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Antibody Response to *Brucella ovis* Outer Membrane Proteins in Ovine Brucellosis

J. I. RIEZU-BOJ,[1] I. MORIYÓN,[1]* J. M. BLASCO,[2] C. GAMAZO,[1] AND R. DÍAZ[1]

Departamento de Microbiología, Universidad de Navarra, Apartado 273, 31080 Pamplona,[1] and Departamento de Producción Animal, Servicio Investigación Agraria, Diputación General Aragón, 50080 Zaragoza,[2] Spain

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Hot saline extracts of *Brucella ovis* were composed of vesicles with outer membrane proteins (OMPs), lipopolysaccharide, and phospholipid as constituents. Extraction with petroleum ether-chloroform-phenol yielded a protein fraction free of detectable lipopolysaccharide, in which group 3 OMPs (28,500 apparent molecular weight [28.5K], 27.0K, and 25.5K) represented 81% of the total. Group 1 OMPs and 67.0K, 22.5K to 21.5K, and 19.5K to 18.0K proteins were also detected. Adsorption of immune sera with whole bacteria suggested that group 3 OMPs and 67.0K, 22.5K to 21.5K, and 19.5K to 18.0K proteins had antigenic determinants exposed on the surfaces of both *B. ovis* and rough *B. melitensis* cells but not on smooth *B. melitensis* cells. Antibodies to group 3 OMPs and the 67.0K protein in the sera of 93 and 87%, respectively, of *B. ovis*-infected rams were found by immunoblotting. Antibodies to other proteins were present in 67% of these animals. Compared with *B. ovis*-infected rams which had not developed lesions, rams with epididymo-orchitis had antibodies to a larger variety of proteins. Although ewes infected with *B. melitensis* also showed antibodies to OMPs, the immunoblot reactions were less intense.

The genus *Brucella* comprises gram-negative intracellular parasites of both humans and animals. In addition to the four species normally isolated as smooth forms, the genus includes two species, *Brucella canis* and *Brucella ovis*, which are naturally rough and fully pathogenic in this form (8). *B. canis* infects dogs and *B. ovis* infects sheep, producing epididymo-orchitis and abortion (8). Except for the lipopolysaccharide (LPS) (2, 13, 32), the immune response to characterized *B. ovis* antigens has seldom been studied (13, 32), and consequently there is little information on the relationship between cell structure and the immune response to this gram-negative pathogen. In addition, a comparative analysis of the immune response to surface antigens in natural hosts infected with smooth and rough *Brucella* spp. has not been carried out.

We have shown previously that hot saline extracts (HS) of whole *B. ovis* cells are rich in rough LPS (R-LPS) and a group (group A) of 32.0K (apparent molecular weight, 32,000) to 25.5K proteins (12, 32). This group is equivalent to the group 3 outer membrane proteins (OMPs) (40), as shown by their similar electrophoretic migration patterns (12, 32) and their reactivities with anti-group 3 antibody (12). We have also shown that sera from *Brucella melitensis*-infected sheep react with *B. ovis* HS in an enzyme-linked immunosorbent assay and that this reaction is stronger than that with purified *B. ovis* R-LPS (32). These observations suggest that *B. ovis* HS are composed largely of materials derived from the outer membrane and that, in addition to LPS, OMPs are important antigens in the ovine immune response to *B. ovis* and *B. melitensis*. In this report, we present data on the characterization of HS which confirm and extend those of previous studies. We have also examined the antibody response of sheep to the protein fraction of HS.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. ovis* REO 198, a CO_2-independent avirulent strain, was used for cell fractionation and antigen extraction. In a previous study, no differences between the antigenic composition of HS obtained from REO 198 and that of HS from virulent strains were found (12). Strain REO 198 was propagated in tryptic soy broth (Oxoid Ltd., London, England) supplemented with 0.5% yeast extract at 37°C with orbital shaking. *B. melitensis* 115 is a rough avirulent mutant whose LPS is devoid of O polysaccharide antigenic determinants (3). *B. melitensis* Rev 1 is a smooth strain used as a live vaccine (4). Both *B. melitensis* strains were grown on tryptic soy agar slants.

