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Enzyme-Linked Immunosorbent Assay with Partially Purified Cytosoluble 28-Kilodalton Protein for Serological Differentiation between *Brucella melitensis*-Infected and *B. melitensis* Rev.1-Vaccinated Sheep

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The problem of differentiating sheep infected with *Brucella melitensis* from those vaccinated or exposed to cross-reacting organisms has not been resolved by conventional serological tests or through the use of the smooth lipopolysaccharide in primary binding assays. We therefore analyzed sera from ewes experimentally infected with *B. melitensis* H38, from ewes naturally infected with *B. melitensis*, and from *B. melitensis* Rev.1-vaccinated ewes by enzyme-linked immunosorbent assay with three antigenic fractions: O polysaccharide, a cytosoluble protein extract (CPE) from the rough strain *B. melitensis* B115, and a partially purified cytosoluble protein of 28 kDa (CP28) from the CPE. Immunoglobulin G anti-O polysaccharide and anti-CPE responses were detected in all groups of animals tested (Rev.1 vaccinated and *B. melitensis* infected). However, false-positive reactions with CPE occurred with sera from *Brucella*-free ewes. The use of partially purified CP28 abolished these false-positive reactions. Furthermore, no immunoglobulin G antibodies against CP28 were detected in sera from vaccinated ewes, whereas 80% (8 of 10) of ewes experimentally infected with *B. melitensis* H38 and 89% (25 of 28) of naturally infected ewes showed various degrees of anti-CP28 reactivity (absorbance values of between 0.5 and 2.5). The results obtained with CP28 showed the potential usefulness of this antigen to permit the detection of *B. melitensis*-infected ewes and their differentiation from *B. melitensis* Rev.1-vaccinated ones.

Ovine brucellosis caused by *Brucella melitensis* is characterized by abortion and early embryonic death in the female and testicular atrophy in the male (1). Standard serological tests (Rose Bengal plate test, agglutination, antiglobulin [Coombs], and complement fixation test), which principally measure antibody to smooth *Brucella* lipopolysaccharide (LPS), have been used routinely for diagnosis (2, 4), but the most difficult task has been to distinguish antibodies of infected animals from those of vaccinated animals. To date, subcutaneous *B. melitensis* Rev.1 vaccination of ewes is done in many parts of the world (5), and it induces high-level antibody responses in all of the standard serological tests (6, 11). However, when young lambs (3 to 8 months) are given the standard dose of Rev.1 vaccine conjunctivally, the serological response is greatly curtailed without a significant reduction in the immunity conferred (6, 11). These differences were less evident in adult vaccinated sheep, so vaccination should be restricted to young animals. In addition, in sheep, infection with *Yersinia enterocolitica* O:9 is likely to cause a cross-reaction in serological tests with smooth *Brucella* antigens in the same way as in cattle (3, 14).

With regard to ovine brucellosis, we have previously attempted to identify the principal polypeptide specificities of the antibody response to protein extracts of *B. melitensis* by using either immunoblotting or competitive enzyme-linked immunosorbent assay (ELISA) with anti-outer membrane protein monoclonal antibodies (16, 18, 21–23). However, antibody responses to outer membrane proteins in *B. melitensis*-infected sheep were low and heterogeneous (18, 23). Therefore, we have focused our research on the identification of immunogenic cytosoluble proteins. In a previous study (16), we identified by immunoblotting five protein bands (of 19, 24, 28, 32, and 54 kDa) from a cytosoluble protein extract (CPE) of *B. melitensis* B115 (rough) that differentiated sera from ewes naturally infected with *B. melitensis*, ewes experimentally infected with *B. melitensis* H38, and conjunctivally Rev.1-vaccinated ewes. Among these proteins, the 28-kDa antigen was the one recognized soonest (at 21 days) after experimental infection and was found to provoke the most consistent humoral responses (100%) during natural infection, and it thus seemed to be the most meaningful to focus upon. We report in this article that this protein could be used in a standard ELISA system to measure antiprotein reactivity in sera from *B. melitensis*-infected ewes and to differentiate between Rev.1-vaccinated ewes and ewes either experimentally infected with *B. melitensis* H38 or naturally infected with field strains.

MATERIALS AND METHODS

Bacterial strain. Rough *B. melitensis* B115 cells were grown in a 20-liter fermentor by the method of the U.S. Department of Agriculture (2).

