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Antibody Response to *Brucella* Outer Membrane Proteins in Bovine Brucellosis: Immunoblot Analysis and Competitive Enzyme-Linked Immunosorbent Assay Using Monoclonal Antibodies

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Sera from *Brucella*-infected bovines were analyzed by immunoblotting by using sonicated cell extracts of *B. melitensis* or *B. abortus* and a competitive enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies against outer membrane proteins (OMPs) with molecular masses of 10, 16.5, 19, 25 to 27, 36 to 38, and 89 kDa. Antibody responses against OMPs were compared with antibody responses against smooth lipopolysaccharide. Immunoblot analysis indicated that the antibody response in infected animals was largely different from one animal to another. The antigens of concern were OMPs with molecular masses of 10, 16.5, 19, 25 to 27, 36 to 38, and 89 kDa and other proteins with molecular masses of between 40 and 80 kDa. According to the specificity of the competitive ELISA, OMPs useful for the detection of infected animals are the OMPs of 10, 16.5, 19, 25 to 27, and 36 to 38 kDa. A competitive ELISA with the anti-89 kDa monoclonal antibody was not specific. Results of the competitive ELISA confirmed the individual variability of the humoral immune response against OMPs. It therefore seems that a combination of several protein antigens is necessary for the development of an immunoassay with a sensitivity comparable to that of the smooth lipopolysaccharide ELISA.

Brucellae are facultative intracellular bacteria which develop mainly in the reticuloendothelial system and occasionally in other target organs, such as joints and placenta, and can cause abortions in cattle. The major species involved in bovine brucellosis is *Brucella abortus*. In many parts of the world, vaccination of cattle is done by inoculating calves with *B. abortus* S-19, an attenuated strain (24). This strain, however, is antigenically similar to virulent strains of *B. abortus*. Serodiagnosis by conventional tests, which principally measure antibody to smooth lipopolysaccharide (S-LPS) (24), does not permit a clear-cut distinction between vaccinated and infected cattle. Other serological methods for the differentiation of vaccinated cattle from field strain-infected cattle have been proposed, such as the radial immunodiffusion test using poly B, a polysaccharide antigen initially prepared from *Brucella melitensis* (10), and an enzyme-linked immunosorbent assay (ELISA) using purified O chain of *B. abortus* (24). The radial immunodiffusion test, however, was insufficiently sensitive, and since then, poly B has been shown to be a cyclic glucan which is serologically inactive (3, 4). The precipitation reactions observed with sera from infected cattle were due to contaminating O polysaccharide in the poly B preparation. Thus, work actually performed in the field of bovine brucellosis identifies protective antigens and antigens useful for diagnosis. S-LPS has been shown to be a protective antigen in mice by passive protection experiments with monoclonal antibodies (MAbs) (8, 18, 20, 21, 25) and by active protection experiments with purified S-LPS (25, 32). MAbs against seven surface-exposed outer membrane proteins (OMPs) with molecular masses of 10, 16.5, 19, 25 to 27, 31 to 34, 36 to 38, and 89 kDa do not react with *Yersinia enterocolitica* O:9 or *Escherichia coli* O:157 (9; unpublished data). The OMPs of 25 to 27 and 36 to 38 kDa are the major *B. abortus* OMPs (13) and are also called group 3 and group 2 proteins, respectively (11, 22, 28, 30, 31). The 31- to 34-kDa OMP is a major OMP in *B. melitensis* strains but a minor one in *B. abortus* strains (12). These three major OMPs have been found to be tightly associated with the peptidoglycan (9, 29). Antibodies to OMPs have been found to be ineffective or less efficacious than S-LPS antibodies for the prevention *Brucella* infections in mice (7, 15, 21). The purpose of the present study was to investigate by immunoblot analysis and competitive ELISA using the anti-OMP MAbs the potential usefulness of these OMPs as diagnostic antigens.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the present study were obtained from the Institut National de Recherches Vétérinaires, Brussels, Belgium (*B. abortus* B3 and 45/20), and from the Institut National de la Recherche Agronomique, Nouzilly, Tours, France (*B. melitensis* B115).

