

Sera against group 3 OMPs were obtained by two biweekly intramuscular injections of 0.8 mg of group 3 proteins

in incomplete Freund adjuvant. The rabbits were exsanguinated 2 weeks after the second injection.

SDS-PAGE. SDS-PAGE was performed in 12.5% acrylamide slabs by the method of Laemmli (21) with a reservoir buffer at pH 8.6 containing 0.3% Tris, 1.8% glycine, and 0.1% SDS. The gels were stained either with Coomassie blue (14) or by the alkaline silver-glutaraldehyde method of Oakley et al. (38). When this stain was used, the background was cleared with a solution containing 0.38 M $Na_2S_2O_3 \cdot 5H_2O$, 0.04 M $CuSO_4 \cdot H_2O$, 0.15 M NaCl, and 0.23 M NH_4OH. The apparent molecular masses (AMM) of the proteins were determined by comparing their electrophoretic mobility with that of the following molecular mass markers: phosphorylase *b* (94 kilodaltons [kDa]), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14.3 kDa).

Enzyme immunoassays. Serum titrations were performed by a plate enzyme-linked immunosorbent assay. Each antigen used was coupled to polystyrene plates (Immulon Mikrotiterplatte; Dynatech Laboratories, McLean, Va.) in carbonate buffer (pH 9.6) overnight at 4°C. LPS was used at 500 ng, and HS and blebs were used at 1 μg per well. The assay was performed by the indirect method of Voller et al. (56), with a peroxidase-conjugated immunoglobulin G fraction of goat anti-rabbit immunoglobulin G, H and L chains (Cooper Biomedical, Inc., West Chester, Pa.), used at a dilution of 1:1,000. The highest dilution with an absorbance 0.2 units greater than the control was taken as the titer.

Immunoblotting was carried out as described by Towbin et al. (51) and Burnette (7), with the following modifications. After SDS-PAGE, the gel was equilibrated in transfer buffer (20 mM phosphate-buffered saline, 10% methanol [pH 6.5]) for 1 h. Proteins were then transferred in that buffer to nitrocellulose papers by using an electroblotter (Bio-Rad Laboratories, Richmond, Calif.), which had been modified by the addition of seven parallel platinum wires to provide a more uniform field (18), with a constant voltage of 13 V (initial amperage, 8 mA/cm), for 90 min at 4°C. Blots were placed in a blocking buffer (1% bovine serum albumin and 0.2% Tween 20 in 10 mM phosphate-buffered saline [pH 7.2]) overnight at 4°C and then incubated for 1 h at room temperature with serum diluted 1:100 in blocking buffer without bovine serum albumin. Development of the reaction with conjugate and substrate followed the same procedure used in the plate enzyme-linked immunosorbent assay.

Chemical analyses. Total protein was determined by the method of Lowry et al. (23), with bovine serum albumin as the standard. Analysis for 2-keto-3-deoxyoctonate corrected for 2-deoxyaldoses was performed by the method of Warren as modified by Osborn (39, 57).

Electron microscopy. Blebs obtained by ultracentrifugation were suspended in distilled, deionized H_2O, fixed with 1% OsO_4 for 30 min, and stained with 2% phosphotungstic acid. Preparations were examined in a transmission electron microscope (Philips 301).

RESULTS

SDS-PAGE analyses. Profiles of HS extracts of representative strains of *B. ovis* and *B. melitensis* are presented in Fig. 1. A maximum of 22 bands was detected in *B. ovis* extracts by Coomassie blue staining, including two major broad bands at 28.5 and 27.0 kDa. In HS extracts from *B. melitensis* there were up to 15 well-resolved bands, of which the most intensely stained were located between 32.0 and 26.5 kDa (Fig. 1). Banding patterns among strains within each species bore a high degree of similarity (Fig. 1). There was also a close resemblance between strains of the two species in the location of bands at 32.0, 28.5, 27.0, 26.5, 18.0, 17.5, 15.5, 15.0, 13.5, and 13.0 kDa. The two bands at 28.5 and 27.0 kDa were major bands in both species (Fig. 1). *B. melitensis* strains exhibited two broad smears between 35 and 90 kDa (Fig. 1), which were caused by the presence of S-LPS (31).