Antigens. The CPE from *B. melitensis* B115 was prepared as described previously (21, 22).

A cytosoluble protein of 28 kDa (CP28) was purified from the CPE of *B. melitensis* B115 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14% polyacrylamide, 1.5-mm thickness) with a Bio-Rad (Ivry sur Seine, France) apparatus. A preparative comb with one reference lane for standards for estimation of apparent molecular mass was used. For protein isolation, 1 mg of protein of CPE in Laemmli buffer (12) was loaded. Electrophoresis was performed at 40 mA per gel for 4 h. The end sections on either side of the gel were cut, stained with Coomassie brilliant blue G (15), and used as a template to excise horizontal strips containing the proteins of interest. Proteins were elec-

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troeluted from the cut gels by using a model electroeluter (Genofit, Geneva, Switzerland) as described by the manufacturer. The eluted protein was stored at −20°C until used.

O polysaccharide (OPS) of *B. melitensis* 16M was purified as described previously (19, 20).

Protein determination. Concentrations of eluted proteins were determined by using the bicinchoninic acid protein determination assay (Pierce Chemical Co., Rockford, Ill.) according to the manufacturer's instructions.

Sera. Sera were collected from 10 ewes conjunctivally vaccinated, at the age of 3 months, with 10^9 CFU of *B. melitensis* Rev.1 vaccine as described by Fensterbank et al. (7). Blood was taken at 4, 13, 33, 36, and 40 weeks postvaccination. All ewes were reared at our station (PII unit, Institut National de la Recherche Agronomique, Nouzilly, France) and monitored for freedom from *Brucella* species by serology (complement fixation test) and bacteriology (2) prior to experimental vaccination with *B. melitensis* Rev.1.

Sera were collected from 10 12-month-old ewes experimentally infected by the conjunctival route, when 133 to 135 days pregnant, with 5.2×10^7 CFU of *B. melitensis* H38. Sera were collected at 11, 15, 18, 21, 25, 28, 32, 35, 39, 46, 53, 60, and 81 days postinfection. These ewes were bacteriologically positive for brucellosis.

Sera from 28 naturally infected sheep were collected from a naturally infected, nonvaccinated flock in an area where *B. melitensis* is endemic (they were a gift from A. Gomez Ferreira, Evora, Portugal). These sera were positive for *Brucella* antibodies in the Rose Bengal plate test and the complement fixation test as described by Alton et al. (2).

Negative control sera were taken from 26 *Brucella*-free sheep from the brucellosis-free flock of the Animal Production facilities, PII unit, Institut National de la Recherche Agronomique. This flock has been officially free of brucellosis for over 25 years.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were performed as described previously (16, 23).

ELISA. Sera were tested for antibody activity in an indirect ELISA. Ninety-six-well polystyrene plates (Greiner, Labortechnic, Frickenhauser, Federal Republic of Germany) were coated by passive adsorption of the antigens OPS, CPE, and eluted CP28 at a concentration of 3, 20, and 0.5 μg/ml, respectively (100 μl per well), diluted in phosphate-buffered saline (PBS) (pH 7.2), for 18 h at room temperature. The wells were emptied and washed five times with 0.15 M NaCl–0.01% Tween 20 (NaCl-T), and then nonspecific binding sites of wells were blocked with a solution of PBS–0.05% Tween 20 (PBS-T) and 0.5% gelatin (Difco, Detroit, Mich.) and washed with NaCl-T. Sera were tested at a dilution of 1/100 in PBS-T, and 100-μl samples were applied in triplicate. Following an incubation of 90 min at 37°C, the wells were again washed and filled with 100 μl of horseradish peroxidase-labeled rabbit anti-sheep immunoglobulin G (IgG) (heavy and light chain specific; Jackson Laboratories) diluted 1/5,000 in PBS-T. After incubation for 60 min at room temperature, the conjugate solution was discarded and the plates were washed with NaCl-T. The wells were filled with 100 μl of substrate solution containing 1 mM ABTS [2,2-azino-di-(3-ethylbenzthiazoline-sulfonic acid)] plus 4 mM H_2O_2 in 50 mM sodium citrate (pH 4.2), and the plates were then shaken continuously at room temperature for 1 h. A_{415} values were then recorded with a Titertek Multiscan MC plate reader (Flow Laboratories, Les Ulis, France) which was interfaced with a computer. For tests of anti-CP28 reactivity, the optimal ELISA conditions were determined by testing one highly positive serum, one weakly positive serum, and one negative control serum, which were diluted in PBS-T (1/50 to 1/6,400) in plates coated with antigen (CP28) at concentrations ranging from 4 to 0.125 μg/ml and conjugate (horseradish peroxidase-labeled rabbit anti-sheep IgG) at a dilution of 1/5,000. Those conditions under which differences in the mean A_{415} values of positive and negative control sera were largest were considered optimal. The optimal conditions were 0.5 μg/ml for the antigen concentration and 1/100 for serum dilution. Sera giving absorbance values of greater than 20% of that of the most reactive negative serum were considered positive.

