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A 37-Kilodalton Glycoprotein of *Babesia divergens* Is a Major Component of a Protective Fraction Containing Low-Molecular-Mass Culture-Derived Exoantigens

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The supernatants of in vitro cultures of *Babesia divergens* Rouen 1987 in human erythrocytes, obtained by using a semidefined medium based on human high-density lipoproteins, were fractionated by gel filtration chromatography into four fractions, F_1 to F_4. The crude supernatant as well as each fraction adjuvanted with Quil-A protected gerbils from mortality due to a homologous infectious challenge. Analysis of the humoral response of the 10 protected gerbils with fraction F_4, containing major proteins with molecular masses lower than 50 kDa, showed that a few antigens (from 50 to 17 kDa) could be important candidates for an improved vaccine against *B. divergens* babesiosis. As an immunodominant response was directed against the 37-kDa antigen (Bd37) in two different *B. divergens* strains tested, a polyclonal antibody directed against Bd37 was produced in a rabbit. In an immunofluorescence assay, the anti-Bd37 antiserum strongly labelled small internal vesicles of the merozoites and the cell surface was diffusely labelled after fixation, whereas on live merozoites, this labelling was not observed. [^{3}H]glucosamine-radiolabelling experiments demonstrate that Bd37 is a glycoprotein. The Bd37 protein can also be labelled with [^{14}C]palmitate but not with [^{3}H]myristic acid. In Triton X-114 temperature phase partitioning of *B. divergens*-infected erythrocyte extracts, Bd37 was exclusively found into the detergent phase, indicating that the palmitoylated Bd37 protein was in the membrane fraction. In the in vitro supernatant, the glycoprotein Bd37 was found in a nonpalmitoylated form, indicating excretion and/or release of the glycoprotein from the merozoite.

Most of the bovine babesiosis in western Europe is due to the intraerythrocytic development of the parasitic protozoan *Babesia divergens* (18). The economic loss due to *B. divergens* bovine babesiosis justifies the cost of a vaccination program (13). Attempts to develop a vaccine against *B. divergens* have been emphasized by the development of long-term in vitro culture (11) and by the production of the crude exoantigens in a semidefined medium containing human high-density lipoproteins (HDL) (38). The use of crude culture-derived exoantigens from a strategy to develop a vaccine against *Babesia* species has been widely used and has successfully reduced the development of infections due to *B. bovis* (19, 26, 27, 35), *B. bigemina* (27), and *B. canis* (25, 28, 29). We demonstrated in previous vaccination trials that the crude culture-derived exoantigens of *B. divergens* induced a well-characterized humoral response apparently correlated with protection in both homologous and heterologous challenges in gerbils (12, 31). For instance, analysis of this humoral response allowed the characterization of the heat shock proteins (especially hsp70) and a 17-kDa merozoite membrane protein as potential interesting immunogens (5, 32). Furthermore, crude and partially purified culture-derived exoantigens were able to protect oxen against babesiosis by inducing both a humoral and cellular immune responses in this natural host of *B. divergens* (37).

In the present work, fractionation of the crude in vitro culture-derived exoantigens of *B. divergens* collected in the HDL semidefined medium was performed by gel filtration chromatography. We demonstrated that the crude exoantigens as well as the fractions were immunogens and were able to protect gerbils from death due to babesiosis. As the simplest protective fraction, named fraction F_4, mainly contained antigens with molecular masses lower than 50 kDa, a detailed analysis of the humoral response was performed by using sera from 10 gerbils protected with fraction F_4. This study showed an immunodominant response against a 37-kDa antigen (Bd37) on both *B. divergens* Rouen 1987 and *B. divergens* Munich 87. Bd37 was characterized by producing a polyclonal antibody in a rabbit (α-Bd37 antiserum). In radiolabelling experiments, the α-Bd37 antiserum was an efficient tool to use to determine that Bd37 was a glycoprotein in a palmitoylated form in the merozoite and in a depalmitoylated form in the supernatants of *B. divergens* in vitro culture. By its biochemical and immunological properties, Bd37 appears to be another potential immunogen in our attempt to develop a vaccine against *B. divergens* babesiosis.

MATERIALS AND METHODS

Animals. Ten- to 12-week-old female gerbils (*Meriones unguiculatus*), an excellent animal model for bovine babesiosis, were obtained from CERJ (Le Genest-Saint-Isle, France), and New Zealand rabbits were obtained from ESD (Romans, France).