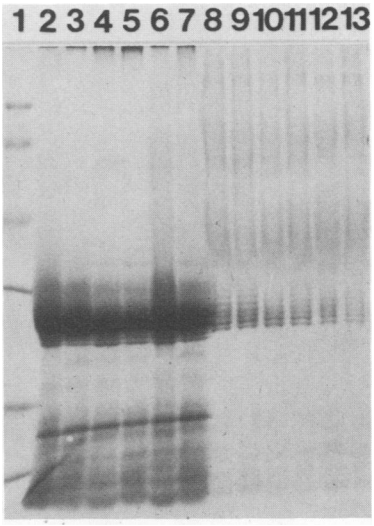

FIG. 1. SDS-PAGE profiles of HS crude extracts from six *B. ovis* (lanes 2 through 7) and six *B. melitensis* strains (lanes 8 through 13). Lanes: 1, standard molecular weight markers; 2, 2987; 3, 2892; 4, 24; 5, 25; 6, 6-1107; 7, Utah no. 4; 8, 1987; 9, 6394/86; 10, 85-7; 11, 83-27; 12, REV 1 (Schurig); 13, REV 1 (Blasco). The load per well was 75 μg (dry weight). Coomassie blue stain was used.

The protein in HS extracts of *B. ovis* can be separated from R-LPS by means of the PCP mixture used for extraction of R-LPS from whole cells (16) (J. I. Riezu-Boj, personal communication). All HS preparations, including those obtained from *B. melitensis*, were therefore extracted by that method. After this treatment the content of protein increased on the average from 12 to 22% in extracts of *B. melitensis* and from 60 to 93% in extracts of *B. ovis*. Although this method is more effective for extracting R-LPS, some S-LPS can also be removed (31). The profiles of HS proteins of representative strains after PCP extraction (HS-PCP fraction) are shown in Fig. 2. The major bands now presented a slightly decreased AMM (25.5 to 32.0 kDa in the crude fraction versus 25.0 to 29.0 kDa in the PCP fractions). The intensity of Coomassie blue staining of the minor bands was increased in the PCP fractions, perhaps due to the elimination of LPS from the major bands. In *B. melitensis* strains the LPS smears between 35 and 90 kDa (Fig. 1) were almost eliminated by PCP extraction (Fig. 2A). Silver-stained preparations of HS-PCP fractions of *B. melitensis* (Fig. 2B, lane 1) confirmed the presence of the regularly spaced doublet profile attributed to the S-LPS of *B. melitensis* (11). Five major groups of proteins could be identified in HS-PCP fractions, which will be designated as follows: proteins of 43 kDa; group A (25.0 to 29.0 kDa); group B (21.5 to 22.5 kDa); group C (18.0 to 19.5 kDa); and group D (13.0 to 15.5 kDa) (Fig. 2A). R-LPS migrated at 13.0 to 15.0 kDa (see Fig. 7D and E) (32, 45), so that any residual R-LPS in the HS-PCP fraction would have comigrated with group D proteins. When the gels were overloaded, other minor bands were demonstrated at 91 and 69 kDa (data not shown).