RESULTS

Partial purification of CP28. Coomassie brilliant blue staining revealed the separation of a fraction enriched for the 28-kDa protein compared with the unfractionated CPE (Fig. 1), which showed a large number of cytosoluble protein bands of between 14.2 and 66 kDa. CP28 contained protein bands of between 26 and 29 kDa.

Immunoblotting of the CPE and partially purified CP28 with sera from the *Brucella*-free ewes showed antibody reactivity of these sera to protein bands of 39 and 50 kDa of the CPE but no antibody reactivity to the CP28 bands (Fig. 1). Immunoblotting of CPE and CP28 with serum from one naturally infected ewe (selected in a previous study from 26 naturally infected ewes [16]), which displayed prominent reactivities against a large number of CPE proteins (19 to 54 kDa), showed

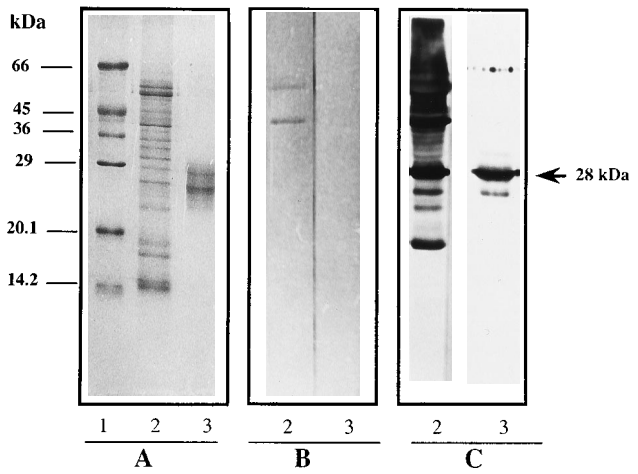

FIG. 1. SDS-PAGE and Coomassie blue staining of CPE and CP28 (A) and immunoblot analysis with serum from either a *Brucella*-free ewe (B) or a naturally *Brucella*-infected ewe (C). Lane 1, molecular mass markers; lanes 2, CPE; lanes 3, CP28.

a strong antibody reactivity against the 28-kDa protein band of CP28. Faint antibody reactivities against bands located at 26 and 29 kDa were also visible.

ELISA with OPS, CPE, and CP28 fractions. (i) Ewes experimentally infected with *B. melitensis* H38. By the OPS indirect ELISA, the sera taken before infection from 10 ewes experimentally infected with *B. melitensis* H38 were all negative. An IgG anti-OPS antibody response was detected by 11 days after experimental infection (Fig. 2A), and this response remained positive for the test period; the antibody reactivities reached, between 15 and 46 days after infection, absorbance values of between 2.0 and 2.5. Only one serum showed weak antibody reactivity to OPS (absorbance values of between 0.5 and 1.3).

In contrast, with the CPE antigen, all sera collected before infection showed a high background IgG response that could not be differentiated from the postinfection antibody response (data not shown).

Last, none of the preinfection sera showed an anti-CP28 response as determined by ELISA with CP28 as the coating antigen (Fig. 2B). Although there were obvious but minor individual differences in the timing of the rise in antibody reactivities, the antibody response of ewes experimentally infected with *B. melitensis* H38 generally increased early, about 21 days after experimental infection. However, 2 of the 10 ewes failed to react serologically, and 1 ewe exhibited increasing ELISA reactivity to CP28 only at day 46 after infection (Fig. 2B). Also, the antibody response against CP28 was lower and delayed in comparison with that against OPS (except for one animal, represented by the large solid triangle in Fig. 2).

