Antibody Reactivity to Omp31 from *Brucella melitensis* in Human and Animal Infections by Smooth and Rough Brucellae

Juliana Cassataro,¹ Karina Pasquevich,¹ Laura Bruno,¹ Jorge C. Wallach,²,³ Carlos A. Fossati,² and Pablo C. Baldi²*¹

Laboratorio de Inmunogenética, Hospital de Clínicas,¹ and Instituto de Estudios de la Inmunidad Humoral, Facultad de Farmacia y Bioquímica,² Universidad de Buenos Aires, and Servicio de Brucelosis, Hospital F.J. Muñiz,² Buenos Aires, Argentina

Received 23 September 2003/Returned for modification 27 October 2003/Accepted 7 November 2003

Group 3 of outer membrane proteins (OMPs) of *Brucella* includes Omp25 and Omp31, which share 34% identity. Omp25 is highly conserved in *Brucella* species, and Omp31 is present in all *Brucella* species, except *Brucella abortus*. Antibodies to *Brucella melitensis* Omp31 have been sought only in infected sheep, and Western blotting of sera from infected sheep did not reveal anti-Omp31 reactivity. We obtained recombinant purified Omp31 (*B. melitensis*) and tested its recognition by sera from humans and animals suffering from brucellosis by an indirect enzyme-linked immunosorbent assay (ELISA). Serum samples from 74 patients, 57 sheep, and 47 dogs were analyzed; brucellosis was confirmed by bacteriological isolation in all ovine and canine cases and 31 human cases of brucellosis. Thirty-five patients (47%) were positive for antibodies to Omp31, including seven cases of *Brucella suis* infection, two cases of *B. abortus* infection, and three cases of *B. melitensis* infection. Of 39 sheep naturally infected with *B. melitensis* (biovars 1 and 3), 23 (59%) were positive for antibodies to Omp31. Anti-Omp31 antibodies were also detected in 12 of 18 rams (67%) in which *Brucella ovis* infection, two cases of *B. abortus* infection, and three cases of *B. melitensis* infection. Of 39 sheep naturally infected with *B. melitensis* (biovars 1 and 3), 23 (59%) were positive for antibodies to Omp31. Anti-Omp31 antibodies were also detected in 12 of 18 rams (67%) in which *Brucella ovis* was isolated from semen. Antibodies to Omp31 were also found in 41 (87%) of the 47 dogs, including 13 with recent infection. These results suggest that an indirect ELISA using recombinant purified Omp31 from *B. melitensis* would be of limited value for the diagnosis of human and animal brucellosis. Nevertheless, the potential usefulness of this antigen in combination with other recombinant proteins from *Brucella* should not be dismissed.

*Brucella* strains are the causative agents of brucellosis, a widespread infectious disease that affects several animal species and is transmitted to humans in several ways. While bacteriological isolation is the most specific diagnostic test, the rate of isolation is generally low, the results are not available immediately, and processing large numbers of samples is cumbersome. Consequently, serological tests are widely used for diagnosing human and animal brucellosis. Classical serological techniques rely mainly on the detection of antibodies to lipopolysaccharide (LPS), giving rise to false-positive reactions because of cross-reactivity with LPS from other bacteria. This and other drawbacks of anti-LPS antibodies have fueled increasing interest in the detection of antibodies to alternative antigens, mainly outer membrane proteins (OMPs) and cytoplasmic proteins.

Major OMPs from *Brucella* have been classified in group 2 (Omp2a and Omp2b [36 to 38 kDa]) and group 3 (Omp25 and Omp31 [25 to 27 and 31 to 34 kDa, respectively]) (4). Omp31 was initially cloned from *Brucella melitensis* 16M, and its predicted amino acid sequence revealed a significant homology (34% identity) with *Brucella* Omp25 (9). Some observations regarding the Omp31 protein, including its ability to form oligomers resistant to denaturation by sodium dodecyl sulfate (SDS) at low temperatures, suggest that it is a porin (4). Omp31 is expressed in all *Brucella* species, except *Brucella abortus*, which has a 25-kb chromosomal deletion comprising omp31 and other genes (10). In addition, some differences between Omp31 from *B. melitensis* and Omp31 from *Brucella ovis* have been reported. Kittelberger et al. (6) showed by Western blotting that only 6 of 10 monoclonal antibodies against Omp31 from *B. ovis* reacted with Omp31 from *B. melitensis*. These results were later confirmed by Vizcaíno et al. (11), who also found that Omp31 from these species differed by 7 amino acids. According to Vizcaíno et al. (11), this 7-amino-acid difference would explain why some monoclonal antibodies to *B. ovis* Omp31 and some sera from *B. ovis*-infected sheep do not react with *B. melitensis* Omp31. An additional and intriguing finding of their work (11) was that none of 11 serum samples from *B. melitensis*-infected sheep reacted with *B. melitensis* Omp31 by Western blotting, leading the researchers to suggest that this protein does not induce an important humoral immune response in infected sheep. However, it is possible that the lack of antibody reactivity against this protein is due to the denaturing conditions of Western blotting.