In vitro culture and vaccine antigens. The in vitro culture of *B. divergens* Rouen 1987 and *B. divergens* Munich 87 was performed on human erythrocytes by using a 10% human serum culture medium (11) or a semidefined medium supplemented with 1 mg of human HDL per ml (38). When parasitemia reached 30 to 40%, the supernatants of *B. divergens* Rouen 1987 cultures were collected, centrifuged (20,000 × *g*, 20 min), filtered (0.2-μm-pore-size filter; Gelman, Ann Arbor, Mich.), and stored at −80°C until use. Such supernatants were designated crude vaccine antigen (CVA) and Ser-CVA or HDL-CVA according to their

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TABLE 1. Mean reciprocal survival times and mortality rates of vaccinated gerbils and controls after virulent challenge with 10^6 *B. divergens* Rouen 1987-parasitized gerbil erythrocytes

Group	Treatment or FPLC fraction	% Mortality	Mean reciprocal survival time (1/survival time) ± SD (no. of gerbils)
Av	Ser-CVA	0	0 (10)
Ac	Ser-CCA	100	0.205 ± 0.017 (10)
Bv	HDL-CVA	0	0 (10)
Bc	HDL-CCA	100	0.222 ± 0.013 (10)
Cv	F_1	0	0 (10)
Cc	Fc_1	100	0.205 ± 0.013 (10)
Dv	F_2	0	0 (9)
Dc	Fc_2	100	0.186 ± 0.025 (10)
Ev	F_3	0	0 (9)
Ec	Fc_3	100	0.176 ± 0.030 (10)
Fv	F_4	0	0 (10)
Fc	Fc_4	100	0.121 ± 0.010 (10)

[a] Measuring the mean reciprocal survival time gives the opportunity to take into account the survival times of gerbils which did not die.

origin from serum or from HDL semidefined medium, respectively. The crude control antigens (CCA) were obtained from unparasitized erythrocytes incubated in serum-containing medium (Ser-CCA) or in semidefined medium (HDL-CCA) and processed as the *B. divergens*-infected erythrocytes. All crude and control antigens were 20-fold-concentrated on Centriflo CF25 (Amicon, Grace, France) and sterilized by filtration on a 0.2-μm-pore size filter.

Radiolabelling of *B. divergens*. *B. divergens* from in vitro cultures was radiolabelled with either [^{35}S]methionine, [^{3}H]glucosamine, [^{14}C]palmitate, or [^{3}H]myristate. All of these radiolabelled compounds were supplied by Amersham International Plc (Little Chalfont, United Kingdom). When parasitemia reached 10%, 12-h incubations were performed at 37°C by adding 1.85 MBq of [^{35}S]methionine per ml (specific activity, 1.15 to 18.50 GBq · mmol^{-1}), 1.85 MBq of D-[6-^{3}H]glucosamine hydrochloride per ml (specific activity, 0.74 to 1.50 TBq · mmol^{-1}), 1.85 MBq of [1-^{14}C]palmitic acid per ml (specific activity, 1.85 to 2.29 GBq · mmol^{-1}), or 1.85 MBq of [9,10(*n*)-^{3}H]myristic acid per ml (specific activity, 1.50 to 2.20 TBq · mmol^{-1}) to fresh culture medium depleted of the corresponding unlabelled component when necessary. After incubation, the labelled cells which exhibited at this time about 20% parasitemia and the corresponding supernatants were collected and processed for immunoprecipitation assays. In some experiments, *B. divergens* radiolabelled proteins were phase separated with Triton X-114 (TX-114) as described by Bordier (1), with some previously described modifications (31).

FPLC and gel electrophoresis. Because of the abundance of serum proteins in Ser-CVA, the gel filtration was performed only on HDL-CVA extracts with a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden), using a Superose 12 HR 10/30 column equilibrated with 25 mM ammonium acetate (pH 7.4). Elution with the same solution was performed at a flow rate of 18 ml · h^{-1}, and 0.3-ml fractions were usually collected. In preliminary experiments, chromatography was performed by injecting [^{35}S]methionine-radiolabelled HDL-CVA to a Superose 12 column. The radioactivity of the each [^{35}S]methionine fraction was determined by using a Flo-One/beta detector (Radiomatic Instruments and Consommable, Tampa, Fla.). These FPLC fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (20) and autoradiographed on X-Omat XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) following separation. Radioactive standards used for molecular mass calibration were from Amersham International Plc. On the basis of [^{35}S]methionine-radiolabelled exoantigen separation profiles, four fractions of the culture-derived exoantigens, designated F_1 (corresponding to 6.5- to 11-ml elution volume), F_2 (11 to 16.5 ml), F_3 (16.5 to 20.1 ml), and F_4 (20.1 to 23 ml), were determined. Then, similar fractions for vaccination trials were produced from nonradiolabelled culture-derived exoantigens. The HDL-CCA supernatants, used as controls, were similarly processed, and the four control fractions were designated Fc_1 to Fc_4.

Immunization and challenge procedures. Ten days before the trial, gerbils were randomly distributed into six groups (A to F) of 24 animals each. On days 0, 21, and 42, half of each group (Av to Fv) received a vaccine injection of Ser-CVA, HDL-CVA, or fractions F_1 to F_4. The other half (Ac to Fc) received on the same days control injections of Ser-CCA, HDL-CCA, or fractions Fc_1 to Fc_4. The different groups were formed as shown in Table 1. All injections were equivalent to 1.5 ml of supernatant adjuvanted with 0.5 mg of Quil-A saponin (Superfos, Vedbaek, Denmark) per ml (18, 31).