(ii) *Brucella*-free ewes and ewes naturally infected with field strains. Of the 26 *Brucella*-free ewes, none was positive for the CP28 or OPS antigen (absorbance values were below 0.1) (Fig. 3). With the CP28 antigen, the sera from the 28 naturally infected ewes showed antibody reactivities with absorbance values that ranged from 0.383 to 2.556 (Fig. 3). Only sera giving absorbance values of greater than 0.5 were considered positive. Eighty-nine percent (25 of 28) of these sera were positive. Most absorbance values were higher in the indirect ELISA with the OPS antigen. Antibody reactivity against CPE was not tested, since as described above for *Brucella*-free ewes prior to experimental H38 infection, their sera showed high-level antibody reactivities.

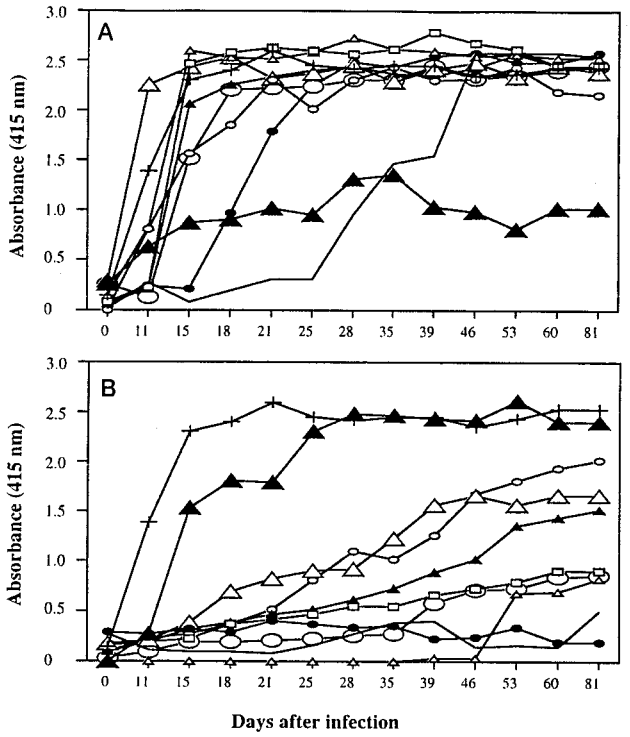

FIG. 2. Evolution of antibody responses against OPS (A) and CP28 (B) in 10 ewes experimentally infected with *B. melitensis* H38. Each point is the mean of triplicate determinations for each ewe. The 10 sera tested are represented by 10 different symbols.

(iii) *B. melitensis* Rev.1-vaccinated ewes. The time courses of the antibody responses against OPS and CP28 for each of the 10 Rev.1-vaccinated ewes tested are shown in Fig. 4. All ewes showed strong OPS antibody responses which increased during 35 days following vaccination, finally reaching absorbance values of between 1.6 and 2.5. This response declined thereafter, especially in five vaccinated animals, but still remained positive during the test period, except for one animal which became negative by 175 days after vaccination (Fig. 4A).

The sera of the 10 Rev.1-vaccinated ewes did not react with CP28 (Fig. 4B) at any time after vaccination.

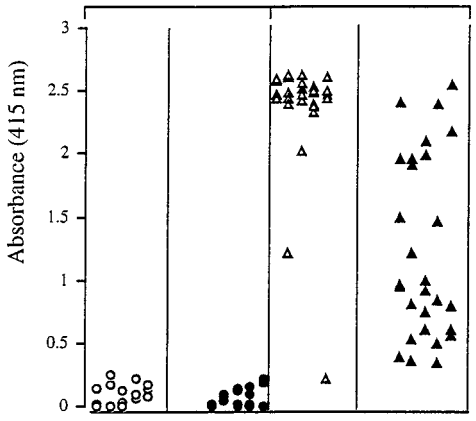

FIG. 3. Antibody reactivities in ELISA of sera from *Brucella*-free ewes (circles) (n = 26) and naturally infected ewes (triangles) (n = 28) to OPS (open symbols) and CP28 (closed symbols).