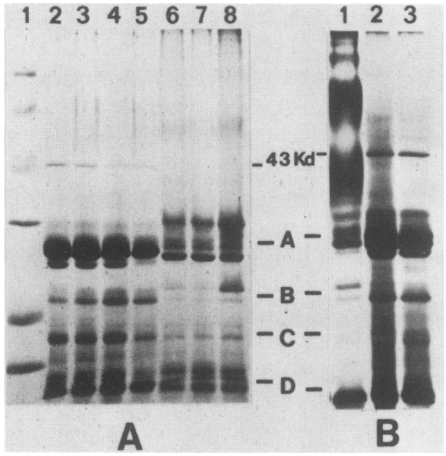

FIG. 2. (A) SDS-PAGE profiles of HS-PCP fractions from four *B. ovis* strains (lanes 2 through 5, 25 μg [dry weight] per well) and three *B. melitensis* strains (lanes 6 to 9, 70 μg [dry weight] per well). Lanes: 1, standard molecular weight markers; 2, 2942; 3, 2895; 4, L81-624-50; 5, L81-624-48; 6, 1408; 7, 5007/85; and 8, 81-35. Stains were performed with Coomassie blue. (B) SDS-PAGE of HS-PCP fractions from *B. melitensis* 4815/85 (lane 1), and *B. ovis* 22 (lane 2) and 2976 (lane 3). Loads were 70 and 25 μg (dry weight) for *B. melitensis* and *B. ovis*, respectively. Silver stain was employed. The gel was destained extensively until the appearance of the LPS doublets in *B. melitensis* between 35 and 42 kDa.

No qualitative differences were demonstrated in the banding patterns of HS-PCP fractions among any of the 41 *B. ovis* strains or 26 *B. melitensis* strains tested. Quantitative differences in the staining of some bands were, however, noted in some strains within each species (Fig. 2A). There was no association in strains of *B. melitensis* between biotype and staining patterns of the PCP-extracted proteins. A comparison of SDS-PAGE profiles between strains of the two species revealed an overall similarity, although quantitative differences were apparent, most notably within the group A proteins. The band at 43 kDa that was uniformly present in *B. ovis* strains was absent in strains of *B. melitensis* (Fig. 2A).

Comparative analyses were also performed on the blebs isolated from the panel of *B. ovis* and *B. melitensis* strains. It had been demonstrated that rough and smooth strains of *B. melitensis* spontaneously release OM fragments when grown in liquid medium (17). We have now demonstrated that *B. ovis* also produces blebs and that this phenomenon occurs with strains of *B. ovis* and *B. melitensis* grown on solid media and in liquid media. Membrane blebs ranging in size from 50 to 284 nm in diameter were found in the buffer used to harvest the plate-grown cells (Fig. 3A). These blebs, of which the majority exceeded 130 nm, were larger than those from *B. melitensis* grown in liquid medium (17). To study this variation, blebs were compared from *B. ovis* REO 198 grown in solid and liquid media. In liquid medium fragments ranged from about 35 to 200 nm in diameter, but the majority were between 117 and 134 nm (Fig. 3B). On solid medium

