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Identification of Major *Babesia divergens* Polypeptides That Induce Protection against Homologous Challenge in Gerbils

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[^{35}S]methionine-radiolabeled proteins from the *Babesia divergens* Rouen 1987 isolate were immunoprecipitated with immune sera from three potential hosts: human, ox, and gerbil. The results showed a constant humoral response against major babesial antigens. Similarly, immunoprecipitation of radiolabeled in vitro culture supernatant demonstrated that the exoantigens of 37, 46, 70, and 90 kDA were the immunodominant polypeptides, whatever the host. The effects of vaccination with concentrated supernatant from *B. divergens* Rouen 1987 in vitro cultures (30 to 40% parasitemia) were examined in gerbils inoculated with the homologous *B. divergens* isolate. Gerbils having received two or three injections of a whole vaccine dose (1.5 ml of parasitized culture supernatant equivalent [PCSE]) or of a 1:5 diluted vaccine dose (0.3 ml of PCSE) showed 100% survival after intraperitoneal challenge with 10^6 *B. divergens*-infected gerbil erythrocytes. Moreover, two or three injections of a 1:25 diluted vaccine dose (0.06 ml of PCSE) or 9‰ NaCl or 1.5 ml of unparasitized culture supernatant equivalent resulted in a mortality rate of 80 to 90% of the infected gerbils. Immunoprecipitation and immunofluorescence assays performed with antisera from vaccinated and control gerbils demonstrated that a single vaccine injection induced a humoral response, which increased slightly after the second or third injection. After challenge, antibody levels increased significantly, although the immunoprecipitation did not display any modification of *Babesia* antigen patterns.

Babesiosis is due to various tick-borne intraerythrocytic parasites of the protozoan genus *Babesia*. These blood parasites occur in many domestic and wild animals, and different *Babesia* species infect cattle throughout the world (12). *Babesia divergens* is the main agent of bovine babesiosis in Europe and is responsible for important economic losses. Human infections have also been observed, especially in splenectomized patients, and a high mortality rate has been reported (5).

Different vaccine strategies have been considered to protect cattle against babesiosis: (i) using live attenuated vaccines (1, 18, 30, 37, 41), (ii) using plasma-derived exoantigens (29), and (iii) using antigens derived from erythrocytic stages (2–4, 13, 20, 21, 35, 36, 38, 42). The attenuated vaccines currently available provide good protection but have several restricting features: a short shelf life, possible variation in virulence, contamination with host erythrocyte material capable of inducing isoantibody-mediated erythrolysis, the risk of transmission of other pathogenic agents, and maintenance of healthy carriers among vaccinated populations. Development of long-term in vitro cultures of *Babesia* species has made it possible to avoid some of these shortcomings through the use of in vitro culture-derived antigens and especially culture supernatant exoantigens (for reviews, see references 10 and 32). In these conditions, good protection has been reported with immunogens derived from in vitro cultures of *B. bovis* (14, 15, 24, 25, 33), *B. canis* (23, 26, 27), and *B. bigemina* (25). Furthermore, only the in vitro culture-derived exoantigens are compatible with the definition of an efficient anti-*Babesia* vaccine according to Mahoney et al. (22).

Recently, we have described a method for the long-term in vitro cultivation of *B. divergens* in human erythrocytes, producing parasitemia routinely reaching 30 to 40% (A. Gorenflot et al., Parasitol. Res., in press). In this study, in vitro culture supernatants were used to prepare a *B. divergens* vaccine which protected the gerbil, a rodent receptive for *B. divergens* infection, against a homologous challenge. The *Babesia* antigens inducing an antibody response in gerbils, oxen, and humans were also identified.

MATERIALS AND METHODS

***B. divergens* isolates.** The *B. divergens* Rouen 1987 isolate was obtained from the blood of a naturally infected human patient (6). It has been maintained by long-term in vitro cultivation since 1987 (Gorenflot et al., in press) as well as in vivo by syringe passage twice weekly in the gerbil (*Meriones unguiculatus*) (19). For maintenance of isolates in gerbils and for vaccine trials, 10- to 12-week-old female gerbils were obtained from CERJ (Le Genest-Saint-Isle, France).

***B. divergens* antisera.** Human antisera were collected from a patient who had recovered from acute *B. divergens* babesiosis (from whom isolate Rouen 1987 originates) (6). Control sera were obtained from healthy donors with a negative indirect fluorescent antibody test for *B. divergens*.

