INFECTION AND IMMUNITY, Feb. 1993, p. 734–741
0019-9567/93/020734-08$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 61, No. 2

Cellular and Humoral Immune Responses Induced in Cattle by Vaccination with *Babesia divergens* Culture-Derived Exoantigens Correlate with Protection

ALEXIS VALENTIN,[1†] ERIC PRECIGOUT,[1‡] MONIQUE L'HOSTIS,[2] BERNARD CARCY,[1] ANDRE GORENFLOT,[3] AND JOSEPH SCHREVEL[1,4*]

Laboratoire de Biologie Cellulaire, URA Centre National de la Recherche Scientifique 290, Pineau, F-86022 Poitiers Cedex,[1] Service de Parasitologie, Ecole Nationale Veterinaire de Nantes, F-44000 Nantes,[2] Laboratoire de Biologie Cellulaire, Faculté de Pharmacie, F-34060, Montpellier Cedex,[3] and Laboratoire de Biologie Parasitaire et Chimiotherapie, URA Centre National de la Recherche Scientifique 114, Museum National d'Histoire Naturelle, Rue Buffon, F-75231 Paris Cedex 05,[4] France*

Received 8 June 1992/Accepted 18 November 1992

Previous results with the *Babesia divergens* gerbil vaccination model were extended in studies with cattle. Two calves were vaccinated with culture-derived *B. divergens* exoantigens, and two others were treated with control supernatant; both preparations were adjuvanted with Quil-A saponin. A parasite-specific humoral response was observed after the first vaccine injection and was boosted by two succeeding vaccine injections. Sera from the two vaccinated calves immunoprecipitated eight major parasitic proteins (with molecular masses ranging between 17 and 110 kDa) whose patterns were close to those observed in gerbil vaccine assays. The cellular immune response, monitored by lymphoproliferation assays, was slightly delayed in comparison with the humoral response; a significant proliferation occurred only after the second vaccine injection. Mononuclear cell proliferation was dose dependent in the presence of (i) lysates of *B. divergens*-parasitized erythrocytes, (ii) exoantigens of the whole supernatant, or (iii) protective exoantigens of two low-molecular-mass fractions obtained after supernatant gel filtration chromatography. An infectious challenge was administered 3 weeks after the third vaccine injection, with 3.6×10^{10} *B. divergens*-parasitized erythrocytes. Erythrocyte count, rectal temperature, and parasitemia of the animals were monitored daily until they returned to initial values. All parameters indicated that the exoantigens induced protection from *B. divergens* infection for the two vaccinated calves.

Among the vaccine strategies developed to control *Babesia* infections, in vitro-culture-derived exoantigens and recombinant parasitic proteins have been the most frequently used (26, 27). In vitro-culture-derived exoantigens have successfully reduced the development of infection due to different babesiosis agents such as *Babesia bovis* (14, 19, 20, 28–30) and *Babesia bigemina* (20, 30); they have also reduced *Babesia canis* infections in dogs (18, 21). Protection from *B. divergens*, the agent of bovine babesiosis in Europe (10), was obtained after exoantigen vaccination in both homologous and heterologous challenges in gerbils. *B. divergens* in vitro-culture-derived exoantigens induced, in gerbils, a well-characterized humoral response apparently correlated with protection (7, 24). However, few data are available on the stimulation of cellular immunity against *Babesia* spp. by exoantigens. Monocytes and lymphocytes are obviously implicated in protection against babesiosis, and protective antibodies are induced primarily as a result of helper T-cell activity (9). Recovery from *Babesia* infections is T lymphocyte dependent, or was at least in a study with rodents in which spleen cells transferred from an animal protected against *Babesia rodhaini* conferred protection to a naive animal (36). Even though some important results about implication of T lymphocytes in *B. bovis* infections have been recently published (1), the involvement of these cells in vaccine protection against *B. divergens* has not yet been investigated. Moreover, most of the studies of *B. divergens* concern immunization of cattle with crude or partially purified lysate of infected erythrocytes rather than with culture-derived exoantigens (31, 32).

In the present work, *B. divergens* exoantigens, tested on calves, were shown to induce parasite-specific humoral and cellular responses. These in vitro-culture-derived exoantigens, injected into splenectomized calves, also conferred good protection from infection as confirmed by weak development of pathology in the vaccinated animals. Moreover, the results here demonstrate the validity of the gerbil model for further studies of vaccination against *B. divergens*. This laboratory model represents a powerful tool in the selection of the best immunoprotective antigens, since the protection from disease observed in the laboratory with gerbils is also found in the field with cattle.

MATERIALS AND METHODS

B. divergens in vitro culture and radiolabelling assays. The *B. divergens* Rouen 1987 isolate was maintained in vitro in human erythrocytes as previously described (6). Radiolabelling experiments were performed after growth of the parasite in vitro in the presence of methionine-free culture medium (RPMI 1640 without methionine; GIBCO, Paisley, Scotland) supplemented with 10% human serum and 1.85 MBq of

* Corresponding author.
† Present address: Laboratoire de Parasitologie et d'Immunologie, Université de Montpellier I, Faculté de Pharmacie, F-34060 Montpellier Cedex 1, France.
‡ Present address: Laboratoire de Biologie Cellulaire, Université de Montpellier I, Faculté de Pharmacie, F-34060 Montpellier Cedex 1, France.

[^{35}S]methionine (specific activity, 37 TBq/mmol; Amersham International Plc, Little Chalfont, Scotland) per ml. After 12 h of incubation, parasitized erythrocytes (PRBC) and supernatants were collected and stored at −80°C (6, 7).

