Antibody response to the 89-kDa outer membrane protein of *Brucella* in bovine brucellosis

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Summary. The antibody response of cattle to the minor 89-kDa outer-membrane protein (OMP) of brucella was measured by indirect ELISA with the purified protein and compared with the antibody response to smooth lipopolysaccharide (S-LPS). Pre-incubating sera with sonicated cell extracts of *Escherichia coli* prevented the binding of antibodies from uninfected animals to the 89-kDa OMP, suggesting the presence of one or more cross-reactive epitopes on this protein. In cattle infected experimentally with *Brucella abortus*, the antibody response to the 89-kDa OMP was later and less intense than that to S-LPS. In naturally infected cattle, 68% of animals showing an antibody response to S-LPS also showed an antibody response to the 89-kDa OMP. Results indicate that specific epitopes of the 89-kDa OMP in combination with those of other OMPs could be useful for diagnosis of brucellosis in cattle.

Introduction

Brucellae are gram-negative facultative intracellular bacteria that can infect many species of animals and that localise mainly in the reticulo-endothelial system and occasionally in other target organs, such as joints and placenta. In cattle, the infection can cause abortion and infertility that result in high economic loss. The major species responsible for bovine brucellosis is *B. abortus* which can be controlled to some extent by vaccination of calves with *B. abortus* S-19, an attenuated strain. This strain is antigenically similar to virulent strains of *B. abortus* and conventional serological tests, such as the Rose Bengal plate test, milk ring test, complement fixation test and serum agglutination test, which principally measure antibody responses to smooth lipopolysaccharide (S-LPS),¹ do not permit precise differentiation of vaccinated from infected cattle. Thus, there is a need to identify other antigens as alternatives to S-LPS for the elaboration of new vaccines and diagnostic tests based on antigens specific for the infected state.

S-LPS has been shown to be a protective antigen in mice by passive protection experiments with monoclonal antibodies (MAbs)^{2–5} and by active protection experiments with purified S-LPS or O-polysaccharide.^{5–7}

Possible alternatives to S-LPS include the major *B. abortus* outer-membrane proteins (OMPs) of 36–38

kDa and 25–27 kDa.^{8–11} They are also called group 2 porin proteins and group 3 proteins respectively.^{12,13} These major OMPs were found to be tightly associated with peptidoglycan (PG)^{14,15} A lipoprotein covalently linked to PG has also been described as a major OMP.^{16–18} Other OMPs identified so far are the minor OMPs of 10, 16.5, 19, 31–34 and 89 kDa.⁸ All these OMPs are exposed on the cell surface, as demonstrated by immuno-electronmicroscopy.^{8,18} The 89-kDa OMP is probably one of the group 1 minor proteins of 88–94 kDa described by others.¹³ MAbs to the seven OMPs (10, 16.5, 19, 25–27, 31–34, 36–38 and 89 kDa) have been found to be ineffective or less efficacious than S-LPS antibodies for the prevention of *B. abortus* infections in mice.^{4,9}

Antibody responses to the 10; 16.5; 19; 25–27; 36–38; and 89-kDa OMPs have been studied previously in brucella-infected cattle by immunoblot analysis and competitive ELISA with MAbs against these proteins.¹⁰ However the binding of anti-89-kDa MAbs was inhibited by sera of healthy animals as well as by sera of brucella-infected animals. This lack of specificity has now been overcome and, in the present study, the specific antibody response to the 89-kDa OMP in experimentally and naturally infected cattle is described.

Materials and methods

Bacterial strains

The bacterial strains were obtained from the Institut National de la Recherche Agronomique, Nouzilly, Tours, France in the case of *B. melitensis* B115, and

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from the Institut National de Recherches Vétérinaires, Brussels, Belgium in the cases of *B. abortus* biovar 3, strain 7608), *B. abortus* biovar 9 (strain 7677-2) and *E. coli* O157.

Monoclonal antibodies (MAbs)

The anti-OMP, anti-rough-LPS (R-LPS) and anti-S-LPS MAbs used were produced as described previously.⁸

Sera from brucella-infected cattle

Serum samples were taken from a bull experimentally infected with 2×10^{10} cfu of *B. abortus* biovar 3 (strain 7608), from cows experimentally infected with 2×10^{10} cfu of *B. abortus* biovar 3 (strain 7608) or 10^7 cfu of *B. abortus* biovar 9 (strain 7677-2), and from 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 farms where brucellosis was confirmed by the isolation of a *Brucella* strain. These animals gave positive results in conventional serological tests (standard tube agglutination test, Rose Bengal plate test and complement fixation test).