To produce bovine antiserum, a 4-month-old calf was inoculated with 10^{11} human erythrocytes infected with *B. divergens* Rouen 1987 and challenged 1 month later by inoculation of 10^{10} gerbil erythrocytes infected with *B.*

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divergens Rouen 1987. The antiserum was collected 3 weeks after challenge, and the preimmune serum of the same calf was used as a control.

Gerbil antisera were collected from the occasional gerbils surviving after inoculation of 10^4 gerbil erythrocytes infected with *B. divergens* Rouen 1987 and from gerbils immunized with *B. divergens* Rouen 1987 culture supernatant. Control sera were obtained from healthy gerbils and from gerbils injected with 9‰ NaCl or unparasitized culture supernatant.

Indirect immunofluorescence test. *B. divergens*-parasitized gerbil erythrocytes were washed in phosphate-buffered saline (0.15 M NaCl, 5 mM Na_2HPO_4, pH 7.2) containing 0.5% bovine serum albumin. The erythrocytes were coated onto wells of Teflonized slides, air dried, and fixed by immersion for 15 min in cold acetone (−20°C). Dilutions of gerbil sera from 1:20 to 1:1,280 were placed on the antigens, and the slides were incubated at 37°C for 30 min in a moist chamber. The slides were washed twice in phosphate-buffered saline and air dried. Fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulins (Biomérieux, Marcy-L'Etoile, France) at a dilution of 1:50 were added for 30 min at 37°C. The slides were washed as above and mounted in Fluoprep (Biomérieux) before fluorescence microscopy examination.

[^{35}S]methionine radiolabeling of *B. divergens*. When parasitemia reached 10%, the culture medium was removed and replaced by methionine-free medium (minimal essential medium without methionine; GIBCO BRL, Paisley, Scotland) supplemented with 10% human serum, 2 mM glutamine, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.35) and containing 15 μCi of [^{35}S] methionine per ml (specific activity, >1,000 Ci · mmol^{-1}; Amersham International Plc, Little Chalfont, United Kingdom). After 12 h of incubation at 37°C, labeled cells (about 20% parasitemia) and supernatant were collected and processed for immunoprecipitation assays.

Immunoprecipitation assays. The radiolabeled cells were extensively washed by centrifugation at 1,000 × *g* in culture medium without serum and incubated in lysis buffer (2% Triton X-100, 0.6 M KCl, 5 mM EDTA, 3 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 2.5% iodoacetamide in Tris-buffered saline [TBS; 0.14 M NaCl, 10 mM Tris hydrochloride, pH 7.8]). The lysate was kept on ice for 1 h and centrifuged at 40,000 × *g* (RC5 Sorvall centrifuge, rotor SS34) for 20 min at 4°C. The Triton X-100-insoluble fraction was discarded, and the supernatant was used immediately or kept at −80°C. The radiolabeled culture supernatant was centrifuged at 10,000 × *g* for 20 min and filtered through a membrane (0.22-μm pore size; Gelman Sciences, Inc., Ann Arbor, Mich.). To remove the unmetabolized [^{35}S]methionine, the culture supernatant was precipitated twice with saturated ammonium sulfate and centrifuged at 4,000 × *g* for 10 min. The pellet was washed twice in 10 mM Tris hydrochloride (pH 7.4) (TB) and dialyzed three times for 12 h against 5 liters of TB. Serum immunoglobulins of the culture medium were removed by adding 2 ml of protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in TBS (1:1, vol/vol) to the remaining sample and incubating for 90 min at 37°C with constant stirring. The beads were centrifuged (3,000 × *g*, 5 min) and discarded. A new incubation was performed to deplete the sample of the major part of serum immunoglobulins.

The radiolabeled *B. divergens* lysate (10^6 cpm) was mixed with 7 μl of serum and incubated overnight at 4°C with constant stirring. The antigen-antibody complexes were precipitated with 70 μl of protein A-Sepharose CL-4B diluted 1:1 in TBS (pH 7.4). The complexes were washed four times with the washing buffer (2% Triton X-100, 0.6 M KCl, 5 mM EDTA in TBS, pH 7.8) and twice with TBS (pH 7.8) with centrifugation at 3,000 × *g* for 5 min at each step. Excess buffer was removed, and the immunoprecipitated antigens were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and run on 10 or 12.5% polyacrylamide gels under reducing conditions as previously described (16). The gels were dried and autoradiographed on X-OMAT XAR-5 films (Eastman Kodak Co., Rochester, N.Y.).