Humoral responses and antigen characterization. Indirect immunofluorescence assays (IFA) were performed with infected gerbil erythrocytes obtained after cardiac puncture (7). Briefly, the erythrocytes were washed twice in phosphate-buffered saline (PBS) (0.15 M NaCl, 5 mM Na_2HPO_4, pH 7.2) containing 0.5% (wt/vol) bovine serum albumin (BSA), coated on glass slides, air dried, and acetone fixed (at −20°C for 15 min). The fixed slides were incubated (at 37°C for 30 min in a moist chamber) with increasing dilutions (from 1:20 to 1:25,000) of calf sera and then washed. Specific antibodies were revealed by using fluorescein isothiocyanate-labelled rabbit anti-bovine immunoglobulins (Nordic, Tilburg, The Netherlands).

[^{35}S]methionine-radiolabelled PRBC were immunoprecipitated with sera obtained from vaccinated or control calves, as previously described (6). During the entire vaccination protocol, the sera were aliquoted and stored at −80°C. They were then tested on the same day for immunoprecipitation experiments. The immune complexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide gels) according to the method of Laemmli (11). The gels were then dried and autoradiographed on X-Omat XAR-5 films (Eastman Kodak Co., Rochester, N.Y.).

Vaccine preparation. For vaccine preparation, the exoantigens were prepared from in vitro culture supernatants. When the parasitemia reached 40%, the whole content of a 75-cm^2 culture flask (Nunc, Roskilde, Denmark) was collected and centrifuged (1,000 × *g* for 10 min). To discard any membrane contaminants from the supernatant, a new centrifugation (40,000 × *g* for 30 min) was performed and the resulting supernatant was filtered through a 0.22-μm-pore-size membrane (Gelman Sciences, Inc., Ann Arbor, Mich.). Controls consisted of supernatants obtained from healthy erythrocytes treated under the same conditions. Parasitized and control supernatants were concentrated 15-fold with Amicon B15 (Amicon, Grace, France) and stored at −80°C until utilization.

A vaccine dose consisted of 2 ml of concentrated supernatants (parasitized or control) mixed with 1 mg of Quil-A saponin (Superfos; Biosector) just before injection.

Vaccination and challenge procedures. Four splenectomized Friesian calves (20 months old) bred in a tick-free cow barn since the age of 8 weeks were injected subcutaneously, two with *B. divergens* exoantigen preparation and the others with control preparation. All calves received three injections at 21-day intervals. From the first injection until challenge, jugular blood was taken twice weekly in order to monitor the cellular and humoral responses. Three weeks after the last injection, the four cows were challenged with 3.6 × 10^{10} bovine PRBC obtained from a splenectomized calf. This calf had been previously infected by intravenous injection of gerbil PRBC (12). After the infectious inoculation, the following parameters were determined daily: parasitemia, packed cell volume (PCV), erythrocyte number (by using a hemacytometer [Coultronics, Paris, France]), and rectal temperature.

Lymphocyte stimulation assay. Venous blood collected in heparinized tubes (F-38241 Meylan; Becton Dickinson) was centrifuged (1,500 × *g* for 20 min). The buffy coat was collected and diluted twice with culture medium (RPMI 1640 [GIBCO] supplemented with 10 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [Sigma], 1 mM L-glutamine [GIBCO], 50 IU of penicillin [GIBCO] per ml, 50 μg of streptomycin [GIBCO] per ml, and 50 μM 2-mercaptoethanol [Sigma]). Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, collected, and resuspended in culture medium with 10% fetal calf serum (GIBCO). After counting, the viability of the recovered cells was estimated by the trypan blue exclusion test. The solution was then adjusted to 4 × 10^6 viable cells per ml, and 100 μl was distributed in each well of a 96-well flat-bottom culture plate (Nunc). After the addition of the antigens or mitogens, the final volume of each well was adjusted to 200 μl. The assays were performed in quadruplicate. Three days (for mitogens) or 5 days (for antigens) later, 37 kBq of [^{3}H]hypoxanthine in 25 μl of culture medium with 10% fetal calf serum was added in each well. Eighteen hours later, the incorporation of [^{3}H]hypoxanthine was estimated by collecting the cells with a Skatron cell harvester (Skatron). Results are presented as the variation of the stimulation index, defined as (mean cpm in the presence of *B. divergens* antigen − blank)/(mean cpm in the presence of control antigen − blank), where cpm is counts per minute.

A stimulation index higher than 10 was considered to indicate a significant stimulation of lymphocytes in the presence of the corresponding antigen (22).

To estimate the biological status of the purified mononuclear cells, their responses to mitogens were tested with concanavalin A (1 and 5 μg/ml in culture medium; Sigma) and phytohemagglutinin (at 1 and 10 μg/ml; Sigma).

Antigen preparation for lymphocyte stimulation. The antigens tested in the lymphocyte stimulation assay were prepared either with infected erythrocytes or with in vitro culture supernatants from the isolate *B. divergens* Rouen 1987. PRBC (parasitemia, 40%) were water lysed at a dilution of 1:10 (vol/vol) for 30 min under constant stirring. The lysates were centrifuged (100,000 × *g* for 1 h), filtered (0.22-μm-pore-size membranes), and stored (−80°C) until utilization. Crude parasitized in vitro culture supernatants (CPSN) were collected (parasitemia, 40%), centrifuged (100,000 × *g* for 1 h), and processed as PRBC lysates. Both parasitic antigens (CPSN and lysed PRBC) were added at final quantities of 1 and 10 μl per well for lymphocyte stimulation.

On the other hand, partially purified exoantigens were tested by the lymphocyte stimulation assay. They were obtained from *B. divergens* in vitro culture in a semidefined medium (34) and partially separated by gel filtration (fast-performance liquid chromatography with a Superose 12 column [Pharmacia] equilibrated with ammonium acetate [25 mM, pH 7.3]; flow rate, 0.3 ml/min) (24a). Of the four defined fractions, only the two (F_3 and F_4) containing low-molecular-mass proteins were tested. Fraction F_3 contained about seven parasitic proteins ranging from 55 to 17 kDa, and fraction F_4 contained four parasitic proteins ranging from 43 to 17 kDa. Each fraction, resulting from the gel filtration chromatography of 200 ml of crude in vitro culture supernatant (parasitemia, 30%), was lyophilized to dryness and solubilized in PBS before being tested as the stimulating antigen. Final protein concentrations of 10 and 25 μg/ml were used for lymphocyte stimulation.