To test this hypothesis, in the present study, we used purified recombinant Omp31 (rOmp31) from *B. melitensis* to assess the antibody response to this protein in sera from *B. melitensis*- and *B. ovis*-infected sheep by an ELISA. Since anti-Omp31 antibodies have been sought only in cases of ovine brucellosis, a second goal of the present study was to assess this reactivity in cases of human and canine brucellosis.
MATERIALS AND METHODS

rOmp31. A 687-bp B. melitensis DNA fragment encoding Omp31 devoid of the putative signal peptide was cloned in pET22+ vector (Novagen, Madison, Wis.) as described previously (5). The resultant plasmid (pET-Omp31) contained the Omp31 gene, with the addition of a poly(H) tail. Competent Escherichia coli BL21(DE3) (Stratagene) was transformed with pET-Omp31. Ampicillin-resistant colonies were grown in Luria-Bertani medium containing 100 μg of ampicillin per ml at 37°C with agitation (300 rpm) until they reached an optical density at 600 nm (OD_600) of 1.0. Five milliliters of this culture was diluted to 500 ml and grown until it reached an OD_600 of 1.0. Protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubating transformed cells for 4 h. Bacteria were pelleted by centrifugation (15,000 × g, 20 min, 4°C) and frozen at −20°C. Bacterial cells were suspended in a solution consisting of 50 mM Tris, 5 mM EDTA, and 1% Triton X-100 (pH 8.0) (suspending solution) and sonicated for three 1-min cycles at 4°C. Inclusion bodies were pelleted at 20,000 × g for 30 min at 4°C and washed twice with suspension solution without Triton X-100. Inclusion bodies were solubilized in a solution containing 50 mM Tris, 5 mM EDTA, and 8 M urea (pH 8.0) at room temperature overnight with agitation. After centrifugation (20,000 × g, 30 min, 4°C), soluble protein was purified by chromatography through Ni-agarose (Qiagen, Dorking, United Kingdom). The presence of rOmp31 in eluates was checked by Western blotting with specific MAb A59/10F09/G010 (3). Purity was assessed by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. rOmp31 was adsorbed with Sepharose-polymyxin B to eliminate LPS contamination. This preparation contained less than 0.05 endotoxin unit per mg of protein (assessed by using a Limulus amoebocyte lysate analysis kit [Sigma, St. Louis, Mo.]).

ELISA with rOmp31. Maxisorp polystyrene plates (Nunc, Roskilde, Denmark) were sensitized with rOmp31 (0.1 μg/well) diluted in phosphate-buffered saline (PBS). Unbound sites in the plates were blocked with 200 μl of PBS containing 3% skim milk well per well. After the wells were washed with PBS containing 0.05% Tween 20 (PBS-T), sera were diluted in PBS-T containing 1% skim milk and dispensed to the wells. Specific antibodies were detected with horseradish peroxidase-conjugated antibodies to human immunoglobulin G (IgG) (Jackson, West Grove, Pa.), or to canine (Sigma) or ovine (Jackson) immunoglobulins. The reaction was developed by adding ortho-phenylenediamine (2 μg ml−1) in 0.1 M citrate-phosphate buffer containing 0.03% H2O2 and was stopped with 4 N H2SO4. To establish the cutoff value of the assays, serum samples from noninfected controls were tested under the same conditions (given above). The cutoff value of each enzyme-linked immunosorbent assay (ELISA) system was calculated as the mean specific OD of control sera plus 3 standard deviations (SD).

Serum samples. (i) Human sera. The study included 74 serum samples from patients with brucellosis, diagnosed on the basis of clinical, serological, and/or bacteriological findings. Blood cultures were performed for 52 patients and were positive for Brucella in 31 cases (11 patients were infected with Brucella suis, 5 were infected with B. abortus, 8 were infected with B. melitensis, and 7 were infected with Brucella spp.). Standard tube agglutination was positive in all but 3 patients and yielded titers equal to or higher than 100 for 50 patients. IgG to Brucella smooth LPS was detected in all patients by ELISA. IgG to total cytosolic proteins of B. abortus (CP) was detected in 64 patients by ELISA. Sera from 38 healthy volunteers with no history of brucellosis were used to calculate the cutoff value of the assay.

