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Protection of *Babesia bigemina*-Immune Animals against Subsequent Challenge with Virulent *Babesia bovis*

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Two groups of cattle, one previously exposed to *Babesia bigemina* and one not, were challenged with *Babesia bovis*. The group previously infected with *Babesia bigemina* was only mildly affected upon challenge with *B. bovis*, whereas four of five of the other group were severely affected. Immunoblotting studies performed in both homologous and heterologous systems showed that there were polypeptides of similar molecular weight in both species, but species-specific polypeptides were demonstrated only in *B. bovis* by the homologous *B. bovis* reaction. *B. bovis* antisera reacted avidly with *B. bigemina*-infected erythrocytes in fluorescent-antibody assays. In contrast, *B. bigemina* antisera did not cross-react with *B. bovis*-infected erythrocytes. Two groups of splenectomized calves were immunized with an enriched antigen fraction of *B. bigemina*. A third group was immunized by infection with *B. bigemina* and treatment with a drug. One of the groups of calves immunized with the antigenic fraction of *B. bigemina*, the group immunized by infection with *B. bigemina*, and a control group were challenged with *B. bovis*. All control calves died, whereas 50% of the calves immunized by infection with *B. bigemina* and 75% of the animals immunized with the *B. bigemina* antigen survived. The second group immunized with the *B. bigemina* antigen and a control group were challenged with *B. bigemina*. All control animals died by day 6, whereas 50% of the vaccinates survived, the deaths occurring on days 8 and 11. The nature of the probable protective mechanism is discussed.

In an early study, Legg (14) reported that cattle infected with *Babesia bigemina* possessed some resistance to subsequent infection with *Babesia argentina* (*Babesia bovis*), a more pathogenic organism. Thus, cattle in areas where both parasites are endemic were intentionally infected with the less harmful parasite, but the practice was discontinued after it was proved unreliable (18). Cross-protection has also been reported between various rodent *Babesia* species (5), and *B. bigemina*-immune cattle have been reported to be resistant to subsequent *Babesia major* challenge (21). More recently, Smith et al. (19) were unable to demonstrate significant protection of *B. bigemina*-immune cattle from *B. bovis* infection. Current vaccination against bovine infections relies, therefore, on the use of either attenuated *B. bovis* alone or attenuated *B. bovis* in combination with *B. bigemina* (2).

Because of the renewed interest in the development of antibabesial vaccines, both living and dead, this study was initiated to determine whether significant protection could be induced against *B. bovis* infection with *B. bigemina* antigens. If this heterologous protection could be confirmed and its mechanism elucidated, this knowledge could be used to produce a bivalent vaccine derived from either of the two organisms; such a vaccine would be extremely valuable.

MATERIALS AND METHODS

Animals. Three-month-old and eighteen-month-old *Bos taurus* animals of mixed breeds and sexes were purchased from an area known to be free of *Boophilus microplus*, the vector of bovine babesia in Australia. The animals were kept under tick-free conditions at the laboratory during the experimental period. Calves were splenectomized 2 weeks prior to infection with either *B. bigemina* or *B. bovis*. Organisms derived from these infected calves were used as sources of challenge material for the adult cattle.

Parasites. Virulent *B. bigemina* (Lismore [L] strain) and *B. bovis* (Samford [S] strain) organisms, previously isolated from field outbreaks, were used to infect animals. These strains have been maintained in our laboratory for a number of years in the vapor phase of liquid nitrogen.

Preparation and analysis of antigenic fractions. (i) Celite antigen. *B. bigemina*-infected erythrocytes (25% parasitemia) were harvested from splenectomized calves by use of disodium EDTA, centrifuged (5,000 × *g*, 5°C), and washed three times in 0.15 M phosphate-buffered saline, pH 7.2. The infected erythrocytes were then diluted twofold with phosphate-buffered saline and stored frozen at −70°C. The frozen cells (50 ml) were thawed under running tap water in the presence of an equal volume of 100 mM imidazole–10 mM disodium EDTA–4 mM benzamidine–2 mM dithiothreitol (pH 7.4).

(ii)Void-volume (VV) antigen. The Celite column was prepared by washing diatomaceous earth for swimming pool filters (Sentry) with water to remove fines and then with 5 N HCl and methanol. The slurry was packed into a column (5.0 by 27.0 cm) and equilibrated with running buffer (50 mM imidazole, 5 mM EDTA, 2 mM benzamidine, 1 mM dithiothreitol [pH 7.4]). The crude antigen was then sonicated with a Braun Labsonic model 1510 apparatus for 1 min at 100 W and 0°C with a small probe. The sonicate was centrifuged for 1 h at 100,000 × *g*, and the supernatant was applied to the Celite column equilibrated with running buffer. The column was washed with the same buffer at 150 ml/h until protein was no longer detectable at 280 nm. The Celite was then

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eluted with running buffer containing 1 M NaCl (total volume, 300 ml), concentrated on an ultrafilter fitted with a YM 10 membrane (Amicon Corp., Lexington, Mass.) to a volume of 50 ml, and dialyzed overnight against 50 mM imidazole–150 mM NaCl–50% glycerol (pH 7.4).