Controls consisted of normal erythrocyte lysates and vitro culture medium collected after one night of incubation with normal erythrocytes. A control supernatant was also prepared with noninfected erythrocytes maintained in semidefined medium overnight and fractionated by fast-perfor-

TABLE 1. Antibody titers of cattle during the vaccination trial and infectious challenge estimated by indirect immunofluorescence

Injection[a]	Day	Antibody titer in:		
		Vaccinated cow 1	Vaccinated cow 2	Controls
None	−10	<1:20[b]	<1:20	<1:20
	0[c]	<1:20	<1:20	<1:20
Vaccine	6	<1:50[c]	<1:100	<1:20
	10	<1:200	<1:400	<1:20
	14	<1:400	<1:400	<1:20
	18	<1:400	<1:400	<1:20
	21	<1:400	<1:400	<1:20
Vaccine	24	<1:400	<1:400	<1:20
	28	<1:400	<1:800	<1:20
	32	<1:800	<1:800	<1:20
	36	<1:1,400	<1:1,200	<1:20
	42	<1:1,400	<1:1,200	<1:20
Vaccine	46	<1:1,200	<1:1,200	<1:20
	50	<1:1,400	<1:1,200	<1:20
	54	<1:1,400	<1:1,200	<1:20
	58	<1:1,600	<1:1,600	<1:20
	63	<1:1,600	<1:1,600	<1:20
Infectious dose	65	<1:1,600	<1:1,600	<1:20
	67	<1:3,200	<1:1,600	<1:50[c]
	69	<1:3,200	<1:3,200	<1:200
	71	<1:6,400	<1:3,200	<1:400
	73	<1:6,400	<1:6,400	<1:800
	75	<1:12,800	<1:6,400	<1:1,400
	77	<1:12,800	<1:12,800	<1:1,600

[a] The vaccine injections were administered on days 0 (after blood collection), 21, and 42; the infectious dose was administered on day 63.
[b] The first dilution tested was 1:20.
[c] Staining at a dilution higher than or equal to 1:50 was considered to be positive.

mance liquid chromatography; elution volume observed for parasitized fractions was used as the reference. Control F_3 and F_4 fractions were lyophilized to dryness and used in stimulation assays at 10 and 25 μg/ml (protein concentrations). The protein concentrations of supernatants (either parasitized or control) and of partially fractionated antigens were determined by the Lowry method, with BSA (1 mg/ml) as the standard; the optical densities of the solutions were read at 660 nm (13).

RESULTS

Humoral response. The humoral response of the calves was monitored by IFA and by immunoprecipitation assays. IFA revealed a constant increase, during the entire vaccination period, in the titers of the antibodies directed against *B. divergens* (Table 1). These titers were always lower than 1:2,000 for the vaccinated calves and never reached 1:20 (first dilution tested) in the controls. The emergence of specific antibodies was observed 10 days after the first injection, and each vaccine injection boosted the humoral response. This boosting effect developed about 2 weeks after the injection. After challenge, titers increased to a high degree in all the animals. However, control titers were always lower than those of vaccinated calves (Table 1).

Immunoprecipitations of [^{35}S]methionine-radiolabelled *B. divergens* proteins were performed with the sera obtained from vaccinated and control calves. Following the first injection, two or four proteins were immunoprecipitated, depending on the vaccinated calf. Their relative molecular masses were 90 and 37 kDa (Fig. 1, V2, lanes 7, 14, and 21) and 90, 47, 37, and 35 kDa (Fig. 1, V1, lanes 7, 14, and 21). Responses against these antigens were highly amplified after the second vaccine injection. Following this second injection, new major antigens were precipitated, a 110-kDa protein and a 70-kDa protein which could be the hsp70 previously described for *B. divergens*-infected erythrocytes (Fig. 1, V1 and V2, lanes 28, 35, and 42) (2). After the second injection, the patterns of the recognized proteins were similar for the two calves, and the third injection only amplified the responses against all parasitic proteins (Fig. 1, V1 and V2, lanes 49, 56, and 63).

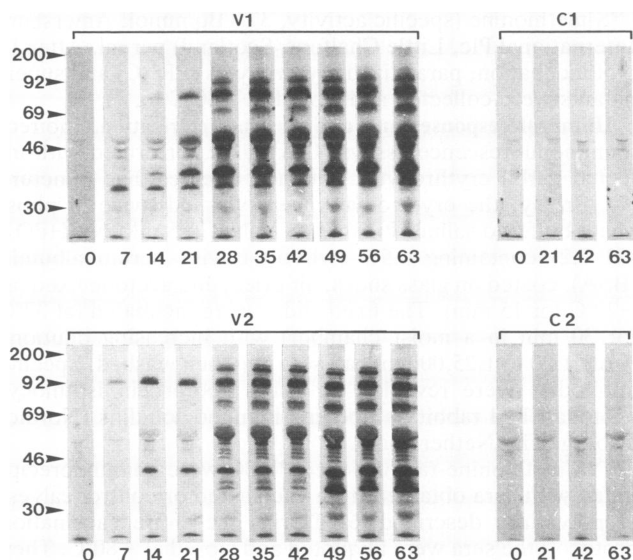

FIG. 1. Analysis of the humoral responses of cattle by immunoprecipitation assays of [^{35}S]methionine-radiolabelled *B. divergens* antigens. Gels contained 10% acrylamide. Lane numbers correspond to the day following the first vaccine injection. V1 and V2 show results for vaccinated calves, and C1 and C2 show results for controls. Numbers on the left are molecular masses (in kilodaltons).