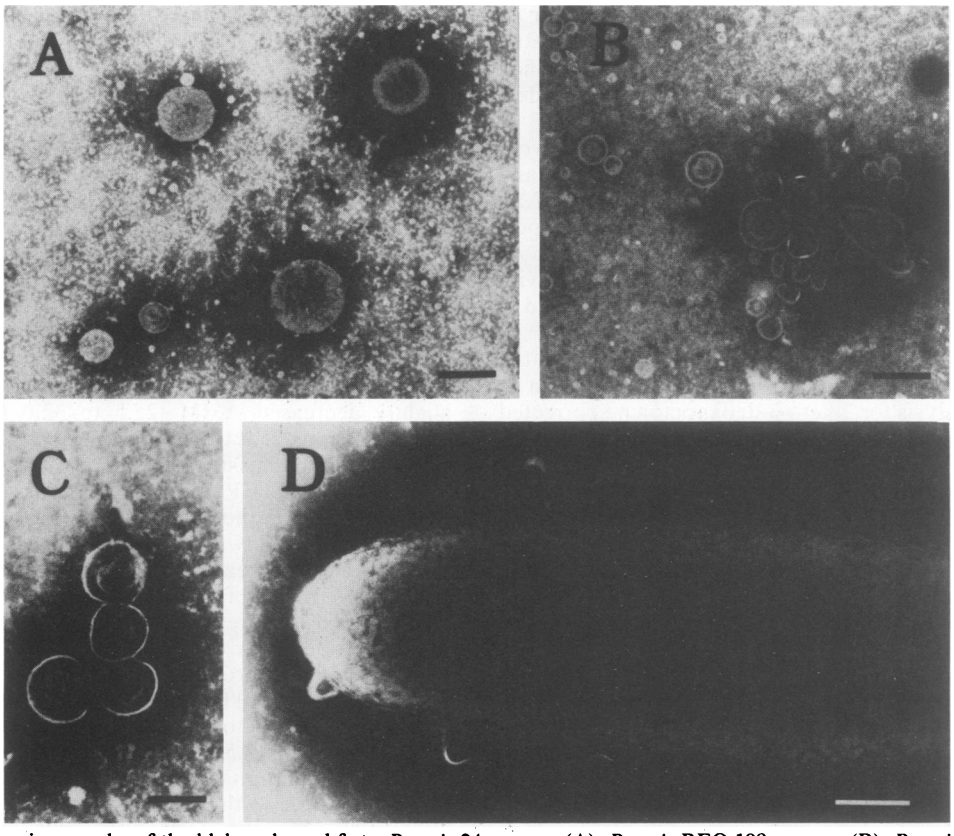

FIG. 3. Electron micrographs of the blebs released from *B. ovis* 24 on agar (A), *B. ovis* REO 198 on agar (B), *B. ovis* REO 198 in liquid medium (C), and *B. melitensis* REV 1 on agar (D). Bars, 50 nm. The normal condition of the cell in panel D is evident by retention of the surface convolutions.

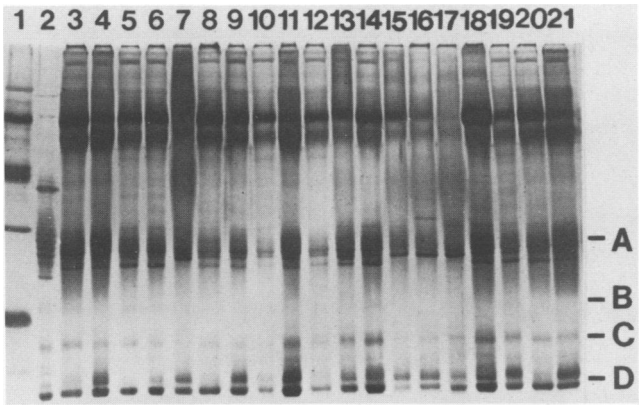

FIG. 4. SDS-PAGE of blebs (lanes 3 through 21) from representative strains of *B. ovis* (lanes 2, 3, 5, 6, 8 through 10, 12, 14, 18, 19 and 20) and *B. melitensis* (lanes 4, 7, 11, 13, 15 through 17, and 21). Lanes: 1, standard MW markers; 2, OMP from *B. ovis* 6-1107; 3, 2950; 4, 9819/85; 5, 2895; 6, 24; 7, 76-303; 8, 414646; 9, 22; 10, 6-1192; 11, 4921/86; 12, Arrata Blue; 13, 2731/85; 14, 2933; 15, 83-183; 16, 80-373; 17, 78-172; 18, Utah no. 4; 19, 26; 20, NB2; 21, 11190/84. Loads per well were 20 and 40 μg (dry weight) for *B. ovis* and *B. melitensis* strains, respectively. Silver stain was employed.

the fragments released from the same strain were larger, between 200 and 267 nm (Fig. 3C). When the buffer employed to harvest the cells was not filtered, whole cells were found in the preparations, and some of these were fixed in the process of forming blebs (Fig. 3D). The size of these blebs was approximately 61 nm, which suggests that the larger blebs formed through reassociation of the smaller ones into multimicelles, as had been proposed previously (17).