VV fractions from distilled water lysates of normal erythrocytes and erythrocytes infected with either *B. bovis* or *B. bigemina* were separated by gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden). The relevant procedures and the fact that only the VV fractions of the *Babesia*-infected erythrocytes had antigenic activity have been described previously (7, 8). Rabbit antisera to *B. bovis* and *B. bigemina* VV fractions were prepared by a series of intramuscular and intravenous injections as described by Gell and Coombs (6). Prior to analysis, the rabbit antisera and antisera from cattle immunized with either *B. bigemina* parasites or *B. bigemina* Celite antigen were absorbed at 4°C for 24 h with an equal volume of glutaraldehyde-polymerized lysate from normal bovine erythrocytes (1). The VV fractions were also absorbed with glutaraldehyde-polymerized normal bovine plasma. Such absorptions were necessary to eliminate or reduce reactions caused by erythrocytic isoantigen-antibody systems.

The absorbed antisera were used in an indirect fluorescent-antibody (IFA) test to stain antigens in acetone-fixed thin blood smears from cattle infected with either *B. bovis* or *B. bigemina*. The procedure and relevant microscopy have been described in detail elsewhere (9). In addition, individual antigens were detected by immunoblotting following electrophoresis. The VV fractions and relevant prestained protein standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were first incubated at 56°C for 30 min with equal volumes of a solution containing 3% sodium dodecyl sulfate and 3% mercaptoethanol and then electrophoretically separated in 3 to 15% linear acrylamide slabs by using the buffer system of Neville (17). Following electrophoresis, the proteins in the slab were immunoblotted by transfer to nitrocellulose sheets as described by Towbin et al. (20), with the exception that methanol was deleted from the transfer buffer. Thereafter, individual antigens were detected by sequential applications, with intermediate buffer washings, of optimally diluted rabbit antiserum or normal rabbit serum, protein A-conjugated horseradish peroxidase (Kirkegaard & Perry), and α-naphthol–hydrogen peroxide.

Experimental technique. (i) Experiment 1. A group of five adult susceptible cattle were infected intravenously with 5×10^8 *B. bigemina*. Six weeks after recovery, this group and a further group of five susceptible adult cattle were infected intravenously with 10^7 *B. bovis* parasites.

(ii) Experiment 2. Five groups, each containing four splenectomized calves, were used. Groups 1 and 3 were immunized on weeks 0 to 4 with 2 ml of Celite antigen plus 2 ml of Freund complete adjuvant. Group 4 was infected with *B. bigemina* on week 0, and the parasitemia was controlled by drug therapy with Diampron (May & Baker) when parasitemia exceeding 1% was observed approximately 7 days later. The animals were then allowed to recover from the infection. Groups 2 and 5 were control groups. On week 8, groups 1 and 2 were infected with 10^6 *B. bigemina* L, and groups 3, 4, and 5 were infected with 10^6 *B. bovis* S.

Jugular blood was collected by syringe daily, and the packed cell volume (PCV) was measured by using a microhematocrit method. Parasitemia was assessed daily by a thick-film method (15). Rectal temperatures were also recorded daily.

RESULTS

IFA asays. The antisera to *B. bigemina* VV antigens stained *B. bovis*-infected erythrocytes very weakly at a 1/1 dilution; staining ceased at dilutions of 1/5 or greater. In contrast, *B. bigemina* VV antisera avidly stained *B. bigemina*-infected erythrocytes and the parasite itself to a dilution of 1/125 (Fig. 1A). The antisera to *B. bovis* VV antigens, on the other hand, stained infected erythrocytes and parasites in both *B. bovis* and *B. bigemina* blood smears to dilutions of 1/125 (Fig. 1B and C). The reaction of *B. bovis* VV antisera against *B. bigemina*-infected erythrocytes could be totally inhibited by absorption with *B. bovis* VV antigens, whereas absorption with *B. bigemina* VV antigens eliminated parasite but not erythrocyte staining (data not shown).

The adult cattle immune to *B. bigemina* in experiment 1 had mean IFA titers of >1/800 against *B. bigemina* antigen and 1/200 against *B. bovis* antigen. Staining was parasite specific.