Two low-intensity bands were seen during the experiment in controls (Fig. 1, C1 and C2, lanes 0, 21, 42, and 63). They could correspond to nonspecific adsorption since they were also precipitated by sera obtained from calves before the experiment began (Fig. 1, C1, C2, V1, and V2, lanes 0).

Cellular response. The lymphoproliferative responses of the calves were determined in parallel to the humoral responses. No alteration of the responses to concanavalin A or phytohemagglutinin compared with results previously obtained from the same calves was observed (data not shown), indicating that the vaccine preparation did not seem to have any immunosuppressive effect. The protein concentration of the crude control supernatant was about 1.7-fold lower than that of the CPSN (6.2 versus 10.5 g/liter, respectively). Whatever the antigen tested (CPSN or lysed PRBC), the first vaccine injection did not lead to a significant stimulation of mononuclear cell growth for either vaccinated or control calves. After the second vaccine injection, the stimulation indices increased to higher than 10 for vaccinated animals, both for lysed PRBC (Fig. 2A) and for the CPSN (Fig. 2B); for lysed PRBC, the stimulation indices reached about 20.

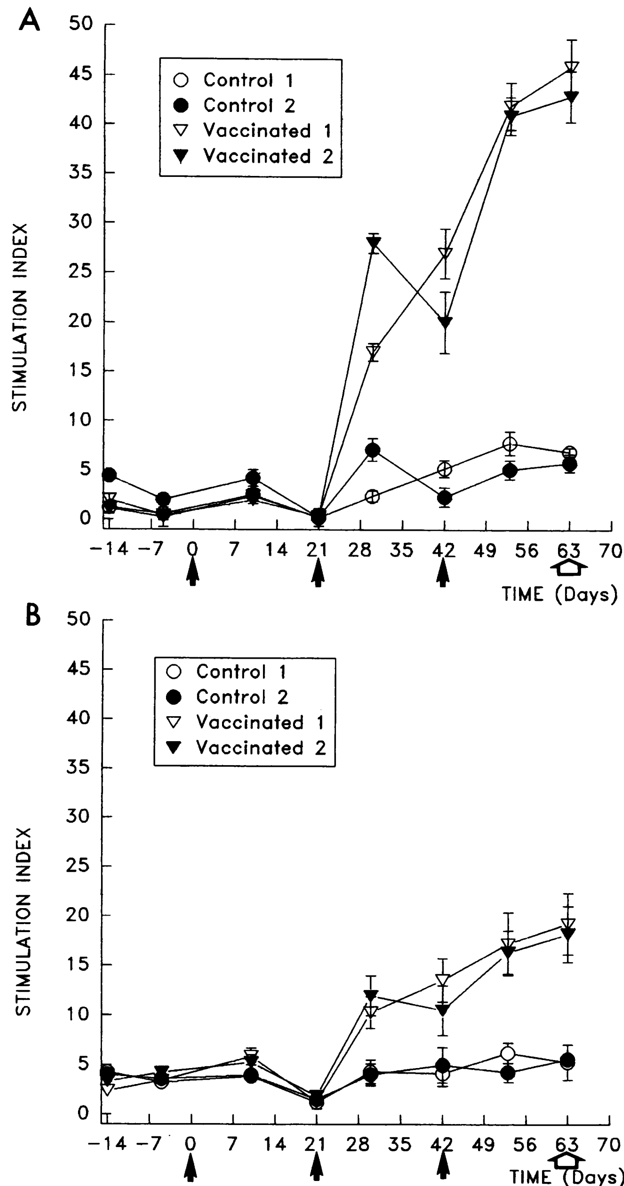

FIG. 2. Cellular responses of cattle analyzed by lymphoproliferation in the presence of crude *B. divergens* antigens. Mononuclear cells were stimulated by crude *B. divergens* antigens (lysed PRBC [A] and CPSN [B]). For clarity, only the response obtained for 10 μl of each antigen has been represented. Values correspond to the mean proliferation indices ± the standard deviations obtained in four wells. Solid and open arrows indicate the days of vaccination and challenge, respectively.

On the other hand, the proliferation values observed for controls increased very weakly. The third vaccine injection also had a boosting effect on lymphocyte proliferation, which reached values slightly lower than 20 for the CPSN and higher than 40 for lysed PRBC (Fig. 2A and B). This boosting effect seemed to be greater than that observed during the humoral response. Stimulation indices remained low for controls even after the third vaccine injection.

A partial dissection of the parasitic proteins that induced lymphoproliferation has been obtained by testing partially fractionated *B. divergens* exoantigens. The two Superose 12 fractions that contained few proteins were tested (24a). As for crude antigen, the first vaccine injection (tested 21 days later) seemed to have no effect on specific lymphoproliferation. Only the second vaccine injection induced detectable stimulation of mononuclear cell growth in the presence of the antigens (tested on day 42), with stimulation indices higher than 10. The third vaccine injection had a boosting effect which appeared to be lower than that observed with PRBC. It also evidenced a higher stimulating effect of proteins (ranging from 35 to 14 kDa) that were present in fraction F_4, since they seemed to be more stimulative for the two vaccinated calves (stimulation index, about 25; Fig. 3B). F_3 proteins (ranging from 50 to 14 kDa) gave a stimulation index which was only about 15 (Fig. 3A).

Response to virulent challenge. During the entire vaccination period, no general or local reactions were observed. On the 63rd day following the first vaccine injection, the four calves were intravenously injected with 3.6×10^{10} bovine PRBC. After 3 days, the four cows stopped feeding; vaccinated calves 2 and 1 stopped for 1 and 2 days, respectively, and controls stopped for 5 days. Hemoglobinuria was found only in controls, for 12 and 7 days in control cows 1 and 2, respectively. However, the more useful data were PCV, parasitemia, and rectal temperature.