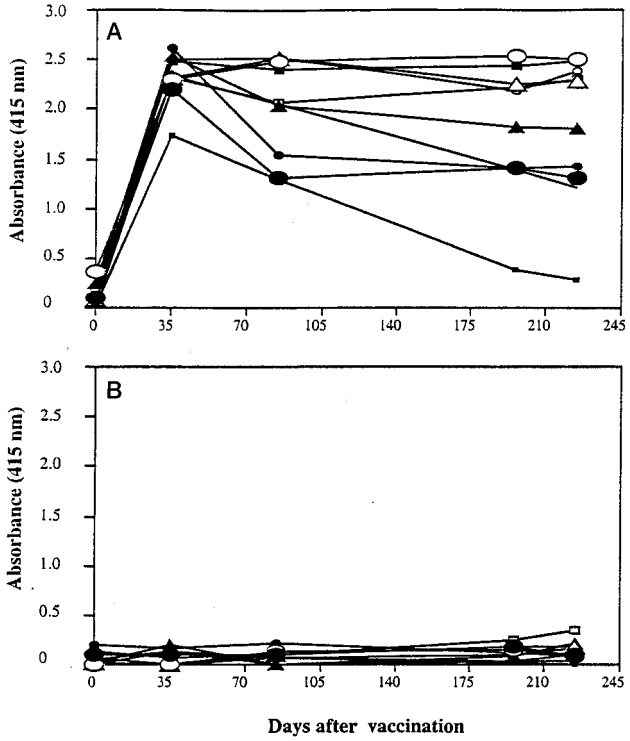

FIG. 4. Evolution of antibody responses against OPS (A) and CP28 (B) in 10 *B. melitensis* Rev.1-vaccinated ewes. Each point is the mean of triplicate determinations for each ewe. The 10 sera tested are represented by 10 different symbols.

DISCUSSION

Our results showed that an ELISA with the CP28 antigen could differentiate antibody responses of Rev.1-vaccinated ewes from those of ewes either naturally or experimentally infected with *B. melitensis*. This suggests that the antibodies of ewes infected with virulent *B. melitensis* differ qualitatively from those of *B. melitensis* Rev.1-vaccinated ewes by their specificity and/or affinity for CP28. Because of a large amount of smooth LPS on the strains, both virulent *B. melitensis* H38 and vaccine strain Rev.1 induce a high-level anti-OPS antibody response. Indeed, the ELISA with the OPS antigen was unable to differentiate infected ewes from those vaccinated with strain Rev.1. Nevertheless, in contrast to what was seen with ewes experimentally infected with *B. melitensis* H38, the anti-OPS antibody response declined by 75 days in five Rev.1-vaccinated ewes.

In preliminary experiments that used CPE in the assay, all preimmune sera from Rev.1-vaccinated ewes and ewes experimentally infected with H38 showed a background reactivity higher than expected, but this problem could not be solved by varying the conditions of the ELISA. Immunoblotting results for the *Brucella*-free ewes showed antibody reactivities to the protein bands of 39 and 50 kDa, suggesting the presence of nonspecific epitopes on these proteins, which may explain the antibody reactivities to CPE observed in the ELISA. These results demonstrate that difficulties may be encountered when mixtures of proteins are used as antigens in the ELISA. Thus, the isolation and characterization of specific antigens were required for the development of a more specific diagnostic test. Immunoblotting previously conducted in our laboratory (16) showed that the 28-kDa protein band was the immunodomi-

nant CPE antigen in either natural infections or experimental infections with *B. melitensis* H38 in sheep. The ELISA results confirmed the potential value of CP28 for the specific detection of *B. melitensis*-infected animals.

The antibody response against CP28, however, was always less intense and more heterogeneous than the antibody response against OPS. In addition, the IgG antibody response against OPS preceded that against CP28 in all of the ewes experimentally infected with H38, which confirmed that smooth LPS is the major immunodominant antigen in smooth *B. melitensis* infections (5, 12, 20). Nevertheless, the CP28 antigen seems to be a better diagnostic antigen than previously reported purified protein antigens such as the 89-kDa outer membrane protein (13), outer membrane lipoprotein (9), Cu-Zn superoxide dismutase (17), and a 17-kDa antigen (8, 10). Antibody responses to these proteins in infected cattle and/or sheep were considerably lower than those against CP28 in infected sheep. The usefulness of CP28 as a diagnostic antigen in bovine brucellosis has yet to be determined.

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