Vaccine preparation. Parasitized culture supernatants from in vitro cultures whose parasitemia reached 30 to 40% were harvested and centrifuged at 4,000 × *g* for 10 min, passed through a filter (0.22-μm pore size; Gelman Sciences), and concentrated 7.5-fold with a Minicon 10 filter (Grace, Epernon, France). Quil A saponin (Superfos Biosector a/s, Vedbaek, Denmark) was added to the concentrated supernatant at a final concentration of 0.5 mg/ml. This preparation was used as a vaccine. Normal erythrocytes from the same donor were cultured in vitro in the same conditions. Supernatants were centrifuged, filtered, and concentrated as described above and used as placebo controls.

Immunization and dose-response evaluation procedure. Ten days before the trial, gerbils were randomly distributed into 20 cages of five animals each and separated into five groups: A, B, C, D, and E.

Group A (20 gerbils) was injected subcutaneously in the flank with 400 μl of full vaccine dose (= 1.5 ml of parasitized culture supernatant equivalent [PCSE]). This group was divided into subgroups A1 and A2. Group A1 (two groups of five gerbils) was injected on days 0, 21, and 42, and group A2 (two groups of five gerbils) was injected on days 0′ and 21′.

Subgroups B1 and B2 (group B) were injected like group A with the vaccine dose diluted 1:5 in placebo control supernatant (0.3 ml of PCSE).

Subgroups C1 and C2 (group C) were injected like group A with a 1:25 diluted vaccine dose (0.06 ml of PCSE).

Subgroups D1 and D2 (group D) were injected like group A with 400 μl of placebo control (1.5 ml of unparasitized culture supernatant equivalent [UPCSE]).

Subgroups E1 and E2 (group E) were injected like group A with 400 μl of saline solution (9‰ NaCl, wt/vol) as the control.

For indirect fluorescent antibody tests and immunoprecipitation assays, blood specimens were obtained by retro-orbital venipuncture from five anesthetized animals of subgroups A1, B1, C1, D1, and E1 on days 14, 35, and 56 (series with three injections) and subgroups A2, B2, C2, D2, and E2 on days 14′ and 35′ (series with two injections).

On days 63 and 42′, all gerbils were injected intraperitoneally with 1.6×10^6 parasitized gerbil erythrocytes in 0.1 ml of RPMI 1640 medium (Gibco BRL). After the virulent challenge, the gerbils were not handled and dead animals were counted daily and removed. Blood specimens were collected by cardiac puncture as soon as possible after death and in gerbils surviving 17 days postinoculation.

Statistical analysis. The reciprocal survival time in vaccinated and control gerbils was the variable analyzed by statistical procedures. Differences were compared by crossed-factor variance analysis. The first factor was the number of injections in gerbils (two or three), and the second factor was the treatment: A, 1.5 ml of PCSE; B, 0.3 ml of PCSE; C, 0.06 ml of PCSE; D, 1.5 ml of UPCSE; and E, 9‰ NaCl.

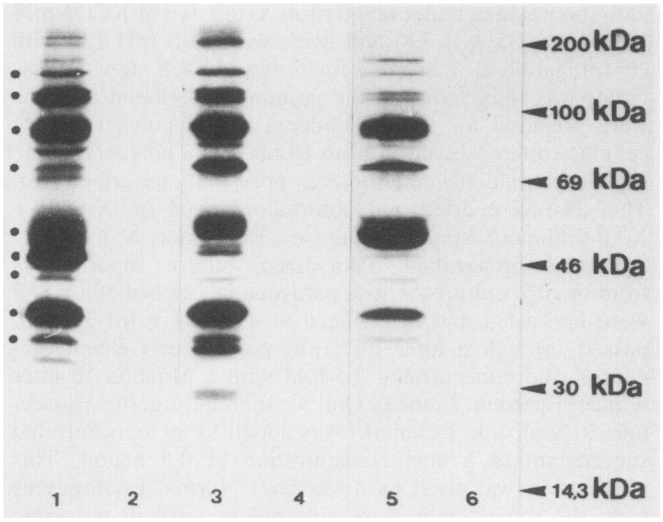

FIG. 1. Immunoprecipitations of [^{35}S]methionine-radiolabeled *B. divergens* Rouen 1987 proteins by immune sera from different hosts: human (lane 1), gerbil (lane 3), and ox (lane 5). No parasitic proteins were precipitated by control sera: human (lane 2), gerbil (lane 4), and ox (lane 6). Dots mark the babesial antigens immunoprecipitated by all immune sera. Molecular mass standards are indicated at the right.