For the two vaccinated cows, the relative PCV decreased to about 20 to 25% of the initial values and came back to initial values within 20 days after infectious challenge (Fig. 4). For controls, the PCV decreased to 40 and 70% of initial values (control cows 1 and 2, respectively), and a return to the initial values was observed 40 days later (Fig. 4). On days 4 and 6 postchallenge, control cow 1 had a PCV under 10%, which is considered to be a lethal value; however, the animal survived. The values obtained from erythrocyte counting correlated with those of PCV (data not shown).

Parasitemia was very low in vaccinated calves, and its course apparently correlated with PCV decreases; parasites were found for a week at a level that was always lower than 1% (Table 2). For controls, results were different according to the animal; control cow 1 (high decrease of PCV) exhibited parasitemia higher than 30%, but, in control cow 2 (low decrease of PCV), it never exceeded 3.5%. However, for both controls, *B. divergens* was present as long as 3 weeks.

Rectal temperatures were determined for a shorter period, as the duration of hyperthermia was shorter than those of the PCV decrease and parasitemia. Vaccinated cows exhibited temperatures higher than 39°C for 3 days (Table 3). On the other hand, the duration of hyperthermia was longer for controls; control cow 1 never reached a high temperature, probably because it was near death.

DISCUSSION

Despite important economic losses in Europe, no efficient immunoprophylaxis has been yet developed against *B. divergens* babesiosis. To extend our previous results on the protection of gerbils against homologous and heterologous challenges after vaccination with exoantigens (6, 24), an assay with the *B. divergens* bovine host was performed. Splenectomized calves injected with *B. divergens* exoantigens exhibited immunological responses similar to those obtained during gerbil assays. Despite the fact that these animals might not represent what might happen in intact cattle, they were splenectomized in order to increase the intensity of all the symptoms of babesiosis as reported for *B. ovata* (3), *B. bovis* (5), and *B. divergens* (23). Thus, splenectomy was used to induce a partial immunosuppression close

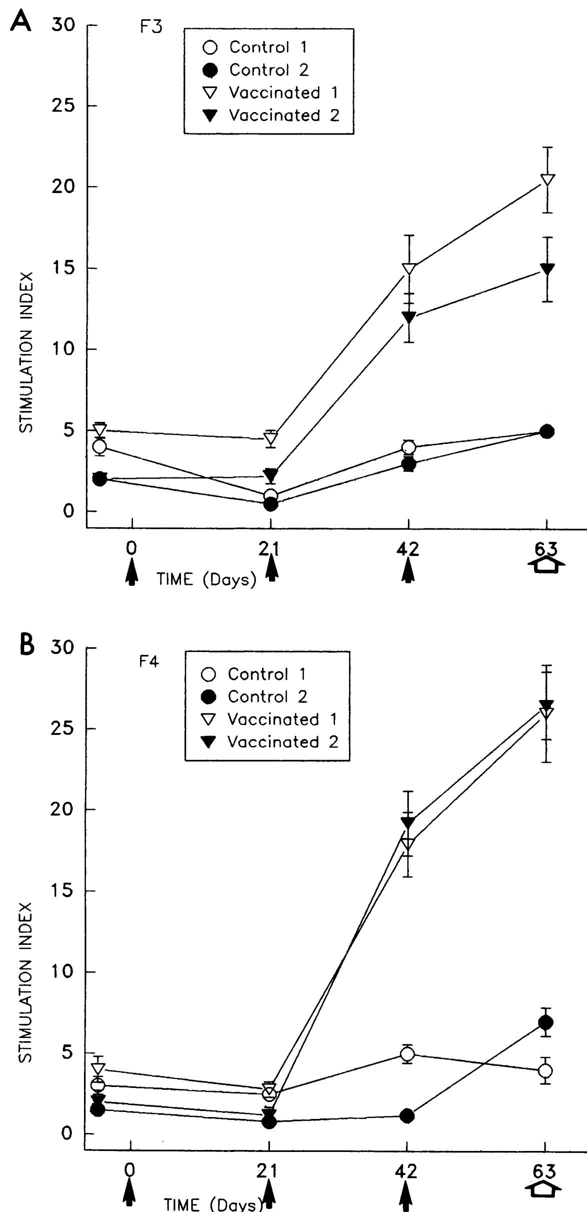

FIG. 3. Lymphocyte proliferation in the presence of *B. divergens* fractionated exoantigens. Proliferation was measured in the presence of the proteins of either F_3 (A) or F_4 (B). Values represent the mean stimulation indices ± the standard deviations obtained in four wells. Only the results obtained for 25 μg/ml are represented. Solid and open arrows indicate the days of vaccination and challenge, respectively.

to what is often found for animals developing a lethal *B. divergens* babesiosis (pregnancy, high yield of lactation, and so on).

Antibodies specific for *B. divergens* were detected in the sera of vaccinated animals after the 6th and 10th days following the first injection. IFA titers were boosted by each vaccine injection but remained low (1:1,600) until challenge. These titers were similar to those obtained with gerbils (6) and consistent with those reported during vaccination against *B. bovis* with culture-derived exoantigens (33). The presence of Quil-A adjuvant in our vaccine preparation confirmed the efficiency of this adjuvant for vaccination against babesiosis (29) since assays previously performed by others with Freund adjuvant led to lower protection from disease (35).

Immunoprecipitation assays with [^{35}S]methionine-radiolabelled *B. divergens* antigens performed just before challenge showed profiles close to those observed for vaccinated gerbils (7). After the first injection, the responses were as fast as those obtained with IFA, with only slight differences between each vaccinated animal. After the second vaccine injection, the responses became similar, and two of the recognized peptides, at least, are characterized: (i) a 70,000-M_r peptide similar to that recently described as hsp70 of *B. divergens* (2) and (ii) a 37,000-M_r membrane-bound glycoprotein (Bd37) (24a). The third vaccine injection amplified the specific response. Although the vaccine preparation contained numerous parasitic proteins, the humoral responses were mainly directed against eight peptides. These results seemed to be in agreement with those reported for *B. bovis* and *B. bigemina*, in which eight and five dominant surface antigens, respectively, were recognized by specific immune sera obtained after immunization with merozoite proteins (4, 15).