Cell fractionation. Exponentially growing bacteria were disintegrated, and the cell envelope and cytosol fractions were separated by ultracentrifugation, with 2-keto-3-deoxyoctulosonic acid plus succinic dehydrogenase (EC 1.3.99.1) and with malic dehydrogenase (EC 1.1.1.37) as the respective markers (23). A fraction rich in OMPs was obtained from the cell envelopes by extraction with sarcosyl (Sigma Chemical Co., St. Louis, Mo.) and Zwittergent 316 (Calbiochem-Behring, San Diego, Calif.) (23, 40). The Zwittergent extract was precipitated with 4 volumes of acetone at −20°C overnight, and the precipitate was delipidated with chloroform-methanol (1:2) and solubilized in 0.7 M 2-mercaptoethanol–10% glycerol–10 mM Tris hydrochloride (pH 6.8) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting.

Antigenic preparations. R-LPS was extracted with petroleum ether-chloroform-phenol, as described elsewhere (11). During R-LPS precipitation, careful and slow saturation of the phenol phase with cold distilled water was found to be critical for obtaining *B. ovis* R-LPS. The final product contained less than 1% protein.

To prepare *B. ovis* HS, freeze-dried cells were suspended in saline at a ratio of 7:100 (wt/vol) and extracted at 100°C for 15 min, and the cell debris was removed by centrifugation at 15,000 × *g* for 30 min. The resulting yellowish fluid was

ultracentrifuged (100,000 × *g* for 5 h), and the pelleted HS were suspended in distilled water, dialyzed, and freeze-dried (9). To remove the R-LPS, the HS were suspended in petroleum ether-chloroform-phenol (5:8:2) (50 to 10 mg per ml of solvent mixture) and homogenized in a blender, and the mixture was centrifuged at 8,000 × *g* for 15 min at 4°C. The insoluble protein fraction was reextracted twice under the same conditions, washed three times with distilled water, and freeze-dried. After flash evaporation of the petroleum ether and chloroform, the R-LPS was recovered from the phenol by precipitation with water (11).

Analytical methods. The total protein content was determined colorimetrically (21), with bovine serum albumin as the standard. The R-LPS content of the HS was estimated by colorimetric determination of 2-keto-3-deoxyoctulosonic acid (42) with R-LPS and with pure 2-keto-3-deoxyoctulosonic acid and deoxyribose as standards. Under the conditions of the assay, 1 μmol of 2-keto-3-deoxyoctulosonic acid gave an optical density of 38.3 to 41.5, with a sensitivity level of 5 μg for purified *B. ovis* R-LPS. SDS-PAGE (13.5% acrylamide) was performed as described by Laemmli (19), and the gels were stained with Coomassie blue or Stainsall (Sigma) (18) or by the periodate-silver method for LPS of Tsai and Frasch (38) (sensitivity, 10 to 15 ng for *B. ovis* R-LPS). The relative proportions of the protein bands were determined by scanning densitometry with Coomassie blue-stained gels.

Gas-liquid chromatography was used to detect lactobacillic acid (*cis*-11,12-methyleneoctadecanoate), a taxonomic marker of *Brucella* phospholipids (7, 16). Samples were saponified with NaOH (27), and methyl esters were obtained by treatment with BF_3 in methanol (26). The analyses were carried out with a Shimadzu GC-9 apparatus (Shimadzu Co., Kyoto, Japan) equipped with a fused silica capillary column (SP-2330; Supelco Inc., Bellefonte, Pa.), a flame ionization detector, and a Shimadzu C-R3A data station. Fatty acid methyl esters obtained from dried *B. ovis* cells by the same methods were used as standards.

Electron microscopy of negatively stained crude HS samples was performed by standard procedures.