LPS fraction

The S-LPS fraction of *B. abortus* biovar 3 (strain 7608) (S-LPS of A > M specificity) was prepared by the phenol-water method.¹⁹

Purification of the 89-kDa OMP

The 89-kDa OMP was purified from rough *B. melitensis* strain B115 cell walls prepared as described previously.¹¹ *B. melitensis* B115 SDS-soluble cell-wall proteins were obtained by boiling 2 g of cell-wall material in 100 ml of SDS 4% solution for 10 min. The proteins were first precipitated by adding two volumes of acetone and recovered by centrifugation (4000 g, 20 min, 20°C) followed by solubilisation in 3 ml of the Laemmli sample buffer for SDS-PAGE. Proteins were separated in 3-mm thick gels and bands were revealed rapidly by copper staining.²⁰ The 89-kDa band was cut from the gels, electro-eluted, dialysed and lyophilised. For large scale preparation, the 89-kDa OMP was purified by gel-filtration. A Sephacryl S200 HR column (85 × 2.5 cm) was equilibrated in 0.1 M ammonium bicarbonate, pH 8, containing SDS 0.1%. Samples of 10–20 mg of SDS-soluble proteins in 2 ml of Laemmli sample buffer containing 1 mM dithiothreitol were boiled for 5 min at 100°C, applied to the column and eluted at a flow rate of 16 ml/h. The eluate was monitored spectrophotometrically at 280 nm and 3-ml fractions were collected. Samples along the first major peak were analysed by SDS-PAGE and silver staining.²¹ The fractions containing the 89-kDa OMP were pooled, lyophilised, resolubilised as above in one-tenth of the collected volume, and re-chromatographed on the same column. Purity of the 89-kDa

OMP was checked by SDS-PAGE and silver staining and by indirect ELISA with an anti-89-kDa MAb and MAbs specific for the other OMPs (10, 16.5, 19, 25–27, 31–34 and 36–38 kDa), S-LPS and R-LPS.

ELISA

ELISA was performed as described previously.^{8,10} S-LPS of *B. abortus* biovar 3 (strain 7608) at a concentration of 1 µg/ml or a mixture of the purified 89-kDa OMP and bovine serum albumin, both at a concentration of 1 µg/ml in glycine-buffered saline (GBS), pH 9.2, were coated on microtitration plates (Greiner Labortechnik-Stuttgart) by overnight incubation at 37°C. After incubation for 1 h, binding of MAbs (hybridoma supernates) or antibody in the bovine sera was revealed by incubation for 1 h more with peroxidase-conjugated goat anti-mouse immunoglobulins (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) or with peroxidase-conjugated anti-bovine IgG1 MAb 1C8 respectively.²² Before ELISA, diluted bovine sera were pre-incubated with sonicated *E. coli* O157 cells (absorbance 1.0 in PBS) for 2 h at 37°C. Some samples were omitted from the pre-incubation stage, for comparison.

Results

Purity of the 89-kDa OMP

Purity of the 89-kDa OMP was checked by SDS-PAGE and silver staining (fig. 1) and by indirect ELISA with an anti-89-kDa MAb and MAbs specific

Fig. 1. Silver staining after SDS-PAGE of SDS-soluble proteins of *B. melitensis* B115 cell wall at 2.5 µg (lane 1), 1.25 µg (2), 0.6 µg (3), and purified 89-kDa OMP obtained by gel filtration (4).

Fig. 2. Binding of anti-89-kDa MAb A53/10B02/A01 (○) and MAbs specific for other OMPs (see text), R-LPS and S-LPS (●) measured by ELISA in wells coated with purified 89-kDa OMP.

Fig. 3. Binding, measured by ELISA, to the 89-kDa OMP-coated wells of antibodies from serum (at 1 in 100 dilution) of a bull taken before (day 0) and 7, 14, 21 and 180 days after infection by *B. abortus* biovar 3 with (□) or without (■) pre-incubation of the serum with *E. coli* O157 sonicated cell extracts.

Fig. 4. ELISA with serum from a bull infected experimentally with *B. abortus* biovar 3 taken 6 months after infection, pre-incubated with *E. coli* O157 sonicated cell extracts, with S-LPS (●) and purified 89-kDa OMP (○) as coating antigens.

for the other OMPs, S-LPS and R-LPS. Only the anti-89-kDa MAb bound to the purified 89-kDa OMP (fig. 2), suggesting the absence of significant contamination by the 10; 16.5; 19; 25–27; 31–34; and 36–38-kDa OMPs, S-LPS or R-LPS.

Fig. 5. Evolution of antibody response against S-LPS and 89-kDa OMP in heifers infected experimentally with brucella. Sera pre-incubated with *E. coli* O157 sonicated cell extracts were tested at 1 in 100 dilution. Open symbols: anti-LPS antibodies; closed symbols: anti-89-kDa antibodies. (a) Heifers infected with 2×10^{10} cfu of *B. abortus* biovar 3 in the conjunctiva; (b) heifers infected with 10^7 cfu of *B. abortus* biovar 9 in the conjunctiva.