Bleb proteins from strains of *B. ovis* and *B. melitensis* separated by SDS-PAGE and stained with silver demonstrated major bands with AMMs of 67.0, 60.0, 25.0 to 30.0 (group A), and 13.0 kDa as well as several minor bands that corresponded to groups B (AMM of 21.5 kDa), C (19.5 and 18.2 kDa), and D (15.0 and 13.5 kDa) (Fig. 4). The high-molecular-weight smear due to S-LPS was apparent in blebs of *B. melitensis* stained with Coomassie blue but was stained minimally with silver stain in the majority of *B. melitensis* strains (Fig. 4). Some differences were noted among the profiles of blebs from the 41 strains of *B. ovis* and the 24 strains of *B. melitensis*. Group B proteins were not detected in blebs from almost half of the strains (Fig. 4). In other samples only one band was observed in group C (Fig. 4, lane 4 versus lane 16). Differences were also observed in the distribution of bands within group A of *B. melitensis* (Fig. 4, lanes 13, 15, 16, and 17). Nevertheless, there was again an overall resemblance of SDS-PAGE profiles both within and between the two species, and none of the differences observed could be used to distinguish the species.

Antigenic analyses. Reciprocal cross-reactions between HS extracts and blebs of *B. ovis* REO 198 and their respective antisera failed to reveal any reactions with anti-bleb sera not detected by anti-HS sera, whereas several bands were developed only by the HS antisera. For this reason all comparisons among strains employed an HS antiserum. Protein groups A, B, C, and D of HS-PCP fractions of all strains of *B. ovis* and *B. melitensis* produced strong reactions, and the 43-kDa band was stained in all strains of *B. ovis*. Results of representative strains are depicted in Fig. 5. A comparison of reactions of protein bands at higher molecular weights between *B. ovis* and *B. melitensis* was obscured by the S-LPS of *B. melitensis* (Fig. 5).

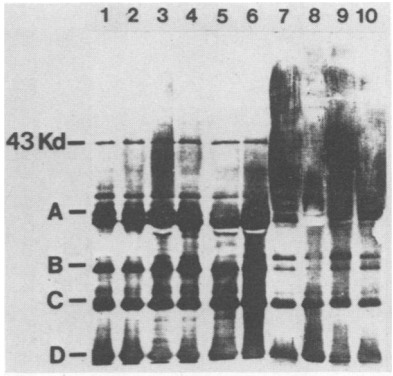

FIG. 5. Western blot analysis with HS antiserum of R-LPS (lane 1, 10 μg per well), and HS-PCP fractions from *B. ovis* (lanes 2 to 6, 10 μg [dry weight] per well) and *B. melitensis* strains (lanes 7 to 10, 30 μg [dry weight] per well). Lanes: 1, 3-1640; 2, L81-624-56; 3, 2889; 4, 2953; 5, 2976; 6, 22; 7, 4815/85; 8, 80-373; 9, 1517/86; 10, REV 1 (Blasco).

A similar analysis was performed on samples of blebs from 37 strains of *B. ovis* and 23 strains of *B. melitensis*. Group A, B, C, and D proteins from all strains of *B. ovis* and group B, C, and D proteins of all *B. melitensis* strains reacted positively, although not always with the same intensity (Fig. 6). Although some of the group A proteins of *B. melitensis* blebs were positively stained, there was a predominance of negative staining in these areas of the blots (Fig. 6, lanes 1, 6, and 9).

Relationship of OMPs to proteins in HS extracts and blebs. Silver stains of detergent extracted OMPs of *B. ovis* REO 198 showed group 3 proteins distributed in five major bands and other faint bands, ranging in AMM from 27.0 to 35.0 kDa (Fig. 7A, lane 2). Porin proteins (10) were found in concentrations lower than those in group 3 (Fig. 7A, lane 2), as reported previously (46). Silver staining demonstrated five