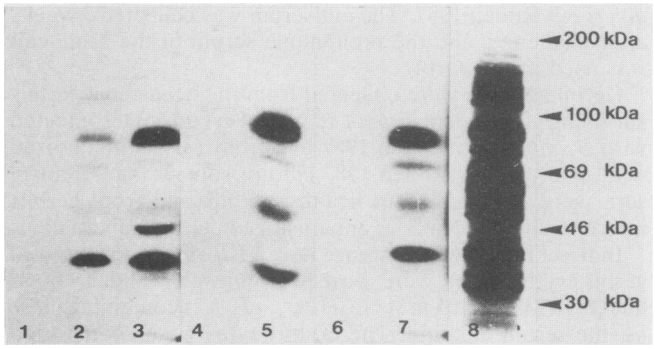

FIG. 2. Identification by immunoprecipitation of the *B. divergens* exoantigens inducing a humoral response in different hosts. Parasitic proteins from *B. divergens* Rouen 1987 in vitro culture supernatants were immunoprecipitated after [^{35}S]methionine radiolabeling. The proteins recognized are quite similar in the three hosts: human (lane 2, day 18 posthospitalization; lane 3, day 124 posthospitalization), ox (lane 5), and gerbil (lane 7). The respective controls (nonimmune sera) are shown in lanes 1, 4, and 6. The total *B. divergens* exoantigens are shown in lane 8. Molecular mass standards are indicated at the right.

RESULTS

Identification of major immunogenic polypeptides in *B. divergens* Rouen 1987. [^{35}S]methionine-radiolabeled *B. divergens* Rouen 1987 total antigens were immunoprecipitated with human, bovine, and gerbil antisera raised against *B. divergens* Rouen 1987 (Fig. 1). These assays clearly demonstrated that humans (Fig. 1, lane 1), gerbils (Fig. 1, lane 3), and oxen (Fig. 1, lane 5) developed similar humoral responses against several babesial antigens. The major immunoprecipitated proteins were observed with all antisera and correspond to 37-kDa, 50-kDa, 70-kDa, 90-kDa, a 105- to 110-kDa doublet, and 150-kDa polypeptides. Other minor polypeptides were also immunoprecipitated by all antisera. In addition, some antigens (29, 34, and 210 kDa) were only immunoprecipitated by one or two antisera, demonstrating intrinsic variations in the humoral responses of the different *B. divergens* hosts (Fig. 1, lanes 1 and 3).

After serum immunoglobulins were removed by protein A-Sepharose CL-4B, the in vitro culture supernatants were immunoprecipitated by *B. divergens* Rouen 1987 antisera from a patient (Fig. 2, lanes 2 and 3), an ox (Fig. 2, lane 5), and a gerbil (Fig. 2, lane 7). These assays demonstrated that the major exoantigens inducing an antibody response were similar in the different hosts, with only quantitative variations in the major immunoprecipitated exoantigens—37-, 46-, 50-, 70-, and 90-kDa polypeptides. The quantitative variation of the exoantigens immunoprecipitated by two serum samples collected from the same patient on day 8 (Fig. 2, lane 2) and on day 124 posthospitalization (Fig. 2, lane 3) is worth emphasizing; with the second serum sample, the specificity or the titer or both of antibodies against the 46- and 90-kDa polypeptides were enhanced.

Statistical analysis of survival rates. Survival rates of vaccinated gerbils and controls (placebo, 9‰ NaCl, wt/vol) were observed daily. To prevent any change in survival rates of animals, no blood specimen was collected during the 17 days after challenge; in our experiments, no animal died later than 11 days after challenge. Cumulative mortality rates of gerbils are indicated in Fig. 3, and reciprocal survival times are shown in Table 1. 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. In the group receiving the 1:25 diluted vaccine dose (group C, 0.06 ml of PCSE) and in control groups D (1.5 ml of UPCSE) and E (9‰ NaCl), gerbil mortality rates varied from 80 to 90% (Fig. 3C, D, and E). In contrast, all gerbils vaccinated with the full vaccine dose (group A, 1.5 ml of PCSE) and 17 of 18 gerbils receiving the 1:5 diluted vaccine dose (group B, 0.3 ml of PCSE) survived (Fig. 3A and B).