Bacterial fractions. Cell walls (CWs) from *B. melitensis* B115 were extracted as described previously (13). Briefly, cells were inactivated by heating at 65°C for 1 h and were broken with glass beads in a Braun MSK homogenizer or a Dyno-Mill apparatus (W. A. Bachofen, Basel, Switzerland). Crude CWs were recovered by centrifugation (at 53,000 × *g*

and 4°C for 1.5 h). CWs were obtained by treating crude CWs with 1% Triton X-100 in 0.2 M NaCl–0.01 M $MgCl_2$ for 30 min at 20°C. After six washings in distilled water, the CWs were lyophilized.

Cell extracts (CEs) of *B. melitensis* B115 and *B. abortus* 45/20 were obtained by ultrasonication. Cells were inactivated by treating them with heat at 65°C for 1 h, washed three times in 0.9% NaCl, and ultrasonicated for 15 min in 1 mM EDTA–30 mM Tris (pH 8). The ultrasonicated cells were treated with lysozyme and centrifuged for 10 min at 4,000 × *g*, and the supernatant was recovered.

S-LPS fraction. The S-LPS fraction of *B. abortus* B3 (S-LPS of A > M specificity) was prepared by the phenol-water method (17).

Sera from *Brucella*-infected cattle. Serum samples were taken from a bull experimentally infected with *B. abortus* B3 (*Brucella* infectious dose, 2 × 10^{10} CFU) and naturally infected herds; the samples were provided by the Centre de Dépistage des Maladies du Bétail of Erpent. Animals from naturally infected herds were from a farm where brucellosis was confirmed by the isolation of a *Brucella* strain. Fourteen of the 20 animals were positive in classical serological tests (seroagglutination test, Rose Bengal plate test, and complement fixation test).

MAbs. Anti-OMP MAbs were produced as described previously (5). The anti-OMP MAbs used were the anti-10-kDa MAb A68/07G11/C10 (immunoglobulin G2a [IgG2a]); anti-16.5-kDa MAbs A68/04G01/C06 (IgG2a) and A68/29E03/C10 (IgG2a); anti-19-kDa MAb A68/25H10/A05 (IgG2a); anti-25- to 27-kDa MAbs A59/05F01/C09 (IgG2a), A68/04B10/A05 (IgG2a), and A68/28G06/C07 (IgG2a); anti-36- to 38-kDa MAbs A63/05A07/A08 (IgA) and A68/25G05/A05 (IgG2a); and anti-89-kDa MAb A53/10B02/A01 (IgG1).

The anti-bovine IgG MAbs used were the anti-IgG2 MAb 3H3 and the anti-IgG1 MAb 1C8; the anti-bovine IgG MAbs were a gift of J. J. Letesson (Facultés Universitaires Notre Dame de la Paix, Namur, Belgium).

Reagents. The following reagents were used: glycine-buffered saline (GBS; 0.17 M NaCl, 0.1 M glycine, 6 mM NaN_3 [pH 9.2]), GBS-EDTA-Tw (GBS containing 50 mM EDTA and 0.1% Tween 80 [final pH, 9.2]), citrate-phosphate buffer (0.051 M Na_2HPO_4, 0.024 M citric acid [pH 5]), NaCl-Tw (0.15 M NaCl, 0.01% Tween 20), Tris-buffered saline (TBS; 0.15 M NaCl, 10 mM Tris-HCl [pH 7.5]), Tw-TBS (TBS containing 0.05% [vol/vol] Tween 20), TBS–3% BSA (TBS containing 3% [wt/vol] bovine serum albumin [BSA]), Tw–TBS–1% BSA (Tw-TBS containing 1% [wt/vol] BSA), PBS-Tw (phosphate-buffered saline [PBS; pH 7.5] containing 0.05% Tween 20), and PBS–3% BSA (PBS containing 3% [wt/vol] BSA).

Antisera. Rabbit anti-mouse immunoglobulin serum was produced by repeated intradermal injections of 100 μg of mouse IgG. Rabbits were injected and bled every 2 weeks during several months. The best bleedings were pooled.

Peroxidase conjugation. Protein A (Sigma, St. Louis, Mo.) and anti-bovine IgG MAbs 3H3 and 1C8 were conjugated with horseradish peroxidase (Sigma) by a modification of the method of Nakane and Kawaoi (23) as described by Dubray and Limet (14). The protein/enzyme ratio (wt/wt) was 1:2.