On day 63, two gerbils of each group were lethally anesthetized and bled by cardiac puncture. The sera obtained were pooled for each group and stored at −80°C. On the same day, all remaining gerbils were inoculated intraperitoneally with 10^6 *B. divergens* Rouen 1987-infected gerbil erythrocytes. During challenge, the gerbils were not handled except for removing dead animals daily from the cages. Sera were obtained after lethal anesthesia from all gerbils surviving on day 80 (α-G antisera).

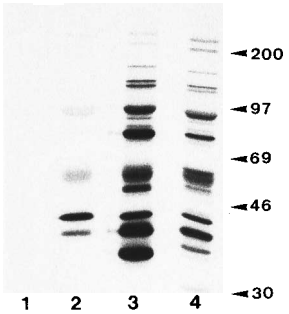

FIG. 1. Immunoprecipitations of [^{35}S]methionine-radiolabelled *B. divergens* Rouen 1987 proteins by α-GI antiserum (lane 2) or of gerbils immunized with HDL-CVA (lane 3) or Ser-CVA (lane 4). Lane 1 corresponds to control sera. ^{14}C-labelled methylated proteins (Amersham) were used as standards; sizes in kilodaltons are shown at the right.

Statistical analysis. The reciprocal survival time in vaccinated and control gerbils was the variable analyzed by statistical procedures. To analyze the effect of the treatment used in our vaccination trials (exoantigens produced as crude in serum or in HDL semidefined medium and as fractions), a Student *t* test was used to analyze differences among the mean reciprocal survival times of vaccinated (Av to Fv) and control (Ac to Fc) groups of gerbils. By statistical analysis, the use of reciprocal survival time gives the opportunity to take into account the survival time of gerbils which did not die.

Production of polyclonal antibodies. As a 37-kDa antigen was one of the antigens strongly recognized by immune sera from a gerbil, which survived a *B. divergens* infection (α-GI antiserum), the α-GI antiserum was used to prepare Bd37. It was incubated with 150 mg of protein from a *B. divergens* Rouen 1987 Triton X-100-soluble fraction. Immunoprecipitated parasitic antigens were separated by preparative SDS-PAGE. The acrylamide gel containing Bd37 was excised, and the protein was electroeluted in 50 mM Tris-borate (pH 8.0), using a Biotrap apparatus (Schleicher & Schuell, Dassel, Germany). After elution, 100 μg of Bd37 was emulsified with complete Freund's adjuvant and injected into the right popliteal node of a New Zealand rabbit (14). Three weeks later, the rabbit was boosted by subcutaneous injections of 100 μg of Bd37 emulsified with incomplete Freund's adjuvant. This rabbit polyclonal antibody was designated α-Bd37 antiserum.

Immunological methods. Indirect immunofluorescence, Western blotting (immunoblotting), and immunoprecipitation assays were performed essentially as previously described (5) except for the resolution of the blots, which was performed by using antibodies conjugated with alkaline phosphatase (Biosys, Compiegne, France). Indirect immunofluorescence labelling with the α-Bd37 antiserum was carried out both on infected erythrocytes permeabilized with a cold acetone-methanol mixture (ratio, 4:1) during 20 min at −80°C and on unfixed suspended live merozoites (cell surface staining). The α-GI antiserum was used as positive control for surface staining. The α-Bd37, α-GI, and α-G antisera were all used at a 1:100 dilution for indirect immunofluorescence and immunoblotting experiments.

For the two-dimensional (2-D) electrophoresis analysis, immunoprecipitation experiments were performed by using complexes bound on protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) which were resuspended in 10 mM Tris-HCl (pH 8). The samples were then lysed in the first-dimension sample buffer (2% Triton X-100, 9.5 M urea, 5% β-mercaptoethanol, 2% 5/7 Ampholyte) and loaded on the gels by using a Mini-Protean II 2-D Cell apparatus (Bio-Rad, Richmond, Calif.) as described by O'Farrell (30). An isoelectric focusing gel of an extended pH range (pH 5 to 7) was used in the first dimension, and an SDS–12% polyacrylamide gel was used in the second dimension.