Immunoblots. Proteins separated by SDS-PAGE were electroblotted on nitrocellulose sheets (type HA [pore size, 0.45 μm]; Millipore Corp., Bedford, Mass.) at either 200 mA for 18 h or 1 A for 1 h in 20% methanol–192 mM glycine–20 mM Tris hydrochloride (pH 8.3) (6). Antibodies in sheep sera bound nonspecifically to blotted proteins; the overnight incubation of blots in 0.05% Tween 20–10 mM phosphate-buffered saline (pH 7.2) was the most satisfactory of several blocking protocols tested. For comparative purposes, all sera were used at a 1:100 dilution, and the developing conditions were standardized as follows. Nitrocellulose strips were incubated for 5 h at room temperature with serum dilutions made in phosphate-buffered saline–Tween solution, washed four times with this same solution, and incubated with peroxidase-conjugated rabbit anti-sheep immunoglobulin G (heavy and light chain specific; Nordic Immunological Laboratories, Tilburg, Netherlands) for 1 h at room temperature. Peroxidase activity was detected by incubation with a solution containing H_2O_2 and 4-chloro-1-naphthol (14) for 20 min in the dark.

Sheep sera. Sera from the following groups of animals were used. The noninfected controls were 40 rams from the brucellosis-free flock of the Department of Animal Production, Servicio Investigación Agraria, Diputación General Aragón. The *B. ovis*-infected controls were 36 rams from whose semen *B. ovis* had been isolated. Animals suspected of being infected with *B. ovis* were 15 rams from flocks in which *B. ovis* had been isolated which were positive in the standard tests for ram epididymitis (4) and negative in the rose bengal and complement fixation tests for *B. melitensis* infection (4). Of these animals, seven had palpable epididymo-orchitis and eight had not developed such symptoms. The *B. melitensis*-infected controls were 36 ewes with *B. melitensis* field infection (biovars 1 and 3) demonstrated by bacteriological culture and serological methods (4).

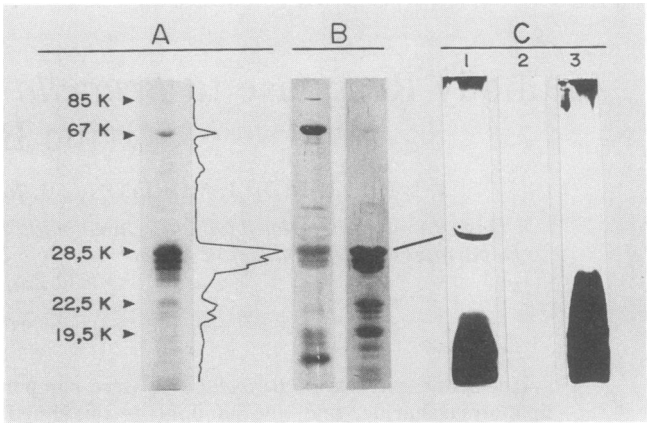

FIG. 1. Analysis of the protein fraction of HS. (A) SDS-PAGE with Coomassie blue staining and scanning densitometry; (B) immunoblot with two sera of *B. ovis*-infected rams; (C) SDS-PAGE with periodate-silver stain (lane 1, crude HS; lane 2, protein fraction of HS; lane 3, purified R-LPS).

Immunoadsorption with whole *Brucella* cells. Ram serum (1 ml) was incubated with 1 to 2 mg of freeze-dried bacteria or an equivalent amount of live bacteria. After incubation for 6 h at 37°C with intermittent shaking, the bacteria and adsorbed antibodies were removed by centrifugation. The adsorption was repeated twice under identical conditions, and the adsorbed and nonadsorbed sera were tested, with the protein and cytosol fractions of the HS as antigens. The sera from three rams, one from the *B. ovis*-infected controls (no. 531) and two from the group with epididymo-orchitis (no. 147 and 130), were used in the first set of immunoadsorptions.

Immunoadsorption with R-LPS. The serum from a ram (no. 638) with a high titer of anti-R-LPS antibodies (measured by enzyme-linked immunosorbent assay [32]) was adsorbed three times with 2 mg of R-LPS under the conditions described above. The removal of anti-R-LPS antibody was confirmed by enzyme-linked immunosorbent assay with R-LPS (32).