The two-crossed-factor variance analysis of the reciprocal survival times showed that the effects of the dilution and the

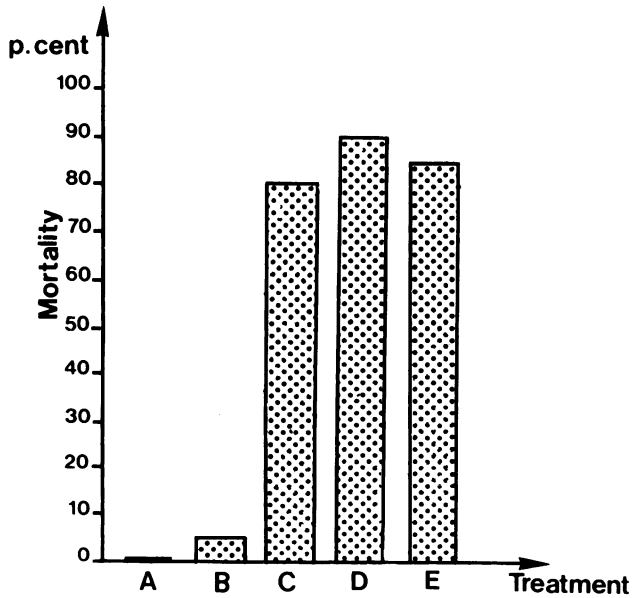

FIG. 3. Cumulative mortality rates of gerbils injected two or three times with *B. divergens* Rouen 1987 culture supernatant. (A) Full-dose vaccine (1.5 ml of PCSE); (B) 1:5 diluted vaccine dose (0.3 ml of PCSE); (C) 1:25 diluted vaccine dose (0.06 ml of PCSE); (D and E) placebo (D, 1.5 ml of UPCSE; E, 1.5 ml of 9‰ NaCl).

TABLE 1. Mean reciprocal survival times of control and vaccinated gerbils after virulent challenge

Group	Treatment	Mean reciprocal survival time[a]	
		Two injections (*n*)	Three injections (*n*)
A	Full vaccine dose (1.5 ml of PCSE)	0 (10)	0 (8)
B	1:5 diluted vaccine dose (0.3 ml of PCSE)	0.017 ± 0.017 (10)	0 (8)
C	1:25 diluted vaccine dose (0.06 ml of PCSE)	0.192 ± 0.014 (10)	0.114 ± 0.032 (10)
D	1.5 ml of UPCSE	0.133 ± 0.027 (10)	0.203 ± 0.014 (8)
E	9‰ NaCl	0.230 ± 0.080 (10)	0.121 ± 0.029 (10)

[a] Mean ± standard deviation; *n*, number of gerbils.

number of vaccinal injections were highly significant ($P < 0.03$); the interactions between the dilution of the vaccine dose and the number of injections were significant at $P < 0.07$ (Table 2).

The statistical analysis demonstrated that (i) the vaccinated groups are significantly different from the control groups, (ii) the dilution of the vaccine dose significantly affects the survival times, and (iii) the number of injections takes a prominent part when the vaccine dose is diluted (this concerns the number of injections for a dilution range between 1:5 and 1:25).

Immunofluorescence assays. Antibody levels of gerbils vaccinated with three injections of a full vaccine dose (1.5 ml of PCSE) or a 1:5 diluted vaccine dose (0.3 ml of PCSE) were measured by using an immunofluorescence assay (Fig. 4A). In gerbils vaccinated with a full vaccine dose, antibodies were observed 14 days after the first vaccinal injection. A second (day 21, detection day 35) or a third (day 42, detection day 56) injection of vaccine did not strongly enhance the antibody levels. Comparatively, only the third injection (day 42) of the 1:5 diluted vaccine dose was able to induce antibody levels as high as those obtained with only one injection of a full vaccine dose. At day 80 (17 days postchallenge), the antibodies reached similar levels in gerbils vaccinated with a full vaccine dose or with a 1:5 diluted vaccine dose.