RESULTS

Purification of *B. divergens* culture-derived exoantigens and their protective properties during gerbil vaccine assays. The humoral response of vaccinated gerbils was assessed by immunoprecipitation on [^{35}S]methionine-radiolabelled antigens of *B. divergens* Rouen 1987 with pooled sera from gerbils immunized with Ser-CVA or HDL-CVA and taken just before challenge (Fig. 1). In both cases, the humoral responses appear similar, since the same major antigens were recognized by the

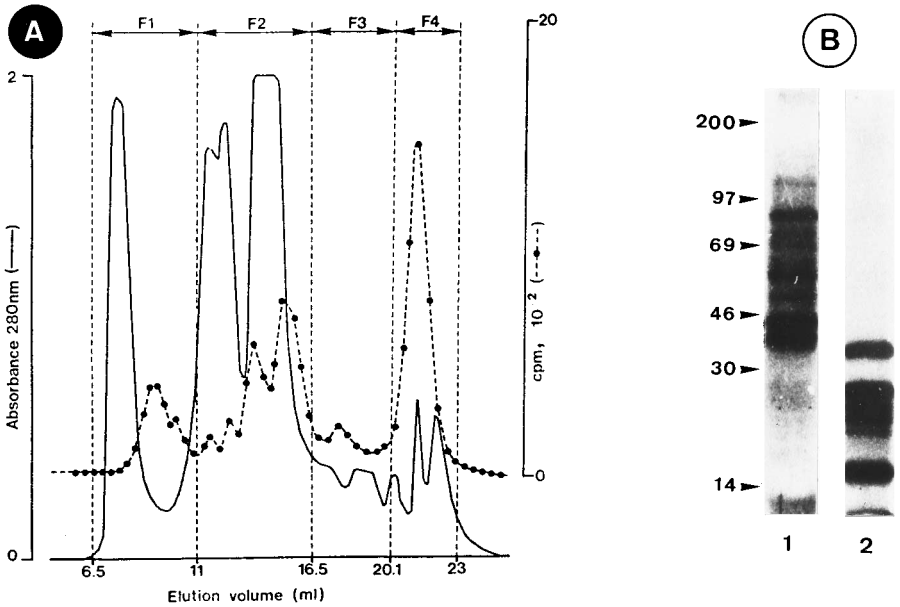

FIG. 2. (A) Gel filtration of [^{35}S]methionine-radiolabelled *B. divergens* Rouen 1987 exoantigens obtained in a semidefined medium. A_{280} and radioactivity were monitored. Fractions F_1 to F_4 were defined according to the radioactivity separation. (B) Exoantigen compositions of fractions F_1 (lane 1) and F_4 (lane 2). ^{14}C-labelled methylated proteins (Amersham) were used as standards. Sizes in kilodaltons are shown at the left.

gerbil sera (Fig. 1, lane 3 for HDL-CVA and lane 4 for Ser-CVA). Control sera failed to immunoprecipitate parasitic proteins (Fig. 1, lane 1), whereas the α-GI antiserum, which corresponds to an immune serum from a gerbil which survived to a *B. divergens* infection, recognized a few antigens, with an immunodominant response against a 37-kDa antigen (Fig. 1, lane 2).

Selected pools of *B. divergens* Rouen 1987 immunogens were prepared from an in vitro culture by using infected human erythrocytes in serum-containing medium (Ser-CVA) and in a semidefined medium (HDL-CVA). The HDL-CVA supernatant was fractionated by FPLC using a Superose 12 gel filtration column; a representative profile reproducible in over 20 FPLC separations is shown in Fig. 2A. The parasitic exoantigens were identified by using [^{35}S]methionine-radiolabelled *B. divergens* cells. The radioactivity profiles allowed us to define four vaccine fractions, F_1 to F_4 (Fig. 2A). The highest fraction, F_1 (Fig. 2B, lane 1), and the lowest fraction, F_4 (Fig. 2B, lane 2), contained proteins with molecular masses ranging mainly from 180 to 50 and 50 to 17 kDa, respectively.

Mortality rates of vaccinated and control gerbils were recorded daily. Cumulative mortality rates and reciprocal survival times are shown in Table 1. Gerbils treated with HDL-CVA (group Bv) or with fractions F_1 through F_4 (groups Cv, Dv, Ev, and Fv) were all protected from mortality as were gerbils treated with Ser-CVA (group Av) and did not exhibit any obvious symptoms. As the mortality was 0% for each group of vaccinated gerbils, their mean reciprocal survival times were not different. All gerbils treated with the control from serum (Ser-CCA [group Ac]) or HDL (HDL-CCA [group Bc]) culture medium, as well as fractions Fc_1 to Fc_4, died, and their mean reciprocal survival times exhibit a slight difference ($P < 0.07$).