Animals immunized with *B. bigemina* Celite antigen (groups 1 and 3) had mean IFA titers prior to challenge of 1/400 when tested with *B. bigemina* antigen and 1/200 when tested with *B. bovis* antigen. Staining was almost entirely of parasites, with only faint staining of infected erythrocytes.

Immunoblotting. Immunoblotting demonstrated that each pool of antisera reacted avidly with both homologous and heterologous antigens, with many antigens being common to both systems. The only major species-specific antigens detected were found in the homologous *B. bovis* system, and they had apparent molecular sizes of 120 and 70 kilodaltons (Fig. 2).

Vaccination challenge. (i) Experiment 1. Primary infection with *B. bigemina* produced detectable parasitemia in all animals (Fig. 3). The mean maximum parasitemia was $29 \times 10^3 \pm 9.3 \times 10^3/mm^3$ of blood 4 days postinfection (DPI); the mean maximum percent PCV fall was 24.9 ± 4.2% 8 DPI; and the mean maximum percent temperature rise was 2.8 ± 1.4°C 4 DPI. None of the animals became clinically affected during the period of patent parasitemia. Six weeks postrecovery, when the PCV of all animals had reverted to preinfection levels, these cattle and the group of susceptible control animals were challenged with virulent *B. bovis*. For control and *B. bigemina*-immune animals, the mean maximum parasitemias occurred at 12 DPI and were, respectively, 51.6 ± 29.9 and 9.0 ± 6.2/mm³ of blood ($P < 0.025$); the mean maximum precent PCV falls were, respectively, 43.5 ± 7.0 and 20.3 ± 3.0% ($P < 0.01$) and occurred at 15 and 14 DPI, respectively; and the mean maximum percent temperature rises occurred at 12 DPI and were, respectively, 4.2 ± 1.1 and 1.3 ± 0.9°C ($P < 0.01$). Parasites were never detected in two *B. bigemina*-immune animals. None of the animals previously infected with *B. bigemina* was clinically affected, whereas four of the five naive animals were severely affected, showing typical tick fever symptoms of dehydration, anorexia, ataxia, jaundice, and hemoglobinuria.

(ii) Experiment 2. All animals in the two control groups (one challenged with *B. bigemina*, the other challenged with *B. bovis*) died from fulminating parasitemia; in the calves of the immunized groups, mortality was 25% in the group which received *B. bigemina* Celite antigen and were challenged with *B. bovis* (group 3) and 50% in the calves immunized with *B. bigemina* Celite antigen and challenged with *B. bigemina* or immunized by infection with *B. bigemina* and challenged with *B. bovis* (groups 1 and 4) (Table 1). The mean maximum parasitemias on day 11 in