From preliminary experiments, the infectious dose for challenge had to be between 10^9 and 10^{10} *B. divergens*-parasitized erythrocytes to induce significant variations of PCV and temperature in splenectomized calves. This dose, equivalent to that reported for *B. bigemina* infectious challenges (16), led to the development of parasitemia in all calves. However, because of the small number of animals in our experiment, no statistical analysis can be made. Nevertheless, the alterations of parameters in controls were drastically higher than in vaccinated animals, which recovered more quickly than the former. The *B. divergens* exoantigens induce a strong protective effect, since alteration of PCV in our vaccination trial is equivalent to that reported for other *B. divergens* vaccination trials in which the challenges were performed with 100-fold-fewer parasites (31, 32).

The cell-mediated immune response during vaccination was measured during the entire assay. In order to clarify the results obtained, we presented them as the stimulation index variation; this could amplify the differences between control and parasitized preparations because of differences in protein concentration between the two preparations. However, this difference is rather low (1.7-fold more in the CPSN than in the control supernatant), and the results obtained with fractions F_3 and F_4 (in which protein concentrations are the same in control and parasitized preparations) may contradict this objection. Although the mononuclear cells of the vaccinated animals were primed only with exoantigens, they responded to both the CPSN and crude PRBC lysates. The cellular response was, however, delayed in comparison with the humoral response, since it became significant only a week after the second vaccine injection. The third injection boosted the cell proliferation in a stronger manner for PRBC than for the CPSN. As the responses were maximal 5 days after presentation and were dose dependent (data not shown), they can be considered to be antigen specific. The difference in stimulation effect between the CPSN and lysed PRBC could be due (i) to the existence of immunosuppressive parasite-derived factors in supernatants, as described for *Plasmodium falciparum* (25); (ii) to a higher concentration of stimulating peptides in PRBC; or, most likely, (iii) to the presence of a larger amount of parasitic membrane proteins in lysed PRBC than in the CPSN. Among the fractions obtained after gel filtration chromatography, we

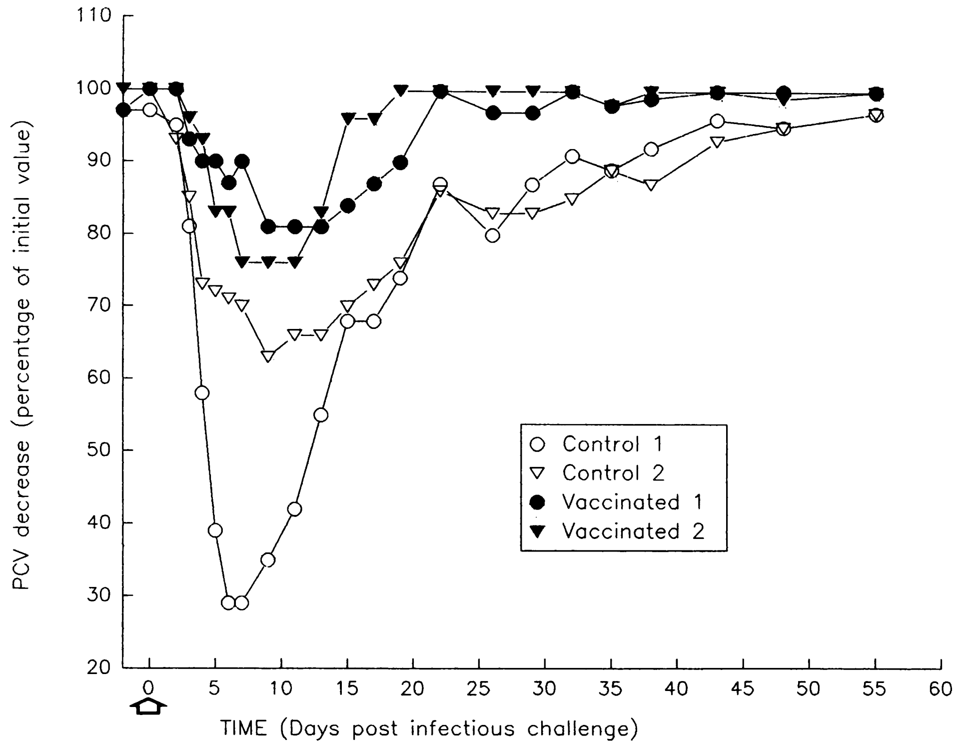

FIG. 4. Alterations of PCV values observed after challenge (day 0). The values were determined daily until day 14 and then every 3 days. Reference values of 100% were calculated as the means of six values obtained before any vaccine injections. The first value on the graph was determined 2 days before challenge (open arrow).

tested the stimulating effect of F_3 and F_4 (which contained Bd37, a palmitoylated membrane-bound glycoprotein [24a]) on calf lymphocytes. Both fractions stimulated mononuclear cell proliferation in an antigen-dependent manner, with a higher stimulation index for F_4, in which only five major (including Bd37) immunogens were present. This agrees with what has been reported for *B. bovis*, with which preparations enriched in parasitic membranes have a higher stimulating effect on proliferation of cells primed in vivo with merozoite antigens instead of soluble proteins (1). Membrane-bound proteins have been reported to be highly immunogenic and to induce immunodominant serological responses during vaccination and infection with *B. bigemina* and *B. bovis* in cattle (4, 8, 15, 17, 20). However, there is no evidence that

TABLE 2. Estimation of the percent parasitemia in cattle after infectious challenge

Day	% Parasitemia[a] in:			
	Control cow 1	Control cow 2	Vaccinated cow 1	Vaccinated cow 2
0	0	0	0	0
1	<0.1	<0.1	<0.1	<0.1
2	4	0.5	<0.1	<0.1
3	11.5	0.75	<0.1	<0.1
4	18	0.9	0.4	<0.1
5	22	1.2	0.7	0.17
6	25	1.8	0.5	0.19
7	29	2.1	0.2	0.2
8	>40[b]	2.5	0.1	
9	40[b]	3	<0.1	U[c]
11	>40[b]	3	U	U
13	>25[b]	2.2	U	U
15	16	2	U	U
17	9	1	U	U
19	3	0.5	U	U
21	1.2	0.2	U	U
25	<0.1	U	U	U
29	U	U	U	U

[a] In all cases, 2,000 erythrocytes were counted.
[b] Blood was hemolysed, so parasitemia was not estimated precisely.
[c] U, undetectable parasites on 2,000 erythrocytes.