RESULTS

Characterization of HS. Crude HS contained 44.7 to 67.1% protein, 10.7 to 55.5% R-LPS, and several fatty acids, the major of which were palmitic and lactobacillic acids (42 to 43% and 33 to 34% of the total fatty acid content, respectively). SDS-PAGE of HS showed a profile in which 85.0K and 67.0K proteins and a group of 32.0K to 25.5K proteins had electrophoretic mobilities similar to those of OMPs extracted with Zwittergent (results not shown). The 32.0K- to 25.5K-protein group had been previously designated group A (12). In addition, 22.5K to 21.5K proteins (group B [12]) and 19.5K to 18.0K proteins (group C [12]) were observed. With Stainsall, group A stained red-green and a short green smear with the same color and mobility as those

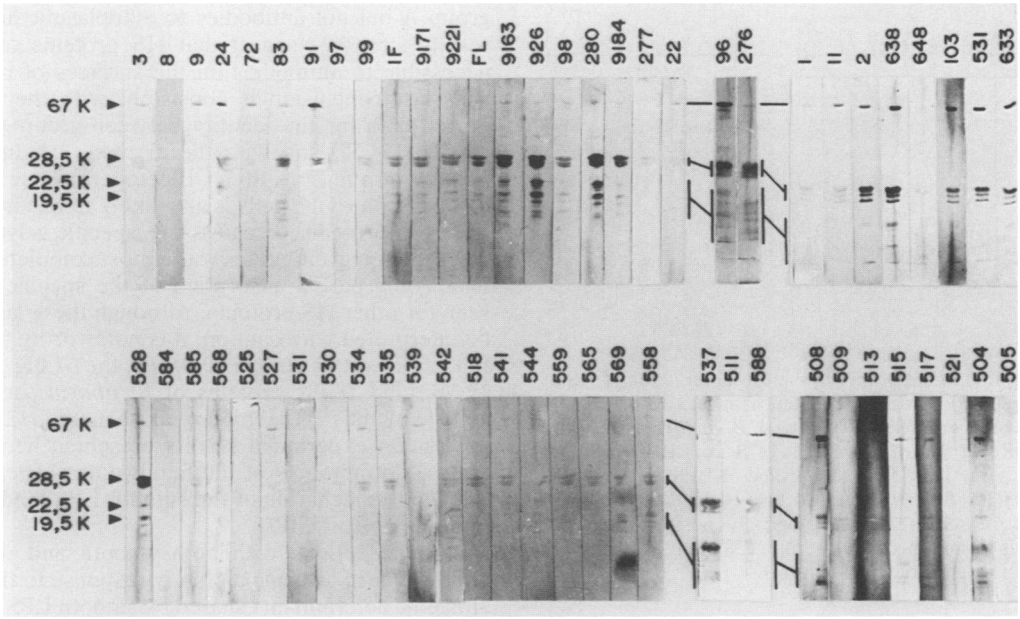

FIG. 2. Immunoblot analysis of the antibody response to the protein fraction of HS in the group of rams in which *B. ovis* infection had been demonstrated by isolation of the microorganism (top) and in ewes infected by *B. melitensis* (bottom). Lane numbers correspond to animal reference numbers.

of an R-LPS control was observed at the running front of the gel. Periodate-silver staining was positive both at the position corresponding to group A and at the running front of the gel but not at the position of other protein bands (Fig. 1C). Finally, electron microscopy of the HS showed that they were made up of vesicles (60 by 70 nm to 13 by 13 nm) (results not shown), confirming previous reports on the micellar structure of this sort of extract (10).

The extraction of the crude HS with petroleum ether-chloroform-phenol yielded a fraction insoluble in the solvent mixture which contained 93% protein. A total of 12 polypeptide bands were detected by SDS-PAGE (Fig. 1A). Group A proteins, which represented up to 81% of the protein (Fig. 1A), had shifted to 28.5K to 25.0K. Immunoblot analysis with sera from *B. ovis*-infected rams revealed additional minor polypeptides up to a total of 21 bands (Fig. 1B).