The antibody levels of the gerbils who had received three vaccinal injections (Fig. 4A) were compared with those of the gerbils who received two vaccinal injections (Fig. 4B). In gerbils injected with a full vaccine dose, the antibodies could be detected after the first injection (day 0′, detection day 14′). Contrary to a second injection of a full vaccine dose (day 21′, detection day 35′), the virulent challenge (day 42′, detection day 59′) strongly enhanced the antibody level in vaccinated gerbils. Gerbils who had received a 1:5 diluted vaccine dose showed lower antibody levels after the first and the second injections than gerbils injected with a full dose, but levels rapidly increased after the virulent challenge.

Antibody responses to *B. divergens* during vaccine assays. Immunoprecipitation assays with [^{35}S]methionine-radiolabeled *B. divergens* Rouen 1987 were performed with sera from gerbils vaccinated with one, two, or three injections of 1.5 ml of PCSE (group A) both before and after challenge. As controls, sera from the gerbils injected with 1.5 ml of UPCSE (group D) or with 9‰ NaCl (group E) were used either before or after challenge.

The first injection of a full-dose vaccine (1.5 ml of PCSE) induced a strong humoral response in the vaccinated animals (Fig. 5, lane 1). A slight amplification was observed after the second injection (Fig. 5, lane 2), but a third injection did not induce major changes (Fig. 5, lane 4), except for the increased amount of high-molecular-weight antigen (200,000) precipitated. In contrast with results obtained by immunofluorescence assays, the virulent challenge after two or three injections did not significantly modify the quantitative and/or qualitative antibody response to the *B. divergens* Rouen 1987 antigens (Fig. 5, lanes 3 and 5).

The immunoprecipitation assays done with the sera from control gerbils injected with 1.5 ml of UPCSE or NaCl demonstrated that these animals did not develop any humoral response, except against a 70-kDa protein that was present before and after challenge (Fig. 5, lanes 6 to 9). This 70-kDa protein corresponds to a parasitic heat shock protein (unpublished data). In addition, a nonvaccinated gerbil surviving *B. divergens* Rouen 1987 infection showed an antibody-mediated response (Fig. 5, lane 10) very similar to that of the gerbils vaccinated with one or two injections of 1.5 ml of PCSE. Identical results were obtained with all specimens of gerbil sera tested in this vaccine trial.

DISCUSSION

Babesial exoantigens can be detected in in vitro culture supernatants as well as in plasma of animals acutely infected with *Babesia* sp. (31, 32). *Babesia* major exoantigens from in vitro cultures were reported to be in the range of 30 to 80 kDa for *B. bovis* (8, 9, 17, 25), 40 to 90 kDa for *B. bigemina* (25, 39), and 15 to 200 kDa for *B. canis* (23, 26; G. Bissuel,

TABLE 2. Variance analysis of the reciprocal survival times in gerbils vaccinated with two or three injections of a full vaccine dose (1.5 ml of PCSE) or a 1:5 diluted vaccine dose (0.3 ml of PCSE) or a 1:25 diluted vaccine dose (0.06 ml of PCSE)

Variation source	Square sum	df	Mean square	Snedecor *F*	Significance
Principal effects	0.3005265	3	0.1001755	36.005	0.0000
Dilution[a]	0.2912647	2	0.1456323	52.344	0.0000
Injection[b]	0.0155924	1	0.0155924	5.604	0.218
Interaction	0.162634	2	0.0081317	2.923	0.630
Dilution × injection	0.162634	2	0.0081317	2.923	0.630
Residue	0.1391116	50	0.0027822		
Total (corrected)	0.4559015	55			

[a] Dilutions of vaccine dose.
[b] Number of injections (two or three).