Analysis of the humoral responses of the gerbils vaccinated with the protective fraction F_4. As fraction F_4 contained only a few major proteins with molecular masses lower than 50 kDa, this simplest protective fraction was used to analyze the humoral response of each of the 10 gerbils immunized with this fraction and challenged with 10^6 *B. divergens* Rouen 1987-infected gerbil erythrocytes. The immunoprecipitation and immunoblotting experiments were performed on extracts from *B. divergens* Rouen 1987. The sera of the 10 protected gerbils constantly immunoprecipitated antigens of 50, 37, and 17 kDa; in contrast, a 85-kDa antigen was immunoprecipitated by seven sera (Fig. 3A). An immunoblot of the same study (Fig. 3B) showed a constant response against these antigens of 50, 37, and 17 kDa (the 17-kDa antigen was detected by eye but difficult to discern on the photograph shown) and also against a supplementary antigen of 25 kDa, whereas no response was noticed against the 85-kDa antigen. In both experiments, the preimmune sera never reacted against babesial antigens (not shown). An immunodominant response was directed against the 37-kDa antigen, as observed with another strain of *B. divergens*, Munich 87 (Fig. 4), in which case the 10 sera constantly immunoprecipitated only a triplet of 35 to 39 kDa, with strong labelling of the 37-kDa antigen in the central predominant position (Fig. 4, lanes 1 to 10). This 37-kDa antigen was also recognized by the α-GI antiserum (Fig. 3B, lane 12).

As the humoral response against the 37-kDa antigen on the two strains was constant and strong and because the humoral response against this protein appeared early in cattle and humans infected with *B. divergens* (5, 12, 37), this 37-kDa antigen was purified for further biochemical characterization.

In the simplest fraction, F_4 the Bd37 antigen is a major component. The rabbit α-Bd37 antiserum recognized strongly the Bd37 protein (Fig. 3 and 4, lanes 11) and weakly a cross-reactive protein of 60 kDa (Bd60) (undetected with sera from the protected gerbils), especially with *B. divergens* Munich 87 (Fig. 4, lane 11, arrows). Pulse-labelling experiments did not identify a precursor form of Bd37.

To confirm that the purified Bd37 was the 37-kDa protein recognized by the sera of the 10 protected gerbils immunized with fraction F_4, an analysis by 2-D electrophoresis was performed by immunoprecipitation of radiolabelled proteins with a serum from an F_4-protected gerbil (α-G antiserum) (Fig. 5A) and with the α-Bd37 antiserum (Fig. 5B). The gels were then

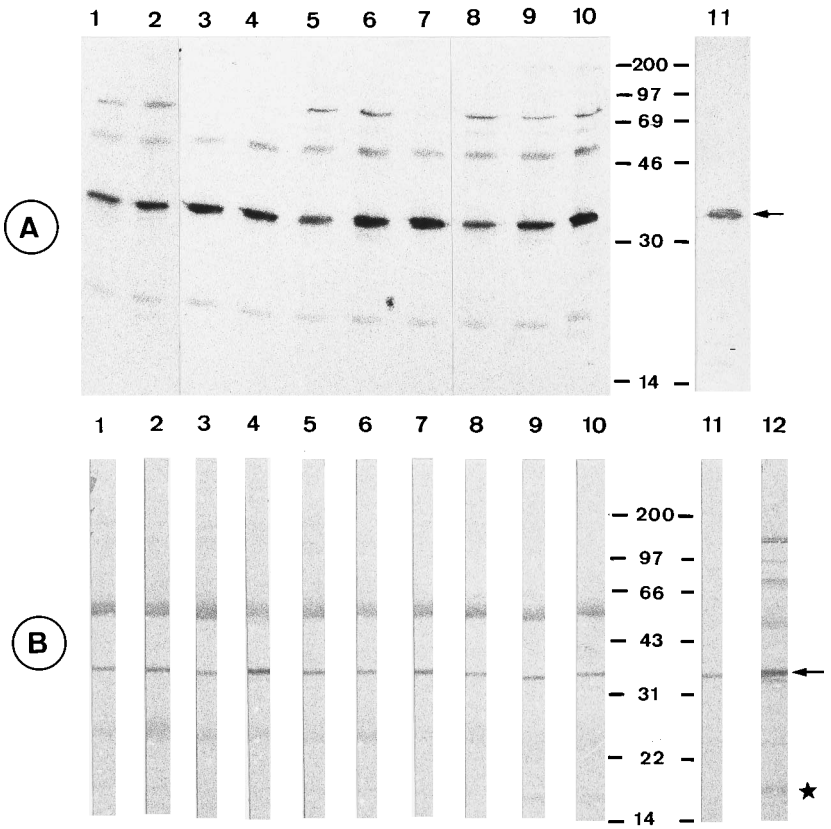

FIG. 3. Analysis of the humoral responses of gerbils immunized with protective fraction F_4 by using immunoprecipitation (A) and immunoblotting (B) experiments on *B. divergens* Rouen 1987 extracts. Lanes: 1 to 10, α-G antisera; 11, α-Bd37 antiserum; 12, α-GI antiserum. The arrow indicates the presence of Bd37, and the star indicates the presence of Bd17. Protein separations were performed on 15% polyacrylamide gels. ^{14}C-labelled methylated proteins (Amersham) and prestained molecular masses (Novex, San Diego, Calif.) were used as standards for the immunoprecipitation and immunoblotting experiments, respectively. Sizes in kilodaltons are shown at the right.