Extraction with petroleum ether-chloroform-phenol removed all the periodate-silver-positive material from protein fraction of the HS (Fig. 1C). On the basis of the amount of material loaded (40 to 60 μg) and the limit of detection of the method (15 to 10 ng), an LPS content of less than 0.03% was estimated. In addition, no green staining was observed with Stainsall. In agreement with these results, no 2-keto-3-deoxyoctulosonic acid was detected, indicating that the R-LPS content was less than 0.2% by this method. In contrast with the results reported above, the material extracted from the HS in the petroleum ether-chloroform-phenol was indistinguishable from the R-LPS obtained directly from dried cells.

Immunoblots with sera from *B. ovis*-infected rams. In a preliminary experiment, detergent extracts rich in OMPs, HS, and the protein fraction of the HS were compared by using the positive control sera from *B. ovis*-infected and *B. melitensis*-infected animals. The best results with respect to the sharpness and intensity of bands on nitrocellulose and the lack of interference of R-LPS were obtained with the protein fraction of the HS. In particular, whereas group 3 OMPs of detergent extracts reacted poorly with sheep antibodies, group A (equivalent to group 3) of the protein fraction of HS yielded strong reactions. Thus, this protein fraction was used in the immunoblot analyses described below.

The immunoblots performed with control sera from rams infected with *B. ovis* are presented in Fig. 2. Most infected rams developed antibodies to group A and to the 67.0K protein, and a large proportion of these rams also had antibodies to group B (22.5K to 21.5K) and C (19.5K to 18.0K) proteins. Adsorption of a ram serum with a high titer of antibody to R-LPS did not diminish the immunoblot reaction with the HS protein fraction (see Fig. 4D), as would be expected if trace amounts of R-LPS bound to the proteins were responsible for the immunoblot reactions.

These results were confirmed and extended by the analyses of sera of rams positive for *B. ovis* in conventional serological tests. With these sera, it was found that rams with epididymo-orchitis had antibodies to a larger variety of proteins than those that had not developed epididymo-orchitis (Fig. 3; compare rams 102, 104, 130, 139, 147, and 154 with rams 103, 111, 112, 123, 106, 160, 161, and 162).

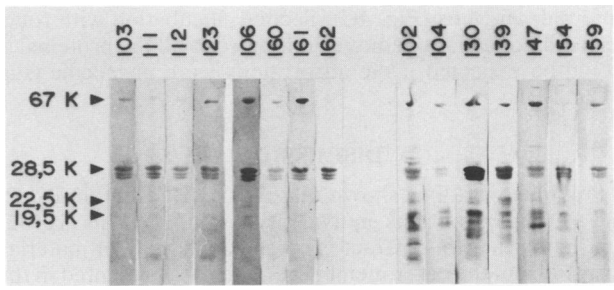

FIG. 3. Immunoblot analysis of the antibody response to the protein fraction of HS in rams which were serologically positive for *B. ovis* and which did (right) or did not (left) have epididymo-orchitis. Lane numbers correspond to animal reference numbers.

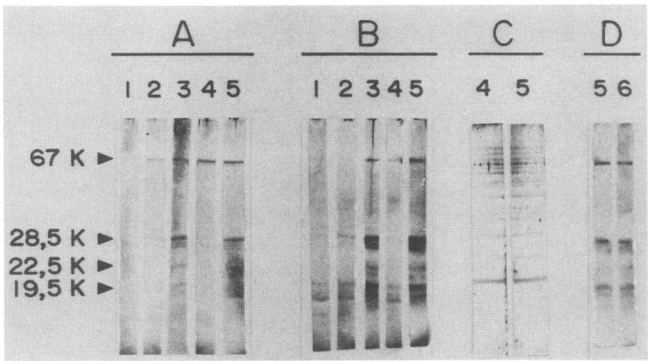

FIG. 4. Adsorption of antibodies to the protein fraction of HS by whole *Brucella* cells. Blots were incubated with sera adsorbed with freeze-dried cells of *B. ovis* REO 198 (lane 1), *B. melitensis* 115 (lane 2), *B. melitensis* Rev 1 (lane 3), live cells of *B. ovis* REO 198 (lane 4), nonadsorbed sera (lane 5), and purified R-LPS (lane 6). (A) Serum 531 (ram with *B. ovis* infection proved by bacteriological culture); (B and C) serum 130 (serologically positive ram with epididymo-orchitis symptoms); (D) serum 638 (ram with *B. ovis* infection proved by bacteriological culture).