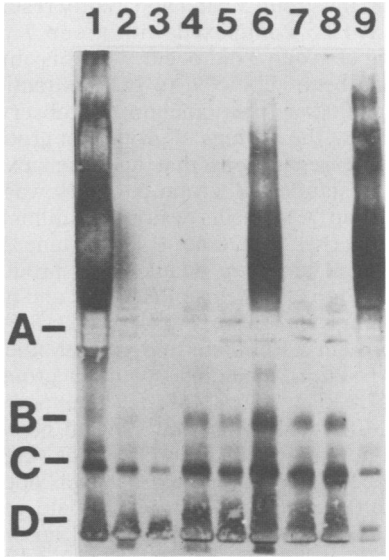

FIG. 6. Western blot analysis with HS antiserum of blebs from strains of *B. ovis* (20 μg per well; lanes 2 through 5, 7, and 8) and *B. melitensis* (40 μg per well; lanes 1, 6, and 9). Lanes: 1, 6394/86; 2, L81-624-48; 3, L81-624-53; 4, 2892; 5, Arrata Y126; 6, 5007/85; 7, 2950; 8, BO 338; and 9, REV 1 (Schurig).

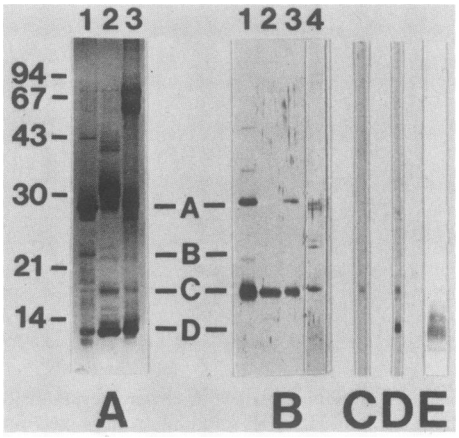

FIG. 7. SDS-PAGE with silver staining (A), and Western blots (B through E) of HS-PCP fraction (lanes 1 of A and B; C and D), detergent-extracted OMPs (lanes 2 of A and B), blebs (lanes 3 of A and B), OMPs after PCP treatment (lane 4), and R-LPS (E). All of the samples were from *B. ovis* REO 198. The blots were developed with the following: B, anti-group 3 serum; C, preimmunization serum from a rabbit vaccinated with group 3 protein; D, anti-R-LPS; E, anti-HS. Loads per well were approximately 10 μg of protein or 10 μg of LPS. In B, lane 2, reactions with bands A2 and A3 were faint and were not reproduced in the photograph.

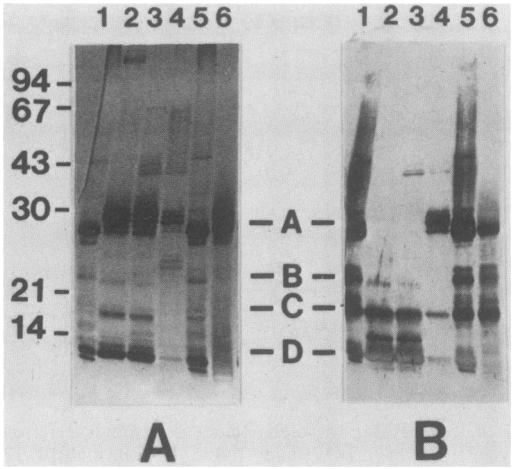

FIG. 8. SDS-PAGE with silver staining (A) and Western blots (B) of the following samples: HS-PCP fraction of *B. ovis* 2892 preincubated at 20°C (A and B, lanes 1) or 100°C (A and B, lanes 5), OMPs from *B. ovis* 6-1107 preincubated at 20°C (A and B, lanes 2), at 100°C (A and B, lanes 3), and at 20°C after PCP extraction (A and B, lanes 4); HS from *B. ovis* REO 198 incubated at 100°C (A and B, lanes 6). The immunoblot was developed with HS antiserum. Loads per well were approximately 10 μg of protein.

bands, ranging from 39.0 to 44.5 kDa, of which the upper two were faintest (Fig. 7A, lane 2).