Indirect ELISA. Bovine sera were assayed for antibody activity by solid-phase ELISA against S-LPS coated on microtiter plates (Greiner Labortechnic, Stuttgart, Germany) at a concentration of 1 μg/ml in fivefold-diluted GBS in water (19). Sera were diluted 50-fold in GBS-EDTA-Tw. Binding of the antibodies to S-LPS was visualized by using peroxidase-conjugated anti-bovine IgG MAbs 1C8 and 3H3 diluted in GBS-EDTA-Tw containing 2% fetal calf serum. Excess reagents between the different incubations were removed by five washings with NaCl-Tw. *o*-Phenylenediamine (0.4%; wt/vol) and 2 mM H_2O_2 in citrate-phosphate buffer were used to visualize peroxidase activity. The difference in light absorbance at A_{492} and that at A_{620} was read in a Twinreader Titertek spectrophotometer. Results were expressed as antibody units in comparison with a standard serum sample as described by Limet et al. (19). Briefly, the results of the ELISA were calculated by comparison with a calibration curve constructed with serial twofold dilutions of a reference serum from a chronically infected animal (nearly only IgG antibodies) that titrated 1,200 units in the complement fixation test. This serum was prepared by P. Dekeyser (Institut National de Recherches Vétérinaires, Brussels, Belgium). It was calibrated with the second international standard *B. abortus* serum, to which the arbitrary value of 1,000 IU/ml was assigned. The assay and calibration were performed as described by Alton et al. (1). Light absorbances were converted to units by interpolation.

Competitive ELISA. The antigens used to coat the microtiter plates consisted of CWs or sonicated CEs at a coating concentration of 20 μg/ml in fivefold-diluted GBS in water. Bovine sera diluted in GBS-EDTA-Tw (several dilutions or a 1/50 dilution) were incubated with these antigens for 2 h at 37°C. Anti-OMP MAbs were added at the appropriate dilution in GBS-EDTA-Tw. After 1 h of incubation at room temperature, binding of the anti-OMP MAbs was visualized by incubation for 1 h at room temperature with peroxidase-conjugated goat anti-mouse immunoglobulins (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1/1,000 in GBS-EDTA-Tw containing 2% fetal calf serum. Excess reagents between the different incubations were removed by five washings with NaCl-Tw. *o*-Phenylenediamine (0.4%; wt/vol) and 2 mM H_2O_2 in citrate-phosphate buffer were used to visualize peroxidase activity. The difference of light absorbance at A_{492} and that at A_{620} was read in a Twinreader Titertek spectrophotometer.

Immunoblot techniques. The protein antigens were separated in a 2001 vertical electrophoresis unit (LKB-Produkter AB, Bromma, Sweden) by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method originally described by Laemmli (16). After electrophoresis, the proteins were transferred to nitrocellulose by using a Transblot apparatus (Biolyon, Dardilly, France) and were electrophoresed at 12 V for 2 h. Unoccupied sites on the nitrocellulose membranes were blocked by a 30-min incubation in TBS–3% BSA at room temperature with agitation. The membranes were then successively incubated overnight at room temperature with hybridoma supernatants (anti-OMP MAbs) diluted 1/2 in Tw–TBS–1% BSA or bovine serum diluted 1/50 in Tw–TBS–1% BSA, 1 h with anti-IgG MAbs 1C8 and 3H3 at a concentration of 1 μg/ml for the bovine sera, 1 h with rabbit anti-mouse immunoglobulin serum diluted 1/250, and 1 h with peroxidase-conjugated protein A diluted 1/1,000 in Tw–TBS–1% BSA. Washings between incubation periods were performed with Tw-TBS. After two washings in Tw-TBS and two washings in TBS, the blots were developed by incubation at room temperature in a solution of TBS containing 0.06% (wt/vol) 4-chloro-1-naphthol (Bio-Rad, Richmond, Calif.) and 5 mM H_2O_2. The reaction was stopped by washing in distilled water.