dried and autoradiographed. In both cases, a 37-kDa antigen with a pI of 6 was immunoprecipitated (Fig. 5), suggesting that the 37-kDa protein recognized by the α-G antiserum could be the Bd37 antigen. This result was assessed by a depletion experiment (Fig. 6). In this case, after an immunoprecipitation experiment with the α-Bd37 antiserum, the pellet of beads with bound Bd37 proteins was processed for SDS-PAGE (Fig. 6, lane 1), and the supernatant was incubated with the α-G antiserum for an immunoprecipitation assay (Fig. 6, lane 2). As control, a basic immunoprecipitation assay was performed with the α-G antiserum (Fig. 6, lane 3). As expected, Bd37 bound to the beads and was immunoprecipitated by the α-Bd37 and α-G antisera (Fig. 6, lanes 1 and 3, respectively), whereas a clear depletion of Bd37 was observed in the supernatant immunoprecipitated with the α-G antiserum (Fig. 6, lane 2). The same results were obtained for the two strains.

Analysis of the Bd37 protein of *B. divergens* merozoites. The localization of Bd37 in *B. divergens*-infected erythrocytes was studied by immunofluorescence assays with the rabbit α-Bd37 antiserum, either on fixed *B. divergens*-infected erythrocytes or on live merozoites. In the first case, a diffused labelling was observed after fixation on the whole parasite surface, with a stronger staining of dots in the merozoites (indicated by arrows) (Fig. 7A). In the second case, no fluorescence was observed on live merozoites with this antiserum, whereas a surface labelling of the live merozoites was observed with the positive control, the α-GI antiserum (data not shown). The α-Bd37 antiserum recognized the parasitic protein Bd37, as confirmed by immunoprecipitation of [^{35}S]methionine-labelled *B. divergens* proteins as well as examination of lysates of parasitized erythrocytes (Fig. 7B, lane 1), in vitro culture supernatants (Fig. 7B, lane 2), and fraction F_4 (Fig. 7B, lane 3).

TX-114 detergent phase separation was performed on [^{35}S]methionine-labelled *B. divergens* proteins, and immunoprecipitation with the α-Bd37 antiserum on each phase demonstrated the hydrophobic nature of Bd37, since the labelling was found only in the TX-114 phase (Fig. 7D, lane 2). Bd37 is a glycoprotein, as shown by the immunoprecipitations of

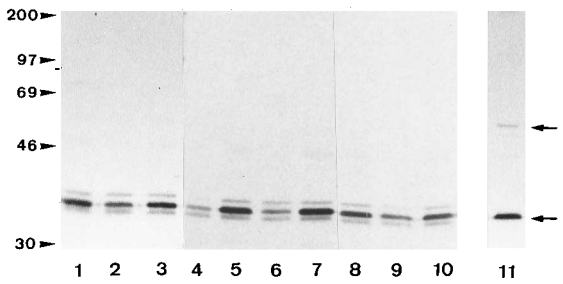

FIG. 4. Immunoprecipitation of [^{35}S]methionine-radiolabelled *B. divergens* Munich 87 proteins by α-G (lanes 1 to 10) and α-Bd37 (lane 11) antisera. The arrows indicate the presence of Bd37 and Bd60. Proteins separation was performed on a 12% polyacrylamide gel. ^{14}C-labelled methylated proteins (Amersham) were used as standards. Sizes in kilodaltons are shown at the left.

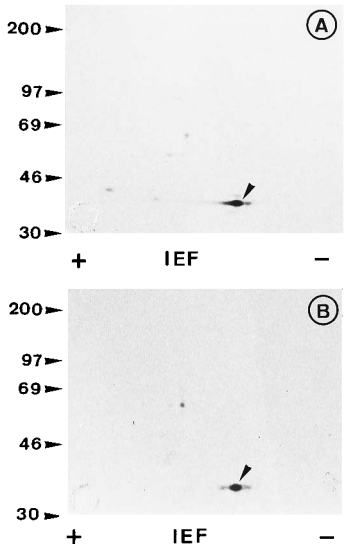

FIG. 5. 2-D electrophoresis gel analysis of Bd37. Proteins immunoprecipitated with the α-G (A) and α-Bd37 (B) antisera were analyzed on a 2-D electrophoresis gel (12% polyacrylamide) and autoradiographed. In both cases, a single 37-kDa antigen with a pI of 6 was the only common protein revealed by the two sera. The arrowheads indicate the presence of Bd37; the isoelectric focusing (IEF) points range from 7 (+) to 5 (−). ^{14}C-labelled methylated proteins (Amersham) were used as standards. Sizes in kilodaltons are shown at the left.