Group A proteins from the HS-PCP fraction and blebs of strain REO 198 migrated in the same region as did group 3 proteins, and some of the lower-molecular-weight bands detected in the OMP preparation by silver staining comigrated with group B, C, and D proteins (Fig. 7A). Western blot analysis of the OMP preparation with the group 3 antiserum produced no reaction with group 3 proteins but did stain a band that comigrated with band C of the HS-PCP fraction or blebs (Fig. 7B, lane 2). It is noteworthy that a much fainter stain of this band was detected with a preimmunization sample of serum from this rabbit (Fig. 7C). The failure to stain the group 3 band apparently resulted from the presence of R-LPS complexed with group 3 proteins, because staining of group 3 did occur with this antiserum after the OMPs had been subjected to PCP extraction (Fig. 7B, lane 4). An analogous phenomenon was observed with HS antiserum, in that the staining of group 3 or group A proteins was intense in preparations that had been extracted with PCP, whereas staining of group 3 OMPs was absent and staining of group A proteins was much diminished before PCP extraction (Fig. 8B, lane 4 versus lane 3 and lane 5 versus lane 6). The group 3 antiserum produced intense staining with group C proteins of HS-PCP and blebs and one group A protein (A1, AMM 28.5 kDa) of HS-PCP and faint stains with protein A1 of blebs and several other proteins of the HS-PCP fraction, including two other group A proteins (A2, AMM 27.0 kDa; A3, AMM 25.5 kDa) and one group B protein (Fig. 7B). The antiserum showed no reaction with R-LPS (data not shown) or group D bands (Fig. 7B).

The presence of porin in HS extracts was not established. In contrast to the SDS-PAGE profile of detergent-extracted OMPs (Fig. 7A, lane 2; Fig. 8A, lane 3), no bands with an AMM similar to that of porin occurred in crude HS extracts of *B. ovis* incubated at 100°C before electrophoresis (Fig. 8A, lane 6). The 43-kDa band in HS extracts of *B. ovis* was present only after PCP extraction and did migrate at an AMM equivalent to that of one of the faint porin monomer bands (Fig. 8A, lanes 3 and 5). In contrast to the stability of the porin trimer in detergent-extracted OMPs incubated at 20°C before SDS-PAGE (Fig. 8A, lane 2), no band in the position of the porin trimer was present in the HS-PCP fraction preincubated at 20°C (Fig. 8A, lane 1). However, it was apparent that the PCP extraction itself disrupted the trimer structure (Fig. 8A, lane 4).

DISCUSSION

The usefulness of HS for extracting antigens from *Brucella* spp. in the rough phase was first reported by Myers et al. (37) and was confirmed and extended by Díaz and Bosseray (9). More recently, Dubray and Plommet demonstrated the presence of vesicles in HS extracts (12), and the results of Riezu-Boj et al. suggested that these extracts contain group 3 OMPs (45). It has also been established that OM blebs are released spontaneously from *B. melitensis* during growth in broth, and SDS-PAGE profiles of blebs indicated that they were rich in group 3 proteins (17). The results of the present study confirm and extend these observations by demonstrating that blebs are released spontaneously from *B. ovis* and *B. melitensis* and that HS causes the release of vesicles in which proteins are tightly complexed with LPS, as occurs in the native state of the cell (36, 40, 52). The removal of LPS from HS extracts or from OM blebs revealed SDS-PAGE profiles which, in the more rapidly migrating regions, bore strong similarities to each other and to OMPs extracted by detergents (Fig. 7A). The overall relatedness of HS extracts, blebs, and OMPs was also affirmed by immunoblots, which demonstrated cross-reactions between a group 3 antiserum and proteins in groups A, B, and C (Fig. 7B). The specificity of this antiserum for group 3 proteins was attested by its nonreactivity with purified R-LPS or components of group D of HS-PCP fractions which contained residual R-LPS (Fig. 7B and D). Moreover, the reaction of this antiserum with group 3 proteins failed to occur unless the proteins had been freed of R-LPS (Fig. 7B, lanes 2 and 4). The same phenomenon was observed in blots developed with HS antiserum

(Fig. 8, lanes 3 and 4). In the latter case it is more difficult to explain this inhibition of staining simply as blockage of group 3 determinants by overlying R-LPS, because HS antiserum also contained antibodies reactive with R-LPS (Fig. 7E). One may speculate that R-LPS may have been complexed with group 3 proteins in a manner that occluded epitopes reactive with R-LPS-specific antibodies in the HS antiserum.