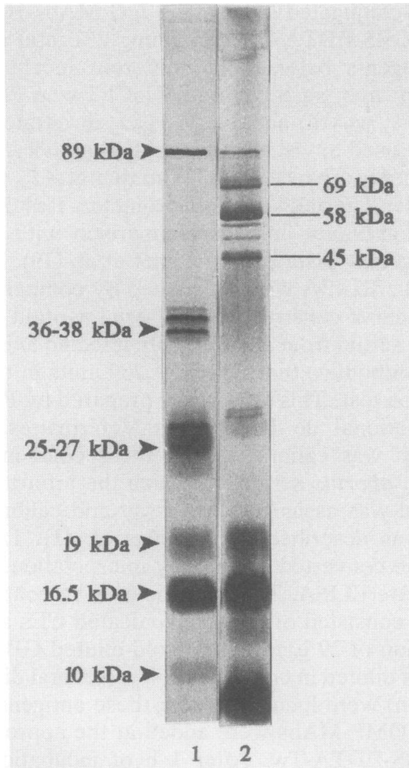

FIG. 1. Immunoblot of *B. melitensis* B115 CE with a mixture of anti-OMP MAbs (lane 1) (anti-10-kDa MAb A68/07G11/C10, anti-16.5-kDa MAb A68/04G01/C06, anti-19-kDa MAb A68/25H10/A05, anti-25- to 27-kDa MAb A59/05F01/C09, anti-36- to 38-kDa MAb A63/05A07/A08, and anti-89-kDa MAb A53/10B02/A01) and serum from a *B. abortus* B3-infected bull 6 months after infection (lane 2).

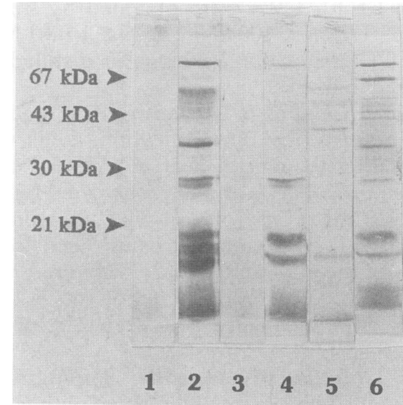

FIG. 2. Immunoblot of *B. abortus* 45/20 CE with serum taken before (lane 1) and 6 months after (lane 2) infection of a bull with *B. abortus* B3 and sera from naturally infected cows with anti-LPS antibodies (lanes 4 to 6) and with no detectable anti-LPS antibodies (lane 3).

RESULTS

Immunoblot analysis. (i) Experimentally infected animal. The CEs of *B. melitensis* B115 or *B. abortus* 45/20 rough strains were used to study the antibody response against proteins by immunoblotting. MAbs were used to identify the OMPs in these extracts. Figure 1 shows the antibody reactivity 6 months after infection against proteins of the *B. melitensis* B115 CE with serum from a bull experimentally infected with *B. abortus* B3. No protein bands were revealed in these extracts by serum taken just before infection. OMPs detected by the antibodies of this serum sample 6 months after infection were the minor OMPs with molecular masses of 10, 16.5, 19, and 89 kDa and the major OMP with a molecular mass of 25 to 27 kDa. Reactivity against the 25- to 27-kDa OMP was weak, however. No reactivity against the major OMP with a molecular mass of 36 to 38 kDa was found. The weakly stained band between 36 and 45 kDa corresponds to S-LPS, which can also be shown by using a S-LPS-specific MAb. Other proteins with high molecular masses with major bands at 45, 58, and 69 kDa were revealed by this serum sample. The heavily stained band below 10 kDa corresponds to rough LPS (R-LPS), which was also revealed by an anti-R-LPS MAb (data not shown). Similar results were observed with *B. abortus* 45/20 CE (Fig. 2), except that no band between 36 and 45 kDa was revealed in the smears and that in this extract a strong reaction was observed against the band at 36 to 38 kDa.

(ii) Naturally infected animals. As shown in Table 1 and Fig. 2, antibodies detected in sera from naturally infected cows were directed against the OMPs of 10, 16.5, 19, 25 to 27, 36 to 38, and 89 kDa and other proteins with molecular masses of between 40 and 80 kDa. However, the antibody response against the proteins varied from one animal to another. No clear-cut correlation was observed between the antibody response against proteins and the anti-LPS antibody titer in sera from these infected animals. Three animals (animals 3, 15, and 20) with no detectable anti-LPS antibodies showed an antibody response against the 25- to 27-kDa OMP and proteins with molecular masses of between 40 and 80 kDa. When using induction of anti-LPS antibodies as a criterion of infection, 17 of 20 animals could be considered positive for brucellosis. Among these animals, 14 presented antibody responses against one or several proteins.