Antibody response in cattle infected experimentally with *B. abortus*

The specific antibody response to the 89-kDa OMP was measured by ELISA with sera from a bull taken before or 7, 14, 21 and 180 days after infection by *B. abortus* biovar 3. In contrast to immunoblotting, with which a specific antibody response against the 89-kDa OMP was observed,¹⁰ ELISA required pre-incubation of sera with *E. coli* O157 sonicated cell extracts to inhibit binding of non-specific antibodies (fig. 3). The antibody titre against the 89-kDa OMP was lower than the titre against S-LPS (fig. 4). Other sera from animals infected experimentally with *B. abortus* were tested for antibody response to the 89-kDa OMP and S-LPS by indirect ELISA. The antibody response to the 89-kDa OMP was weaker than the antibody response to S-LPS and was delayed by up to several weeks depending on the infection conditions (fig. 5).

Antibody response in healthy cattle and cattle infected naturally with brucella

When pre-incubated with *E. coli* O157 sonicated cell extracts, negative control sera from 30 healthy animals

Table. Antibody response determined by ELISA* against S-LPS and the 89-kDa OMP in naturally infected and healthy cattle

Animals	S-LPS response		89-kDa OMP response	
	Absorbance range	Number of animals	Absorbance range	Number of animals
Brucella infected	1.8–2.0	27	1.0–1.2	5
	1.6–1.8	15	0.8–1.0	2
	1.4–1.6	3	0.6–0.8	7
	1.2–1.4	2	0.4–0.6	10
	1.0–1.2	2	0.2–0.4	9
	< 1.0	1	0.1–0.2	1
Healthy†	0.046 (0.021)‡		0.048 (0.023)	

* Sera were tested at 1 in 100 dilution.

† Absorbance values of infected animals below the mean absorbance + 3 SD of these values were considered to be negative.

‡ Mean absorbance value of 30 negative control sera (SD).

showed no reactivity against either S-LPS or the 89-kDa OMP. Among the 50 anti-S-LPS-positive sera from infected animals, 34 (68%) showed variable reactivity against the 89-kDa OMP in indirect ELISA (table). However, the absorbances observed were lower than those observed for the anti-S-LPS antibodies.

Discussion

The 89-kDa OMP was purified from cell walls of *B. melitensis* B115 to study the antibody response in infected cattle. This rough strain is currently used for extraction of R-LPS^{8, 23} and protein antigens^{8, 10, 14, 24–26} for testing humoral and cellular immune responses of cattle and small ruminants.

The antibody response to S-LPS has been studied extensively, and it has been shown that the polysaccharide moiety of this molecule bears the main antigenic determinants involved in the standard serological tests for smooth brucellae.¹ Antibody responses against purified OMPs in animals infected with brucella have been reported for the lipoprotein described by Gomez-Miguel *et al.*¹⁷ They found a serological cross-reaction between the lipoproteins of *Brucella* spp. and *E. coli* but considered that the anti-brucella lipoprotein antibody detected in infected animals could not be the result of antigenic stimulation by *E. coli*. A correlation between the antibody response against the lipoprotein and S-LPS was observed which demonstrated that the anti-lipoprotein antibody was related to brucella infection and not to infections with

other gram-negative bacteria. In contrast with their results, to detect a specific antibody response with the purified 89-kDa OMP in our study, sera had to be pre-incubated with *E. coli* cell extracts, suggesting the presence of epitopes of this OMP cross-reactive with *E. coli* antigens. However, MAbs specific for the 89-kDa OMP did not show reactivity against *E. coli* antigens in immunoblotting and ELISA (data not shown) and probably recognised brucella-specific epitopes. Therefore, inhibition of these MAbs in competitive ELISA¹⁰ by sera from healthy animals was probably due to cross-reactive epitopes similar enough to those recognised by the anti-89-kDa MAbs to prevent their binding to the 89-kDa OMP.

As in the case of the lipoprotein, the antibody response to S-LPS was always more intense than the antibody response to the 89-kDa OMP. MAbs specific for the 10; 16.5 and 19-kDa minor OMPs have been used to develop a competitive ELISA.¹⁰ When applied to the sera of 20 animals from an infected farm, these tests detected antibodies in six of the 17 sera containing anti-LPS antibodies. The indirect ELISA against the 89-kDa OMP detected antibodies in 13 of these sera, again *c.* 65% of sera containing anti-LPS antibodies.

These data indicate that specific epitope(s) of the 89-kDa OMP in combination with those of other OMPs¹⁰ merit further evaluation for the diagnosis of brucellosis in cattle.

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