Immunoblots with sera from *B. melitensis*-infected ewes. Many *B. melitensis*-infected ewes developed antibodies to group A, and some also developed antibodies to the 67.0K protein and to proteins with molecular weights lower than 24,000 (Fig. 2). However, the immunoblot reactions were generally less intense than those in *B. ovis*-infected rams. Only the sera of ewes 528, 537, and 508 produced immunoblot reactions comparable to those of rams 83, IF, 9171, 9221, FL, 9163, 926, 98, 280, 9187, 96, 276, 2, 638, 103, 531, and 633 or the 15 ram sera whose results are shown in Fig. 3.

Adsorption of antibodies by whole cells of *B. ovis* and *B. melitensis*. Representative results of antibody adsorption experiments are presented in Fig. 4. Freeze-dried cells of *B. ovis* REO 198 and the rough mutant *B. melitensis* 115 almost completely adsorbed the antibodies to the 67.0K protein and to group A (28.5K to 25.0K) and B (22.5K to 21.5K) proteins. Essentially the same results were obtained with live *B. ovis* REO 198 cells for both groups A and B; with some sera, this adsorption also diminished the reaction with the 67.0K protein. Antibodies to group C (19.5K to 18.0K) proteins were only partially removed by either freeze-dried or live cells. In contrast to the results obtained with rough bacteria, adsorption with freeze-dried cells of smooth *B. melitensis* Rev 1 did not remove antibodies to any of the HS proteins (Fig. 4).

The specificities of these immunoadsorptions were shown in two ways. First, the adsorptions did not diminish the immunoblot reaction of antibodies specific for the cytosol (internal) antigens (Fig. 4C). Second, incubation with rough cells did not equally remove antibodies to all the proteins, as would be expected if the adsorptions were unspecific (Fig. 4A and B).

DISCUSSION

Previous work has shown that *B. ovis* HS contain R-LPS and a group of proteins equivalent to group 3 OMPs (12, 32), suggesting that this extract is composed largely of materials derived from the outer membrane. The data presented in this article confirm this hypothesis, since they show the membranous structure of the HS and the presence of phospholipid markers (such as lactobacillic acid). In addition, whole rough cells (but not smooth cells) removed antibodies to group A but not antibodies to cytoplasmic antigens. These results strongly suggest that HS proteins of group A are accessible to antibodies on the surfaces of rough bacteria. This last conclusion is consistent with the previously reported data for the identity between group A and group 3 OMPs (12, 32) and with the extrinsic labeling of *Brucella abortus* group 3 with [^{125}I]lactoperoxidase (23). Surface exposure on rough cells is also likely to occur for 67.0K and group B proteins, because the specific adsorption of the corresponding antibodies was almost complete. On the other hand, only partial adsorption of the specific antibody was seen for other HS proteins. Although these last results must be interpreted with caution, it is noteworthy that in addition to the three main groups of OMPs, the 67.0K, 22.5K, 21.5K, 19.5K, and 18.0K proteins of *B. abortus* are extrinsically labeled by the [^{125}I]lactoperoxidase method (23). Finally, the adsorption experiment results presented here also suggest that, as in members of the family *Enterobacteriaceae* (39), the O polysaccharide of the smooth LPS hinders the access of antibody to OMPs.