The number of different protein species within groups A, B, C, and D and the interrelationships among proteins of the different groups have yet to be determined. Similarities in migratory properties in SDS-PAGE and immunological cross-reactions strongly support the view that group A proteins constitute the group 3 OMPs. The reason why one of these proteins (A1) reacted much more strongly than two others (A2 and A3) with group 3 antiserum (Fig. 7B) is unclear but does not exclude the possibility that all of the bands represent group 3 proteins. The cross-reactions noted between group 3 antiserum and proteins of groups B and C derived from HS extracts, blebs, or detergent-extracted OMPs (Fig. 7B) were surprising and suggest either that these are all derivatives of group 3 proteins or that they represent separate sets of structural proteins with a common epitope. Low concentrations of serum antibodies reactive with group C protein were present in normal rabbits used in this study (Fig. 7C) and in about 50% of normal lambs tested, which were indigenous to New York State (C. Gamazo and A. J. Winter, unpublished data). Since it is unreasonable to suppose that these antibodies were induced by prior contact with *Brucella* species, they probably represent cross-reactive antibodies to OMPs of a related bacterial species.

Aside from minor variations in the quantitative distribution of proteins from HS-PCP fractions or blebs, proteins of groups A, B, C, and D were remarkably conserved among the 41 strains of *B. ovis* originating in several European countries and in the western United States. These results are in accord with those of a similar study conducted on the OMPs of a large number of *B. abortus* strains (55). When comparisons were made between strains of *B. ovis* and *B. melitensis*, variations in banding patterns were more obvious (Fig. 2 and 4). Nevertheless, proteins antigenically related to groups A, B, C, and D of *B. ovis* were demonstrated by Western blotting in all 26 strains of *B. melitensis* tested. These findings indicate that a single representative strain of *B. ovis* could be used with confidence as a source of protein reagents for the diagnosis or immunoprophylaxis of *B. ovis* infections.

A protective role for antibodies specific for OMPs may be readily envisaged for ovine brucellosis caused by *B. ovis*. It has been well established in the murine model system that antibodies exert a protective effect against infection with *B. abortus* (4, 22, 25, 34, 41, 43, 48). In *B. abortus*, a naturally occurring smooth species, the O polysaccharide of the S-LPS dominates the surface of the organism, and it has been demonstrated that O-polysaccharide-specific antibodies are responsible for protective immunity (22, 34). This will most likely be found to apply as well to infections caused by *B. melitensis*, since absorption of infected ram sera with smooth *B. melitensis* failed to remove antibodies reacting with group 3 proteins. In contrast, absorption with whole cells of either rough *B. melitensis* or *B. ovis* removed antibodies to proteins of groups 3, B, and C (J. I. Riezu-Boj, Ph.D. thesis, University of Navarra, Pamplona, Spain, 1988). Therefore, in *B. ovis*, a naturally occurring rough species, OMPs have ready access to the bacterial surface, thus insuring that specific antibodies can interact with them and mediate various protective functions.

Brucellae are facultative intracellular parasites, and protective immunity in ovine brucellosis will in all probability be found to require a combination of humoral and cell-mediated immune responses, as is now considered to be the case for infections caused by *B. abortus* (3, 33, 42, 44). Cell-mediated immune responses to porin proteins have been demonstrated in cattle or mice infected with *B. abortus* (A. J. Winter, G. E. Rowe, and W. L. Castleman, unpublished data), and it has been hypothesized that OMPs of *B. abortus* incorporated into subcellular vaccines may induce protective cell-mediated immune responses (59). The efficacy for lambs of vaccines composed of HS extracts, blebs, or cell walls of *B. ovis* in adjuvants which enhance both cell-mediated and humoral immune responses is currently under investigation.

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