ELISA. (i) Experimentally infected animal. Anti-OMP MAbs were selected by ELISA as a function of the inhibition of their binding on *Brucella* CWs or CEs by serum antibodies of a bull experimentally infected with *B. abortus* B3. Serum taken just before infection and sera from healthy cattle were tested under the same conditions to determine the specificity of the inhibition reaction. Inhibition of the binding of the MAbs that recognized the OMPs of 10, 16.5, 19, and 25 to 27 kDa was observed only with infectious sera (Fig. 3A to D). The inhibition of the anti-25- to 27-kDa MAb A68/04B10/F05 (Fig. 3B) was the weakest. With the anti-36- to 38-kDa MAb A68/25G05/A05, inhibition was already observed with the serum taken before infection, but inhibition of binding of the anti-36- to 38-kDa MAb was about 20 times greater with the serum of the infected bull (Fig. 3E). No difference between inhibition by serum taken before and that taken 6 months after infection was observed for the anti-89-kDa MAb A53/10B02/A01 (Fig. 3F). Another anti-89-kDa MAb was tested under the same conditions and gave the same result (data not shown). Sera taken from other noninfected animals were also tested with these anti-89-kDa MAbs and gave the same results (data not shown).

(ii) Naturally infected animals. The 20 serum samples from naturally infected cows analyzed by immunoblotting were tested in a competitive ELISA by using the anti-OMP MAbs described above, but the anti-89-kDa MAb was not used since no specific inhibition test could be observed with this

TABLE 1. Analysis by immunoblotting of sera from naturally infected cows

Animal no.	Classical test result[a]	Anti-LPS antibody (ELISA units)	Staining of bands with the following molecular mass (kDa):								Protein recognized by each animal
			<10	10	16.5	19	25–27	36–38	40–80	89	
3	–	–	±[b]	–	–	–	±	–	+	–	1
15	ND[c]	–	–	–	–	–	+	–	+	–	2
20	ND	–	++	–	+	–	+	–	+	–	4
1	ND	11.4	+	–	–	–	±	–	+	–	2
2	++++	>60	+++	+	+++	+++	+++	±	+	++	7
4	ND	2.9	–	–	–	–	–	–	–	–	0
5	+	8.5	–	–	–	–	–	–	+	–	1
6	++	>60	+	–	–	–	–	–	–	–	1
7	++++	>60	–	++	+	++	+	–	++	–	5
8	+	49.1	–	–	–	–	–	–	–	++	1
9	+++	>60	–	++	++	++	+	–	+	–	5
10	++	>60	–	+	–	+	–	–	+	+	4
11	+	>60	–	±	–	–	–	–	–	–	0
12	++	>60	–	+	+	+++	±	–	±	+++	4
13	++	>60	–	–	–	–	–	–	–	–	0
14	++	ND	+	–	±	±	–	–	+	+	3
16	–	4.6	–	–	–	–	–	±	+	++	2
17	++	>60	+	+	+	+	+	±	++	+	7
18	++	>60	±	+	+	+	+	–	+	–	5
19	+++	>60	+++	++	±	+++	++	++	++	+++	7
No. of animals recognizing one or more OMPs	14	17	9	9	9	9	11	4	15	8	17

[a] The classical tests were the seroagglutination test, the Rose Bengal plate test, and the complement fixation test.
[b] ±, weakly stained bands.
[c] ND, not determined.

MAb. The sera were tested at a 1/100 dilution to avoid nonspecific inhibition of the anti-36- to 38-kDa MAb A68/25G05/A05. The antibody response against the minor OMPs of 10, 16.5, and 19 kDa observed by immunoblot analysis was confirmed by competitive ELISA by using the MAbs; this was not the case for serum sample 12, however, which showed a reactivity for these OMPs in immunoblots but not in the competitive ELISA (Table 2). A more important antibody response against the major OMPs of 25 to 27 and 36 to 38 kDa was observed by competitive ELISA than by immunoblot analysis. For antibody responses against OMPs, the largest number of infected animals were detected by competitive ELISA by using the anti-25- to 27-kDa and anti-36- to 38-kDa MAbs, and as was the case for immunoblot analysis, sera from animals 3, 15, and 20 presented antibodies against the 25- to 27-kDa OMP, whereas they did not contain detectable anti-LPS antibodies. Of the 17 serum samples containing anti-LPS antibodies, 13 serum samples had antibodies against one or several OMPs.