[^{3}H]glucosamine-radiolabelled Bd37 protein from both parasitic and exoantigen fractions (Fig. 7C, lanes 1 and 2, respectively). The presence of at least one palmitoyl residue in Bd37 was evidenced by radiolabelling experiments with [^{14}C]palmitate. Bd37 was labelled with this radioelement, as shown by immunoprecipitations with the α-Bd37 antiserum (Fig. 7E, lane 1), providing further evidence of the hydrophobic nature of Bd37. The same type of experiment performed with [^{3}H]myristate did not show any Bd37 labelling (data not shown). When immunoprecipitations with the α-Bd37 antiserum were performed by using in vitro culture supernatants after [^{14}C]palmitate radiolabelling, it was not possible to detect the Bd37 palmitoylated moiety (Fig. 7E, lane 2), although the presence of the protein in the same preparation could be demonstrated by immunoblotting (Fig. 7E, lane 3).

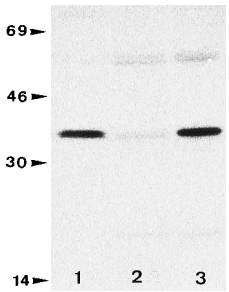

FIG. 6. Depletion experiment demonstrating that the 37-kDa antigen recognized by the sera of protected gerbils is Bd37. After immunoprecipitation with the α-Bd37 antiserum, the pellet of beads with bound Bd37 was processed for SDS-PAGE (lane 1). The supernatant was incubated with the α-G antiserum and processed for the immunoprecipitation assay (lane 2). As a control, a basic immunoprecipitation assay was performed with the α-G antiserum (lane 3). ^{14}C-labelled methylated proteins (Amersham) were used as standards. Sizes in kilodaltons are shown at the left.

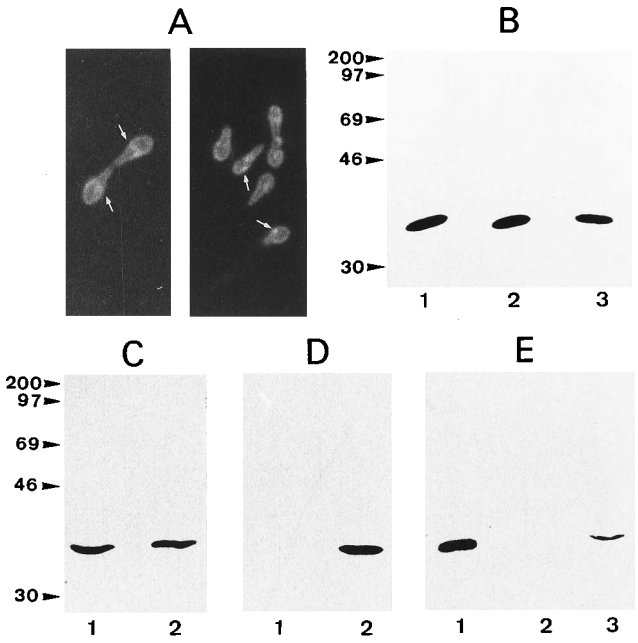

FIG. 7. Localization and biochemical characterization of Bd37 with the α-Bd37 antiserum. (A) Immunofluorescence staining of *B. divergens*-infected erythrocytes revealed the cell surface localization of Bd37 and also dot staining in the merozoites (indicated by arrows). (B) Immunoprecipitations of [^{35}S]methionine-radiolabelled *B. divergens* Rouen 1987 proteins revealed the presence of Bd37 in the whole parasitic proteins (lane 1), in the in vitro culture supernatant (lane 2), and in the F_4 radiolabelled proteins (lane 3). (C) Immunoprecipitations of [^{3}H]glucosamine-radiolabelled *B. divergens* Rouen 1987 proteins present in the parasitic lysate (lane 1) and in the culture supernatant (lane 2) revealed that Bd37 is a glycoprotein. (D) TX-114 phase separation of [^{35}S]methionine-radiolabelled *B. divergens* Rouen 1987 proteins followed by immunoprecipitation experiments showed that Bd37 was present in the detergent phase (lane 2) and undetectable in the aqueous phase (lane 1). (E) [^{14}C]palmitate-radiolabelled *B. divergens* Rouen 1987 proteins followed by immunoprecipitation experiments demonstrated the presence of the palmitoylated form of Bd37 in the crude parasitic antigen (lane 1) and its lack in exoantigens (lane 2), although the presence of Bd37 was confirmed by Western blotting of the same sample (lane 3). ^{14}C-labelled methylated proteins (Amersham) were used as standards. Sizes in kilodaltons are shown at the left of panels B and C.