(ii) Sheep sera. Eighteen serum samples from rams naturally infected with B. ovis were included. In all cases, the infection was confirmed by isolation of the bacterium from semen. All these animals were positive by the Brucella complement fixation test. The assay also included 39 serum samples from sheep naturally infected with B. melitensis (biovars 1 and 3), which was isolated from blood or tissue. Most of these animals (34 sheep) were positive by the Rose Bengal test, and all but one had a positive delayed-type hypersensitivity reaction to Brucella antigens. Sera from infected sheep were kindly provided by Ignacio Moriyo M. Wanke, University of Buenos Aires.

RESULTS

Ovine samples. By ELISA, sera from healthy sheep yielded ODs between 0.115 and 0.304 (mean, 0.172; SD, 0.042), resulting in a cutoff value of 0.298 (Fig. 1). Samples from sheep naturally infected with B. ovis yielded ODs from 0.164 to 1.068 (mean, 0.470; SD, 0.256), and those from B. melitensis-infected animals ranged from 0.057 to 1.301 (mean, 0.407; SD, 0.267). Antibodies to Omp31 were detected in 23 (59%) sheep infected with B. melitensis and in 12 (67%) infected with B. ovis.

Using Western blotting with lysates of transformed E. coli, other researchers have found differences in the reactivity of B. ovis-infected and B. melitensis-infected sheep to Omp31. However, in this study, we did not find a significant difference between the two groups in terms of the percentage of positive reactions or signal level by an ELISA. To assess whether this discrepancy was due to the non-denaturing conditions of ELISA as opposed to the denaturing conditions of Western blotting, the reactivity of our sera against the purified rOmp31 was also tested by Western blotting. As shown in Fig. 2, sera from B. melitensis- and B. ovis-infected animals that had been positive in an ELISA also reacted with Omp31 by Western blotting, albeit sera from the B. ovis-infected animals gave stronger signals.
**Canine samples.** Sera from healthy dogs yielded ODs between 0.030 and 0.360 (mean, 0.105; SD, 0.068), resulting in a cutoff value of 0.309 (Fig. 3). Antibodies to Omp31 were detected in 41 (87%) of the 47 infected dogs. The reactivity of samples from dogs infected with *B. canis* ranged from 0.100 to 2.450 (mean, 0.578; SD, 0.643). All 13 serum samples from dogs infected for less than 30 days were positive for antibodies to Omp31, including 7 cases of *B. abortus* infection, 2 cases of *B. suis* infection, 2 cases of *B. melitensis* infection, and 2 cases of infection with *Brucella* spp. Patients were classified according to the length of their illness as recent brucellosis patients (up to 1 year since diagnosis) or past brucellosis patients (more than 1 year since diagnosis). Forty-two patients were classified as recent brucellosis patients, including 37 with current clinical manifestations. Thirty-two patients were classified as past brucellosis patients, including 27 with current clinical manifestations. Antibodies to Omp31 were detected in 20 recent brucellosis patients (47.6%) and 15 past brucellosis patients (46.9%). Thus, no significant differences for anti-Omp31 reactivity were found for patients infected with different *Brucella* species or for different lengths of time.

**Human samples.** By ELISA, sera from healthy controls yielded ODs between 0.025 and 0.406 (mean, 0.156; SD, 0.097), resulting in a cutoff value of 0.483 (Fig. 4). Samples from patients with brucellosis yielded ODs between 0.168 and 1.528 (mean, 0.578; SD, 0.256). Overall, 35 patients (47.3%) were positive for antibodies to Omp31, including 7 cases of *B. suis* infection, 2 cases of *B. abortus* infection, 3 cases of *B. melitensis* infection, and 2 cases of infection with *Brucella* spp. Patients were diagnosed according to the length of their illness as recent brucellosis patients (up to 1 year since diagnosis) or past brucellosis patients (more than 1 year since diagnosis). Forty-two patients were classified as recent brucellosis patients, including 37 with current clinical manifestations. Thirty-two patients were classified as past brucellosis patients, including 27 with current clinical manifestations. Antibodies to Omp31 were detected in 20 recent brucellosis patients (47.6%) and 15 past brucellosis patients (46.9%). Thus, no significant differences for anti-Omp31 reactivity were found for patients infected with different *Brucella* species or for different lengths of time.

**DISCUSSION**

While Omp31 is expressed in all *Brucella* species, except *B. abortus*, previous studies using Western blotting have shown a partial or total lack of recognition of *B. melitensis* Omp31 by sera from *B. ovis- or B. melitensis*-infected sheep, respectively (6, 11). The apparent lack of anti-Omp31 antibodies in *B. melitensis*-infected sheep was attributed to the presence of O polysaccharide chains in *B. melitensis* LPS, which could reduce the immunogenic properties of Omp31 (11). However, the absence of antibody reactivity against this protein could also be due to the elimination of conformational B-cell epitopes under the denaturing conditions of Western blotting. We hypothesized that the number of positive reactions would be increased by the use of purified rOmp31 under the nondenaturing conditions of the ELISA.