TABLE 3. Pattern of rectal temperatures of challenged cattle

Day	Rectal temp[a] in:			
	Control cow 1	Control cow 2	Vaccinated cow 1	Vaccinated cow 2
−2	38.5	38.6	38.6	38.6
0	38.5	38.6	38.5	38.5
1	38.8	38.8	39.8	39.2
2	38.7	40.7	38.6	40.5
3	39.2	40.8	39.7	39
4	39.5	40.2	39.4	38.6
5	40	40.6	39.8	39.2
6	39.9	40.7	38.7	38.5
7	39.1	40	38.5	38.8
8	39.2	39.3	—[b]	38.5
9	38.9	39.5	—	—
11	38.3	39.5	—	—
13	—	38.5	—	—
15	—	—	—	—

[a] Brackets show periods of fever. The duration of fever was longer in controls than in vaccinated animals.
[b] —, Values are ±0.1°C within normal rectal temperature (38.5°C).

this humoral response confers protective immunity, but such a similarity among different *Babesia* species can emphasize the importance of these proteins as candidates in a vaccine preparation.

In this article, we report the first vaccination trial against *B. divergens* in cattle conducted with exoantigens. We clearly demonstrate that both cellular and humoral responses were induced by *B. divergens* in vitro-culture-derived exoantigens in cattle. These responses are correlated with protective immunity in immunized cattle. The characterizations of parasitic peptides inducing protective immunity are in progress (with attention to the membrane-bound glycoprotein Bd37), and the cellular immunogenicities of the peptides could be confirmed by the establishment of specific bovine T-cell lines.

ACKNOWLEDGMENTS

We are very grateful to Anne-Marie Marchand (Ecole Nationale Vétérinaire, Nantes, France) and Francoise Chevalier and Joel Derisson (URA CNRS 290, Poitiers, France) for efficient technical assistance.

This work was supported by grants from ANVAR (A88.03.041.T. 037.0), Rhône Mérieux S.A., the Ministère de la Recherche et de L'Enseignement Supérieur, the Centre National de la Recherche Scientifique, and the Fondation pour la Recherche Médicale. Alexis Valentin was a Ph.D. student supported by a fellowship from the Centre National de la Recherche Scientifique (BDI/CNRS).

REFERENCES

1. **Brown, W. C., K. S. Logan, G. G. Wagner, and C. L. Tetzlaff.** 1991. Cell-mediated immune responses to *Babesia bovis* merozoite antigens in cattle following infection with tick-derived or cultured parasites. Infect. Immun. **59:**2418–2426.
2. **Carcy, B., E. Precigout, A. Valentin, A. Gorenflot, R. T. Reese, and J. Schrevel.** 1991. Heat shock response of *Babesia divergens* and identification of the hsp70 as an immunodominant early antigen during ox, gerbil and human babesiosis. Biol. Cell **72:**93–102.
3. **Fujinaga, T.** 1982. Effect of splenectomy and dexamethasone administration on cattle experimentally infected with *Babesia ovata*. Jpn. J. Vet. Sci. **44:**71–80.
4. **Goff, W. L., W. C. Davis, G. H. Palmer, T. F. McElwain, W. C. Johnson, J. F. Bailey, and T. C. McGuire.** 1988. Identification of *Babesia bovis* merozoite surface antigens by using immune bovine sera and monoclonal antibodies. Infect. Immun. **56:** 2363–2368.
5. **Goodger, B. V., I. G. Wright, D. J. Waltisbuhl, and G. B. Mirre.** 1985. *Babesia bovis*: successful vaccination against homologous challenge in splenectomized calves using a fraction of haemagglutining antigen. Int. J. Parasitol. **15:**175–179.
6. **Gorenflot, A., P. Brasseur, E. Precigout, M. L'Hostis, A. Marchand, and J. Schrevel.** 1991. Cytological and immunological responses to *Babesia divergens* in different hosts: ox, gerbil, man. Parasitol. Res. **77:**3–12.
7. **Gorenflot, A., E. Precigout, G. Bissuel, O. Lecointre, P. Brasseur, E. Vidor, M. L'Hostis, and J. Schrevel.** 1990. Identification of major *Babesia divergens* polypeptides that induce protection against homologous challenge in gerbils. Infect. Immun. **58:** 4076–4082.
8. **Hines, S. A., T. F. McElwain, G. M. Buening, and G. H. Palmer.** 1989. Molecular characterization of *Babesia bovis* merozoite surface proteins bearing epitopes immunodominant in protected cattle. Mol. Biochem. Parasitol. **37:**1–10.
9. **James, M. A.** 1988. Immunology of babesiosis, p. 119–130. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla.
10. **Kuttler, K. L.** 1988. World-wide impact of babesiosis, p. 1–22. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
12. **Lewis, D., and H. Williams.** 1979. Infection of the Mongolian gerbil with the cattle piroplasm *Babesia divergens*. Nature (London) **278:**170–171.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
14. **Mahoney, D. F., I. G. Wright, and B. V. Goodger.** 1984. Immunization against babesiosis: current studies and future outlook. Prev. Vet. Med. **2:**401–418.
15. **McElwain, T. F., L. E. Perryman, W. C. Davis, and T. C. McGuire.** 1987. Antibodies define multiple proteins with epitopes exposed on the surface of live *Babesia bigemina* merozoites. J. Immunol. **138:**2298–2304.
16. **McElwain, T. F., L. E. Perryman, A. J. Musoke, and T. C. McGuire.** 1991. Molecular characterization and immunogenicity of neutralization-sensitive *Babesia bigemina* merozoite surface proteins. Mol. Biochem. Parasitol. **47:**213–222.
17. **Mishra, M. S., E. B. Stephens, J. B. Dame, L. E. Perryman, T. C. McGuire, and T. F. McElwain.** 1991. Immunogenicity and sequence analysis of recombinant p58: a neutralization-sensitive, antigenically conserved *Babesia bigemina* merozoite surface protein. Mol. Biochem. Parasitol. **47:**207–212.
18. **Molinar, E. M., M. A. James, I. Kakoma, C. J. Holland, and M. Ristic.** 1982. Antigenic and immunogenic status of cell culture derived *Babesia canis*. Vet. Parasitol. **10:**29–40.
19. **Montenegro-James, S., M. Ristic, M. Toro-Benitez, E. Leon, and R. Lopez.** 1985. Heterologous strain immunity in bovine babesiosis using a culture-derived soluble *Babesia bovis* immunogen. Vet. Parasitol. **18:**321–337.
20. **Montenegro-James, S., M. Toro-Benitez, E. Leon, R. Lopez, and M. Ristic.** 1987. Bovine babesiosis: induction of a protective immunity with culture-derived *Babesia bovis* and *Babesia bigemina* immunogens. Parasitol. Res. **74:**142–150.
21. **Moreau, Y., S. Martinod, and G. Fayet.** 1988. Epidemiologic and immunoprophylactic aspects of canine babesiosis in France, p. 191–196. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla.
22. **Muscoplat, C. L., A. W. Chen, D. W. Johnson, and I. Alhaji.** 1974. *In vitro* stimulation of bovine peripheral blood lymphocytes: standardization and kinetics of the responses. Am. J. Vet. Res. **35:**1557–1567.
23. **Phillips, R. S.** 1984. *Babesia divergens* in splenectomized cattle. Res. Vet. Sci. **36:**251–255.
24. **Precigout, E., A. Gorenflot, A. Valentin, G. Bissuel, B. Carcy, P. Brasseur, Y. Moreau, and J. Schrevel.** 1991. Analysis of immune responses of different hosts to *Babesia divergens* isolates from different geographic areas and capacity of culture-derived exoantigens to induce efficient cross-protection. Infect. Immun. **59:**2799–2805.