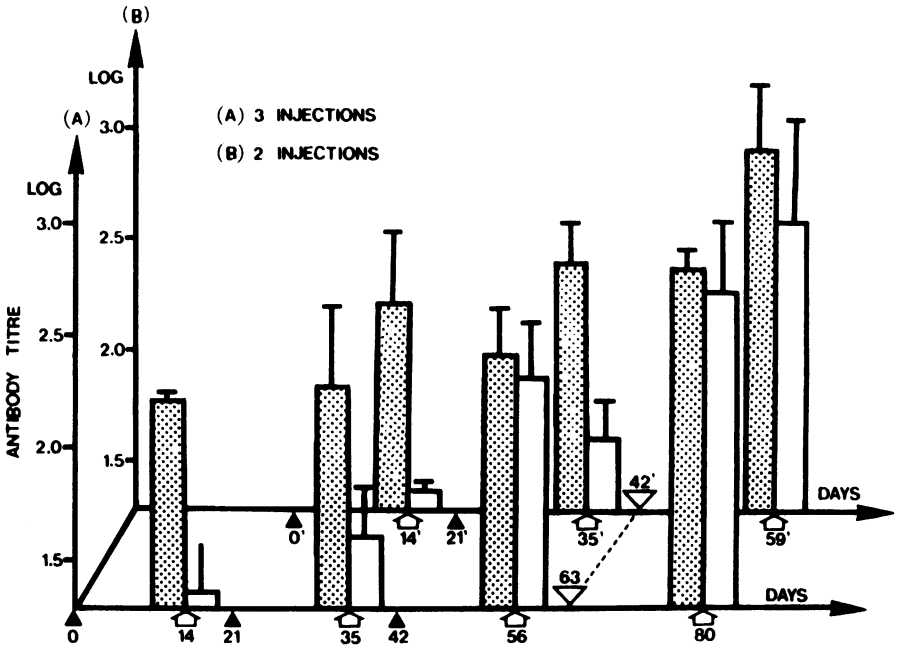

FIG. 4. Antibody titers in gerbils immunized with *B. divergens* Rouen 1987 in vitro culture supernatants. In protocol A, gerbils were vaccinated (▲) at days 0, 21, and 42, blood was collected for serology (⌓) at days 14, 35, 56, and 80, and gerbils were challenged by inoculation of 10^6 gerbil erythrocytes infected with *B. divergens* Rouen 1987 (▽) at day 63. In protocol B, gerbils were vaccinated (▲) at days 0′ and 21′, blood was collected (⌓) at days 14′, 35′, and 59′, and gerbils were challenged (▽) at day 42′. The shaded bars show the antibody levels of the gerbils vaccinated with a full vaccine dose (1.5 ml of PCSE), and the open bars show the antibody levels of the gerbils vaccinated with a 1:5 diluted vaccine dose (0.3 ml of PCSE).

thesis, Conservatoire National des Arts et Métiers, Lyon, France, 1986).

Winger et al. (40) reported that in their *B. divergens* in vitro culture system, the exoantigens were not able to induce protection against a challenge with the homologous strain. Our experiments, however, emphasized that a high degree of protection of gerbils against the homologous *B. divergens* isolate can be obtained with exoantigens from our long-term