DISCUSSION

To extend our previous results on the protection of gerbils against homologous and heterologous challenges after vaccination with exoantigens of *B. divergens* Rouen 1987 (12, 31), an assay was performed by using the same exoantigens produced in HDL-CVA. We observed that gerbils immunized with HDL-CVA and challenged with *B. divergens* Rouen 1987 were protected from mortality and did not present secondary side effects, such as hemoglobinuria. The production of exoantigens in a semidefined medium presented the major advantage to avoid contamination with serum components (albumin, immunoglobulins, etc.) (23, 38). In this vaccine strategy, we also prepared fractions of exoantigens of *B. divergens* Rouen 1987 by gel filtration chromatography of the whole exoantigens recovered in HDL semidefined medium. The goal was to identify a protective fraction containing a few exoantigens which could be then cloned in order to produce a recombinant vaccine. On the basis of their molecular masses, the exoantigens of HDL-CVA were fractionated in four fractions, F_1 to F_4, and all of these fractions were able to protect gerbils from mortality after a challenge with *B. divergens* Rouen 1987. The antigenic composition is different in each fraction; however, the gel filtration used cannot exclude in the highest-molecular-mass fractions

the presence of smaller proteins which could be trapped or form complexes. Given this situation, we focused our investigations on fraction F_4 for the additional reasons that fraction F_4 protects all of the gerbils from mortality; contains only a few major antigens with molecular masses lower than 50 kDa, and protects oxen against a homologous challenge and induces an in vitro lymphocyte proliferation in this natural host (37). Analysis of humoral responses of the 10 protected gerbils with fraction F_4 showed that a few antigens from 50 to 17 kDa could be potential candidate immunogens since they were constantly detected with strain Rouen 1987. Detection of a 85-kDa antigen was unexpected since in the native fraction F_4, such a protein was not detected even after [^{35}S]methionine radiolabelling. Of course, the presence of small amount of the 85-kDa antigen in fraction F_4 cannot be excluded; alternatively, this antigen could correspond to a precursor of one of the F_4 components.

Among the antigens of 50, 37, 25, and 17 kDa found in fraction F_4, only the 17-kDa antigen (Bd17) had previously been characterized. Bd17 was shown to be a merozoite membrane protein, and monoclonal antibody DG7 directed against this antigen was able to inhibit parasite growth in vitro (32). Obviously, even if protection could be due to antigens which did not elicit an immunodominant response, as determined from studies of the humoral responses of the three hosts of *B. divergens* (5, 11, 12, 37) and from the cellular immune responses of oxen vaccinated with fraction F_4 inducing an in vitro lymphocyte proliferation (37), selection of the Bd37 antigen for further characterization was justified for the following reasons: (i) a strong humoral response was directed against Bd37, suggesting an immunodominant response against this antigen in various strain of *B. divergens* (this work); (ii) Bd37 was present in all the *B. divergens* strains so far tested (31); and (iii) in different hosts surviving a *B. divergens* infection, the chronology of the appearance of antibodies showed that Bd37 was among the first immunodominant antigens (5, 11, 12, 37).

Bd37 was shown to be glycosylated, but as in other *Babesia* spp. glycoproteins (16, 24), the contribution of the glycan moieties, in contrast to glycan moieties of *Plasmodium falciparum* antigens (33), to immunogenicity remains to be determined. The exclusive partitioning of Bd37 in the detergent phase in a TX-114 phase separation experiment and the palmitoylation of this antigen strongly suggest an anchorage of Bd37 in the membrane via a palmitoyl residue. Thus far, four classes of covalent lipid modification of proteins have been identified in eukaryotic systems (22): glycosyl-phosphatidylinositol (GPI) anchorage, myristoylation, palmitoylation, and isoprenylation. As fatty acid and glucosamine have been described as characteristic components of a GPI structure (2, 3, 6–10, 15–17, 21, 34, 36), our data may indicate that Bd37 is linked to a GPI moiety. However, palmitate is rather infrequent in GPI anchors (4, 7). Palmitoylation most often refers to acylation of membrane proteins found on the cytoplasmic side of the plasma membrane (22). The molecular structure of Bd37 requires further investigation.

Immunofluorescence assays on fixed *B. divergens*-infected erythrocytes showed that Bd37 is localized both in internal vesicles and on the surface of merozoites; the same assays performed on suspended live merozoites by using the α-Bd37 antiserum were negative. Furthermore, Bd37 was found in a palmitoylated form inside the merozoites and in a nonpalmitoylated form in the culture medium. Together, these results strongly suggest that Bd37 is excreted by or released from merozoites and/or infected erythrocytes. This secretion or release would be associated with a specific cleavage of the palmitate from the membrane form, as indicated by presence of depalmitoylated Bd37 in the culture medium.

In conclusion, the efficient vaccination against *B. divergens* Rouen 1987 with defined fractions of in vitro culture supernatants produced by using a semidefined medium led us to characterize the major proteins present in the simplest one obtained, fraction F_4. Among these exoantigens, the Bd17 antigen was previously characterized and was considered an interesting immunogen (32). By its biochemical and immunological properties, Bd37 appears to be another candidate for future development of recombinant vaccines against *B. divergens* bovine babesiosis.

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