DISCUSSION

In a previous study (6), seven surface-exposed *Brucella* OMPs were identified by using MAbs. The data presented in this report indicate the usefulness of these MAbs for identifying OMPs of diagnostic significance in cows with brucellosis. Immunoblot analysis of sera from *Brucella*-infected bovines indicated the presence of antibodies against the major OMPs with molecular masses of 25 to 27 and 36 to 38 kDa and minor OMPs with molecular masses of 10, 16.5, 19, and 89 kDa. Other proteins with molecular masses of between 40 and 80 kDa were recognized by these sera, yet the nature of these proteins is not known, i.e., whether they are CW or cytoplasmic proteins, since immunoblot analysis was performed with *Brucella* whole-cell sonicated extracts. The same results were obtained with *B. abortus* 45/20 or *B. melitensis* B115 CE, suggesting at least a partial immunological identity between *B. abortus* and *B. melitensis* proteins at least for the OMPs identified by the MAbs. However, whereas in an immunoblot analysis of *B. melitensis* B115 CE no reaction by serum from a bull experimentally infected with *B. abortus* B3 was observed against the 36- to 38-kDa OMP (porin), in *B. abortus* 45/20 CE a strong reaction was observed against this OMP by the same serum sample. This could be due to antigenic differences between the porin proteins of *B. melitensis* and *B. abortus* detected by the bovine serum but not by the MAb and/or to the well-established fact that the relative quantity of porin in rough strains of *B. melitensis* is much lower than that in rough strains of *B. abortus* (28). The antibody response against OMPs has already been studied by Riezu-Boj et al. (26, 27) for ovine brucellosis. Their results are partially in accordance with our results. They also observed by immunoblotting an antibody response against group 3 OMPs (25 to 27 kDa) and other surface-exposed proteins with molecular masses of 67, 22.5 to 21.5, and 19.5 to 18 kDa. The OMP of 19.5 to 18 kDa probably corresponds to the 19-kDa surface-exposed OMP described by us. We were able to demonstrate with our MAbs that group 3 OMPs consist, in fact, of a single protein which appears after immunoblotting as multiple bands by association of peptidoglycan subunits of different sizes (9). The same observation has been made for group 2 OMPs (porin proteins) or the 36- to 38-kDa OMP.

Immunoblot results of the antibody responses of most of the infected animals are in accordance with the competitive ELISA results for the 10-, 16.5-, and 19-kDa OMPs. Substantial differences between immunoblotting and competitive ELISA were observed for the antibody response against