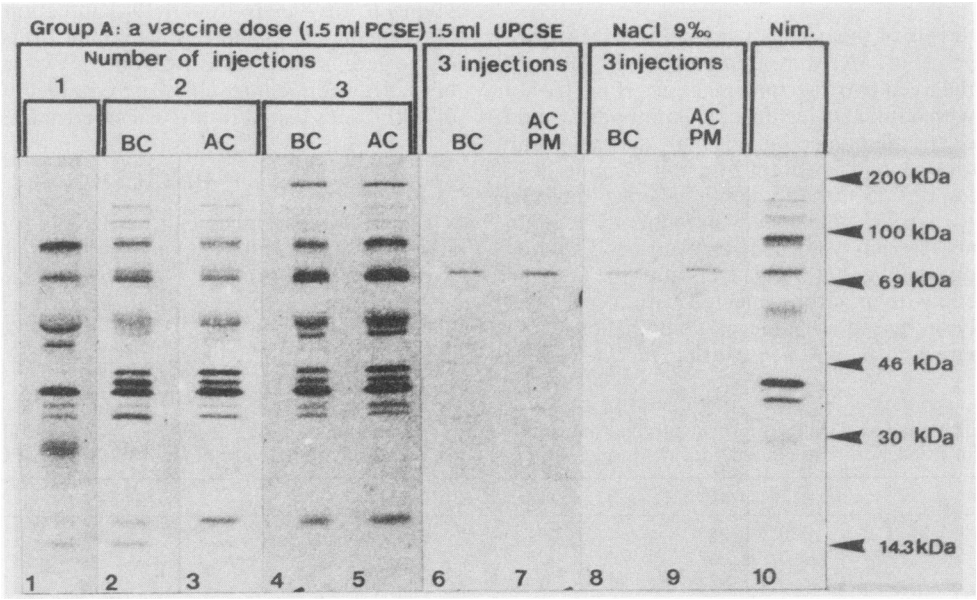

FIG. 5. Immunoprecipitations of *B. divergens* Rouen 1987 proteins by sera from vaccinated and control gerbils. The proteins were immunoprecipitated with sera from gerbils which received one injection of 1.5 ml of PCSE (lane 1), two injections of 1.5 ml of PCSE (lane 2, before challenge [BC]; lane 3, after challenge [AC]), three injections of 1.5 ml of PCSE (lane 4, before challenge; lane 5, after challenge), three injections of 1.5 ml of UPCSE (lane 6, before challenge; lane 7, after challenge and postmortem [PM]), or three injections of 9‰ NaCl (lane 8, before challenge; lane 9, after challenge and postmortem). Lane 10 shows the *B. divergens* proteins immunoprecipitated by naturally immune gerbil sera (Nim). Molecular mass standards are indicated at the right.

in vitro culture. Furthermore, the immunoprotective potential of the supernatant exoantigens did not decrease after they were frozen for several months (data not shown). High protection of gerbils that was obtained with culture supernatants did not correlate with high levels of anti-*B. divergens* serum antibodies, as detected by immunofluorescence assays. These data are in contrast to those previously reported, in which high *B. divergens* antibody titers were obtained without a good immunoprotection of the vaccinated animals (40). In this last case, the use of Freund adjuvant might be responsible for the high humoral response of gerbils since it has been demonstrated that this adjuvant induced high levels of antibodies of very low affinity (11). In our experiments, Quil A saponin was selected because this adjuvant induces the production of high-affinity antibodies against undenatured antigens and is, therefore, preferable to Freund adjuvant in a vaccination regimen using exoantigens (11). Furthermore, injection of cattle with Freund adjuvant alone may produce a false-positive serological reaction when the cattle are tested for tuberculinization; so, in our experience, the use of this particular adjuvant in a vaccine against *Babesia* sp. was not further explored. It is suggested that the very high protection observed in gerbils (≈100% survival in vaccinated animals) after a homologous challenge was achieved because of the concomitant use of culture supernatants with high contents of parasite proteins and Quil A saponin as the adjuvant. The *B. divergens* exoantigens confer a strong protection against babesiosis as previously observed for *B. bovis*, *B. bigemina* (15, 25, 32, 34), and *B. canis* (26, 27) exoantigens. Such a vaccine, free of *Babesia* cells, cannot initiate severe clinical disease by reversed virulence as sometimes observed with multiple-passaged or irradiated vaccines (R. D. Harrington et al., Anaplasmosis Babesiosis Network Newsl. **1**:4–5, 1989).

Within the framework of this vaccination trial with culture-derived exoantigens of *B. divergens*, it was important to identify the exoantigens which induced the antibody-mediated immune response. The immunoprecipitation performed with the immune sera from three different hosts, using [^{35}S]methionine-radiolabeled in vitro culture supernatants of *B. divergens* Rouen 87, demonstrated that the antibody responses against the babesial exoantigens were very similar in these three hosts. Five immunodominant exoantigens of 37, 46, 50, 70, and 90 kDa were immunoprecipitated by all sera. Immunoprecipitation assays performed on radiolabeled *B. divergens* in vitro cultures with polyvalent anti-*B. divergens* Rouen 87 sera from different hosts surviving natural or experimental babesiosis infections demonstrated that the immunodominant polypeptides were recognized whatever the serum tested. The few immunoprecipitated exoantigens observed in our experiments are in agreement with the data of Hines et al. (7), who showed that among the merozoite proteins of *B. bovis*, only some were immunodominant and likely to be implicated in the protective immunity. The conservation of the antibody response against *B. divergens* proteins in naturally and experimentally infected hosts suggests the importance of these immunodominant exoantigens in the development of an anti-*B. divergens* vaccine.

Since only a little information is available about the antigenic diversity of *B. divergens* (28), we will in a future study determine whether the differences in geographical origin of *B. divergens* isolates are or are not correlated with variation in the pattern of immunodominant parasitic polypeptides. Further immunization trials in gerbils and in cattle will monitor the effectiveness of *B. divergens* exoantigens against heterologous challenge to confirm the usefulness of these *B. divergens* exoantigens for the development of a vaccine in Europe.

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