24a. **Precigout, E., et al.** Submitted for publication.

25. **Riley, E. M., O. Jobe, M. Blackman, H. C. Whittle, and M. Greenwood.** 1989. *Plasmodium falciparum* schizont sonic extracts suppress lymphoproliferative responses to mitogens and antigens in malaria-immune adults. Infect. Immun. **57:**3181–3188.
26. **Ristic, M., and I. Kakoma.** 1988. Exoantigens of *Babesia*, p. 131–142. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla.
27. **Ristic, M., and S. Montenegro-James.** 1988. Immunization against *Babesia*, p. 163–189. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla.
28. **Smith, R. D., J. Carpenter, A. Cabrera, S. D. Gravely, E. E. Erp, M. Osorno, and M. Ristic.** 1979. Bovine babesiosis: vaccination against tick-bovine challenge exposure with culture-derived *Babesia bovis* immunogens. Am. J. Vet. Res. **40:**1678–1682.
29. **Smith, R. D., M. A. James, M. Ristic, M. Aikawa, Y. Vega, and C. A. Murgia.** 1981. Bovine babesiosis: protection of cattle with culture-derived soluble *Babesia bovis* antigens. Science **212:** 335–338.
30. **Smith, R. D., E. Molinar, F. Larios, J. Monroy, F. Trigo, and M.**

Ristic. 1980. Bovine babesiosis: pathogenicity and heterologous species immunity of tick-borne *Babesia bovis* and *Babesia bigemina* infections. Am. J. Vet. Res. **41:**1957–1965.

31. **Taylor, S. M., C. T. Elliott, and J. Kenny.** 1986. *Babesia divergens* sequential exposure to heterologous tick-borne challenge of cattle immunized with a fraction of parasitized erythrocytes. J. Comp. Pathol. **96:**101–107.
32. **Taylor, S. M., J. Kenny, T. Mallon, and C. T. Elliott.** 1984. The immunization of cattle against *Babesia divergens* with fractions of parasitized erythrocytes. Vet. Parasitol. **16:**235–242.
33. **Timms, P., R. J. Dalgliesh, D. N. Barry, C. K. Dimmock, and B. J. Rodwell.** 1983. *Babesia bovis*: comparison of culture-derived vaccine, non-living antigen and conventional vaccine in the protection of cattle against heterologous challenge. Aust. Vet. J. **60:**75–77.
34. **Valentin, A., D. Rigomier, E. Precigout, B. Carcy, A. Gorenflot, and J. Schrevel.** 1991. Lipid traffic between high density lipoproteins and *B. divergens*-infected human erythrocytes. Biol. Cell **73:**63–70.
35. **Winger, C. M., E. U. Canning, and J. D. Culverhouse.** 1987. Induction of protective immunity to *Babesia divergens* in Mongolian gerbils, *Meriones unguiculatus*, using culture-derived immunogens. Vet. Parasitol. **26:**43–53.
36. **Zivkovic, D., W. Seinen, H. Kuil, C. M. G. Albers-van Bemmel, and J. E. Speksnijder.** 1984. Immunity to *Babesia* in mice. I. Adoptive transfer of immunity to *Babesia rodhaini* with immune spleen cells and the effect of mediation on the protection of immune mice. Vet. Immunol. Immunopathol. **5:**343–358.