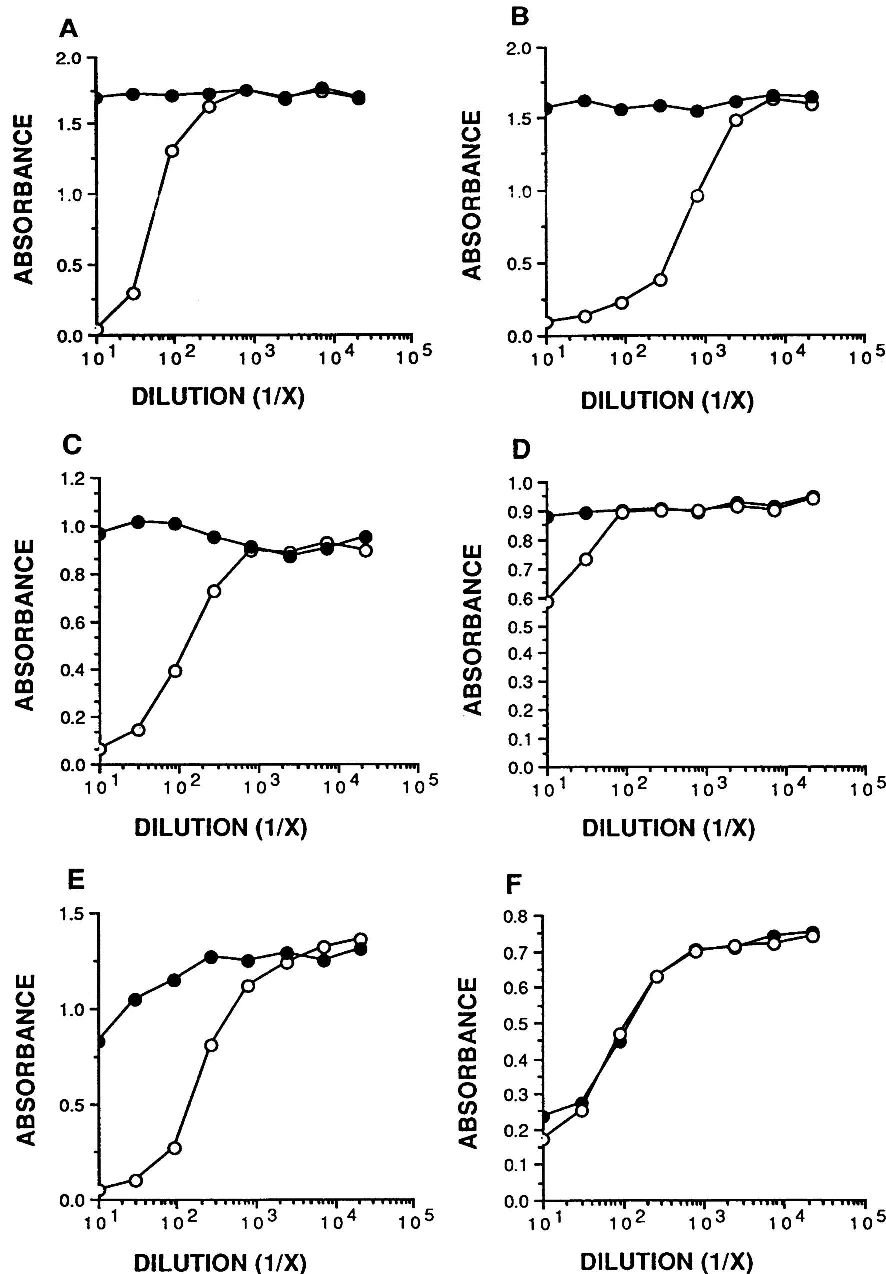

FIG. 3. Competitive ELISA between bull sera (several dilutions from 1/10 to 1/21,870) taken before (●) and 6 months after (○) infection with *B. abortus* B3 and the following anti-OMP MAbs (at one dilution): anti-10-kDa MAb A68/07G11/C10 (A), anti-16.5-kDa MAb A68/29E03/C10 (B), anti-19-kDa MAb A68/25H10/A05 (C), anti-25- to 27-kDa MAb A68/04B10/A05 (D), anti-36- to 38-kDa MAb A68/25G05/A05 (E), and anti-89-kDa MAb A53/10B02/A01 (F). The antigens used, according to the relative abundances of the OMPs determined by ELISA binding of the MAbs and to optimal conditions of competition, were *B. abortus* 45/20 CE for the anti-10-, anti-16.5-, anti-19-, and anti-36- to 38-kDa MAbs (A to E) and *B. melitensis* B115 CW for the anti-25- to 27- and anti-89-kDa MAbs (D and F). Binding of the MAbs was revealed by use of goat anti-mouse peroxidase-conjugated immunoglobulins.

the 36- to 38-kDa OMP (Tables 1 and 2). In contrast to the competitive ELISA with anti-36- to 38-kDa MAb A68/25G05/A05, in most of the sera tested no response was observed against this OMP by immunoblotting. The same antigen (CE of *B. abortus* 45/20) was used for the competitive ELISA and immunoblotting. A discordance between the immunoblotting and competitive ELISA results could be due to the fact that antibodies elicited by the 36- to 38-kDa OMP recognize conformational epitopes with low or no binding on the denatured protein form obtained after SDS-PAGE. Indeed, the anti-36- to 38-kDa MAb A68/25G05/A05 that we used, which recognizes a surface-exposed epitope, has a very weak reactivity in immunoblots but a strong one in ELISA (6). The same discordance was observed for the antibody response against the 25- to 27-kDa OMP. The anti-25- to 27-kDa MAbs used in the competitive ELISA also

TABLE 2. Comparison between anti-OMP titer and anti-LPS titer in naturally infected cows by ELISA[a]