As shown in Fig. 1, antibodies to Omp31 were detected by ELISA in roughly 60% of infected sheep regardless of the infecting species (*B. melitensis* or *B. ovis*). These results clearly show that sheep infected with *B. melitensis* develop antibodies against the homologue Omp31 and that the proportion of sheep with positive reactions is similar to that found among *B. ovis*-infected animals. Notably, the percentage of *B. ovis*-infected sheep that reacted with *B. melitensis* Omp31 by ELISA was higher than that found in previous studies by Western blotting (61% versus 33%) (11). The finding of antibodies to *B. melitensis* Omp31 in *B. ovis*-infected animals clearly shows the existence of shared epitopes between the Omp31 proteins from these species. This is also supported by the finding that sera from mice immunized with *B. melitensis* Omp31 react with *B. ovis* whole cells by ELISA and with *B. ovis* Omp31 by Western blotting (5). Moreover, this immunization protects mice from infection with *B. ovis*.

To test our hypothesis that the failure to detect anti-Omp31 antibodies in previous studies was due to the denaturing conditions of Western blotting, some sera yielding high reactivity by ELISA were tested against the same protein by Western blotting. Surprisingly, antibodies to Omp31 were detected in
both *B. melitensis*- and *B. ovis*-infected animals, unlike the results reported by Vizcaíno et al. (11). This discrepancy may be due to differences in the experimental conditions used for Western blots. For example, while a lysate of recombinant *E. coli* was used in that previous study, purified Omp31 was used in the present investigation, which could translate into differences in the mass of Omp31 per lane. Overall, our results show that Omp31 from *B. melitensis* shares immunogenic epitopes with its homologue from *B. ovis* and that it can be used to diagnose brucellosis in both *B. melitensis* - and *B. ovis*-infected sheep.

To our best knowledge, the presence of antibodies to Omp31 has been tested only in sheep and experimentally infected mice. Thus, a second goal of the present study was to assess the potential diagnostic usefulness of rOmp31 in humans and animals other than sheep.

As shown in Fig. 3, antibodies to Omp31 were detected in 87% of dogs infected with *B. canis*, thus confirming the presence of the protein in this *Brucella* species (11). While previous studies have shown the presence of antibodies to a mixture of outer membrane components (hot-saline extract of *B. canis*) (8), this is the first study to identify serum reactivity to a particular OMP in canine brucellosis. The present findings confirm the diagnostic usefulness of antibodies to *Brucella* proteins in canine brucellosis, as has been shown for cytosolic components (1).

Antibodies to *B. melitensis* Omp31 were also detected in brucellosis patients by ELISA, although the percentage with positive reactions was lower than in dogs and sheep. The frequency of detection was similar for patients with recent or past brucellosis (47.6% and 46.9%, respectively). While Omp31 is absent in *B. abortus*, some sera from patients infected with this species reacted with *B. melitensis* Omp31 by ELISA. This could be due to cross-reactivity with Omp25, which is expressed in *B. abortus* and exhibits 34% amino acid identity with Omp31.

The frequency of antibodies to Omp31 in sheep was similar or higher than that reported for other OMPs. In a previous study using ELISA with recombinant proteins, 33% of sheep naturally infected with *B. melitensis* had antibodies to Omp25, and 50% had antibodies to Omp36 or Omp16 (7). To our best knowledge, the diagnostic value of isolated OMPs has not been assessed in dogs or humans.

In summary, this study shows that antibodies to *B. melitensis* Omp31 can be detected in patients and animals with brucellosis. An indirect ELISA using rOmp31 allowed the detection of brucellosis in 48% of patients, 61% of infected sheep, and 87% of infected dogs. The usefulness of an ELISA combining Omp31 and other *Brucella* proteins should be addressed, since previous studies have shown an improved diagnostic sensitivity by the simultaneous measurement of antibodies to different *Brucella* proteins (2, 7). Studies are under way in our laboratory to test this possibility.

ACKNOWLEDGMENTS

This work was supported in part by grant PICT99-0506324 from the Agencia Nacional de Promocion Cientifica y Tecnologica (ANPCYT) and by a grant from Fundacion Antorchas. J.C. was supported by a fellowship of the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET). C.A.F. and P.C.B. are members of the Research Career of CONICET. K.P. was supported by a fellowship of the Universidad Nacional de La Plata. C.A.F. is also a member of the Facultad de Ciencias Exactas, Universidad Nacional de La Plata.

REFERENCES