Since infections with both smooth and rough *Brucella* strains induce a strong antibody response to the core-lipid A antigenic determinants shared by smooth LPS and R-LPS (2, 3, 13, 32), the removal of R-LPS to levels at which it would not interfere with the detection of antibodies to OMPs was essential. This was achieved by extraction with petroleum ether-chloroform-phenol, as shown by the results of the immunoblot with ram serum adsorbed with R-LPS. This conclusion is in agreement with the results of both the periodate-silver staining and the 2-keto-3-deoxyoctulosonic acid analysis, which indicated R-LPS contamination beyond the detection limits of either method. Obviously, the removal of LPS explains the shift of group 3 from the 32.0-to-25.5K range to the 28.5-to-25.0K range brought about by the solvent extraction. As in other gram-negative bacteria, there are tight protein-LPS-phospholipid interactions in the outer membrane of *Brucella* spp. which are partially resistant to the action of detergents and urea (25), even under the stringent conditions of SDS-PAGE (24). The more efficient removal of LPS shown in this report is due in all likelihood to a more efficient action of the organic solvents compared with that of detergents or chaotropic agents.

Under the experimental conditions used, group 3 OMPs (group A) and the 67.0K protein were the proteins which reacted most often with antibodies elicited by *B. ovis* infection. It is remarkable that although the 67.0K protein represented only 2.9% of the total protein before electroblotting, 87% of the sera reacted with it. Since the 67.0K protein is a minor one in the detergent extracts of cell envelopes, it should be highly immunogenic in vivo. A similar conclusion cannot be reached with certainty with respect to group 3 proteins, since they represented 81% of the protein before electroblotting and since the frequency of reaction could also reflect the amount of antigen blotted. However, it is interesting that patients afflicted with brucellosis also develop antibodies to group 3 OMPs (F. J. Alvarado, D. Delgado, and P. Rice [The Maxwell Finland Laboratory for Infectious Diseases, Boston University School of Medicine, Boston Mass.], personal communication, 1989; C. Gamazo, I. Moriyón and R. Díaz, unpublished results). Afzal et al. (1) have studied the antibody response to *B. ovis* cell envelope components extracted with detergents and claim that antibodies to group 1 but not groups 2 (porins, 39.0K to 35.0K) and 3 were present in the sera of infected rams. Since group 3 represents a high proportion of the proteins in the detergent extracts of *B. ovis* cell envelopes (32, 35), the discrep-

ancy between our results and theirs is difficult to explain. The presence of LPS in detergent extracts, which could interfere with the detection of antibodies to group 3 (12), and differences in the methods of extraction and in the criteria used to identify the major groups could explain such a discrepancy.

Sera from infected rams that had developed epididymo-orchitis showed stronger reactions with blotted proteins than sera from infected rams without symptoms did, an observation also made previously for the outer membrane lipoprotein (13). Since those symptoms appear late after infection, a possible explanation is that those rams that had developed lesions experienced a more intense antigenic stimulation. Alternatively, the antibody response could play a role in the pathogenesis of the disease, a hypothesis which could be studied by histopathological examination of the lesions.

Regardless of the group of rams considered, sera from *B. melitensis*-infected ewes generally showed a less-intense antibody reaction with blotted HS proteins and with group 3 OMPs in particular. This could result from differences in the intensity of the overall antigenic stimulation caused by *B. ovis* and *B. melitensis* infections or from the use of heterologous antigens to test the immune response to *B. melitensis* or from differences in the antigenic structures of smooth and rough *Brucella* species. However, it is not possible to decide on the relative importance of these groups of factors with the data available. Obviously, the importance of both OMPs and R-LPS explains why HS are the antigen of choice for the serodiagnosis of *B. ovis* ram epididymitis (5, 15, 17, 28, 29, 32–34, 36, 41, 43).

Both cell- and antibody-mediated responses are important in brucellosis immunity. With the mouse model, it has long been established that antibodies to smooth LPS are necessary to provide immunity to *B. abortus* and *B. melitensis* (30, 31, 37); this has been confirmed with monoclonal antibodies to the O polysaccharide (20, 22). In contrast, immunization with porins from rough strains and passive transfer of antiporin monoclonal antibodies fail to provide protective immunity in the same animal model (22, 44). However, the absence of the O polysaccharide and the differences found in the antibody response to OMPs show that the conclusions drawn from studies on the role of anti-OMP antibodies in immunity to smooth *Brucella* strains cannot be extended to infections caused by the rough species. Thus, antibodies to OMPs could play an important role in immunity to *B. ovis*.

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