Animal no.	Clasical tests[b]	Anti-LPS antibody (ELISA units)	% Inhibition						No. of proteins recognized by each animal
			10-kDa A68/07G11/C10	16.5-kDa A68/29E03/C10	19-kDa A68/25H10/A05	25–27-kDa A68/04B10/F05	25–27-kDa A68/28G06/C07	36–38-kDa A68/25G05/A05	
3	–					23.0		13.9	2
15	ND[c]						22.0		1
20	ND						26.8		1
1	ND	11.4							0
2	++++	>60	56.5	38.4	45.1		55.0	16.2	5
4	ND	2.9					26.9		1
5	+	8.5					35.5	16.2	2
6	++	>60							0
7	++++	>60	15.5	84.0	10.0	8.2	15.9	78.6	6
8	+	49.1				52.5		23.2	2
9	+++	>60	51.6	27.5	13.8	52.5		22.8	5
10	++	>60	53.5					30.5	2
11	+	>60	10.7			31.6		31.3	3
12	++	>60							0
13	++	>60				5.4	19.5		2
14	++	ND		14.5	32.2	31.2	34.3	24.0	5
16	–	4.6					31.7		1
17	++	>60		10.8	13.0	32.6	31.7	21.8	5
18	++	>60							0
19	+++	>60	86.7	80.6	65.5			21.6	4
Total no. of infected cows[d]	14	16	6	6	6	8	10	11	16

[a] The antigens used were the same as those described in the legend to Fig. 3.
[b] The classical tests were the seroagglutination test, the Rose Bengal plate test, the complement fixation test.
[c] ND, not determined.
[d] A total of 20 cows were tested.

showed weak reactivities in immunoblots (data not shown). Another explanation could be that antibodies against these OMPs are of the IgM isotype and, therefore, are not detected in immunoblots by the anti-bovine IgG MAbs used to reveal the binding of the anti-*Brucella* antibodies. The differences in infected cows detected by the two anti-25- to 27-kDa MAbs in the competitive ELISA could be due to the different epitopes recognized by these MAbs.

The antibody response against *Brucella* OMPs determined by ELISA has been studied by others (2, 27). The methods used, however, were based on an indirect ELISA with several antigenic preparations, with the problem of interfering anti-LPS antibodies which can bind to contaminating LPS. The competitive ELISA with anti-OMP MAbs avoids this interference, but inhibition by steric hindrance because of the binding of anti-LPS antibodies to the LPS of the antigenic preparations we used cannot be excluded. However, MAbs to S-LPS, R-LPS, and OMPs did not hinder the binding of peroxidase-conjugated anti-OMP MAbs (data not shown). Since MAbs are specific for one epitope, only the antibody response against this epitope can be measured. This may be the reason that one of the serum samples from a naturally infected cow (cow 12) showed reactivity in immunoblots against the 10-, 16.5-, and 19-kDa OMPs but was negative in the competitive ELISA with MAbs against these three OMPs.

The specificity of the competitive ELISA was controlled by the use of sera taken before experimental infection or sera from noninfected animals. The test was specific for the antibody responses against the OMPs of 10, 16.5, 19, and 25 to 27 kDa; less specific for the OMP of 36 to 38 kDa; and not specific for the OMP of 89 kDa. However, all MAbs used in the present study did not react with *E. coli* O:157 or *Y. enterocolitica* O:9 CE in the ELISA or by immunoblotting (9; data not shown) and thus seemed to be specific for *Brucella* spp. One or several epitopes of the 89-kDa OMP close to the epitope recognized by the anti-89-kDa MAb are probably not specific for *Brucella* spp., and binding of antibodies from noninfected animals to these epitopes may hinder the binding of the anti-89-kDa MAb to its epitope. Antibody reactivities of sera from noninfected animals against the 89-kDa OMP were, however, not observed in immunoblots (data not shown). Those antibodies might be of the IgM isotype, however.

Our results suggest that the antibody response to OMPs is different from one animal to another. A combination of several OMPs will be necessary for detection of all infected animals that have anti-LPS antibodies. This first approach to the study of the antibody response against *Brucella* OMPs in bovine brucellosis by immunoblot analysis and competitive ELISA indicates the potential usefulness of the OMPs of 10, 16.5, 19, 25 to 27, 36 to 38, and 89 kDa as diagnostic antigens. These OMPs have been cloned in *E. coli*, and expression is undertaken to develop an indirect ELISA, with the latter method being generally more sensitive and easier to set up.

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