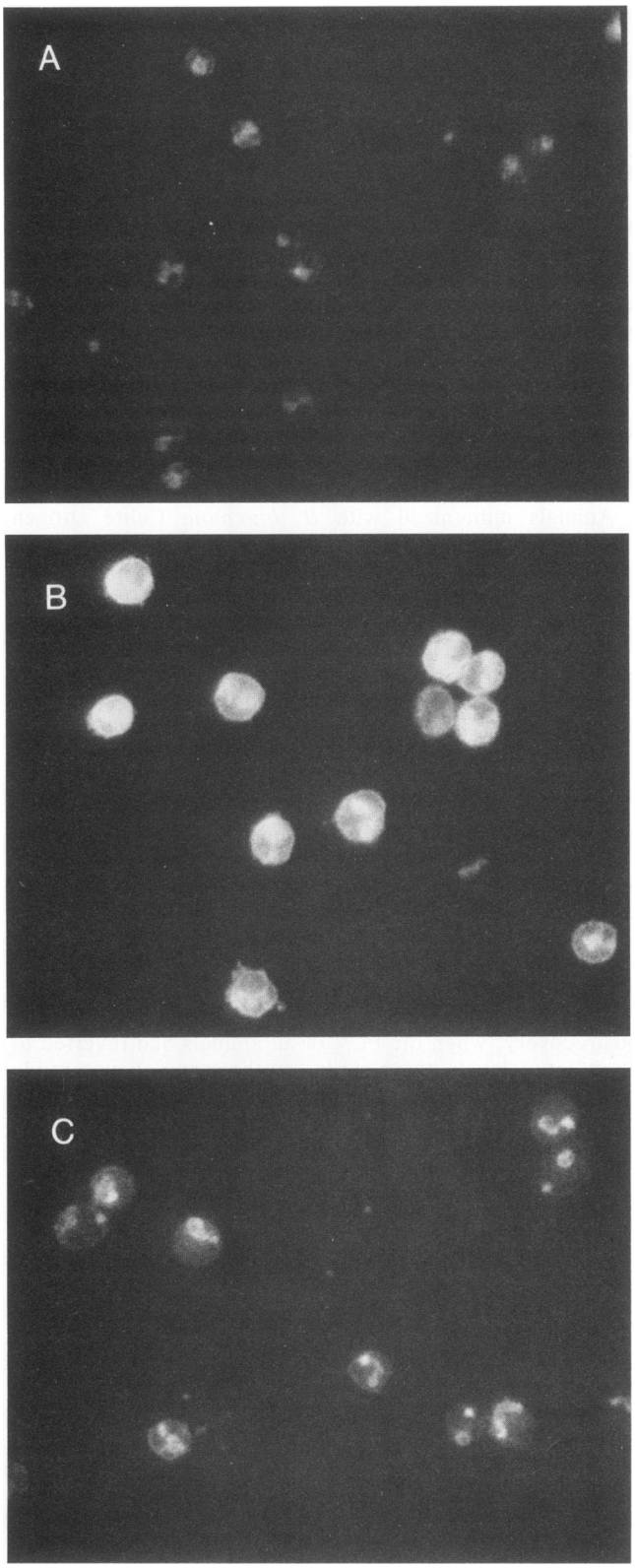

FIG. 1. (A) IFA assay of thin films of *B. bigemina*-infected blood with pooled rabbit antisera to the VV fraction of *B. bigemina*. Magnification, ×1,000. (B) IFA assay of thin films of *B. bigemina*-infected blood with pooled rabbit antisera to the VV fraction of *B. bovis*. Magnification, ×1,000. (C) IFA assay of thin films of *B. bovis*-infected blood with pooled rabbit antisera to the VV fraction of *B. bovis*. Magnification, ×1,000.

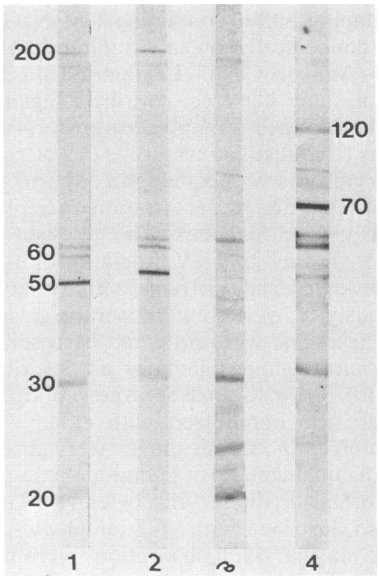

FIG. 2. Immunoblots of the crude soluble fractions of *B. bigemina*-infected erythrocytes (lanes 1 and 3) and *B. bovis*-infected erythrocytes (lanes 2 and 4). Lanes 1 and 2 were stained with bovine antiserum to *B. bigemina* from naturally infected cattle; lanes 3 and 4 were stained with bovine antiserum to *B. bovis* from naturally infected cattle. Common bands and the 120- and 70-kilodalton antigens specific to *B. bovis* are indicated.

animals challenged with *B. bovis* were 72,233/mm^3 in control animals (group 5), 30,833/mm^3 in Celite antigen-immunized animals (group 3), and 16,346/mm^3 in *B. bigemina* drug-controlled animals (group 4). The respective percent PCV falls on the same day were 37, 31, and 22%.

In *B. bigemina*-challenged animals (groups 1 and 2), the mean maximum parasitemias on day 6 were 469,000/mm^3 (control group 2) and 117,500/mm^3 (Celite antigen group 1) ($P < 0.003$). The mean percent PVC falls were −32.16 and −17.14%, respectively.

DISCUSSION

These experiments confirm that a high level of immunity against *B. bovis* is generated by immunization with *B. bigemina*. From the reports of other authors (2, 10), it may be concluded that protection is one way only, between a primary infection with *B. bigemina* and a subsequent challenge with *B. bovis*. The converse does not occur (14). This same pattern of protection has been demonstrated by vaccination with dead antigenic material (D. F. Mahoney, unpublished results).

The data obtained in experiment 2 clearly indicate that the cross-protection is not due to nonspecific factors. Nonspecific induction of cross-protective immunity has been reported in mouse hemoprotozoan infections (3). In addition, mice infected with *Babesia microti* were shown to be immune to a subsequent challenge with *Plasmodium vinckei* (4). However, although it could be argued that prior infection with *B. bigemina* induced strong cross-protection not as a result of protective antibody production but as a result of nonspecific factors, the data obtained in experiment 2 clearly negate this premise. An antigenic extract derived from *B. bigemina* (group 3) induced an immune response against heterologous challenge of similar magnitude to that induced by drug-controlled *B. bigemina* infection (group 4). Although

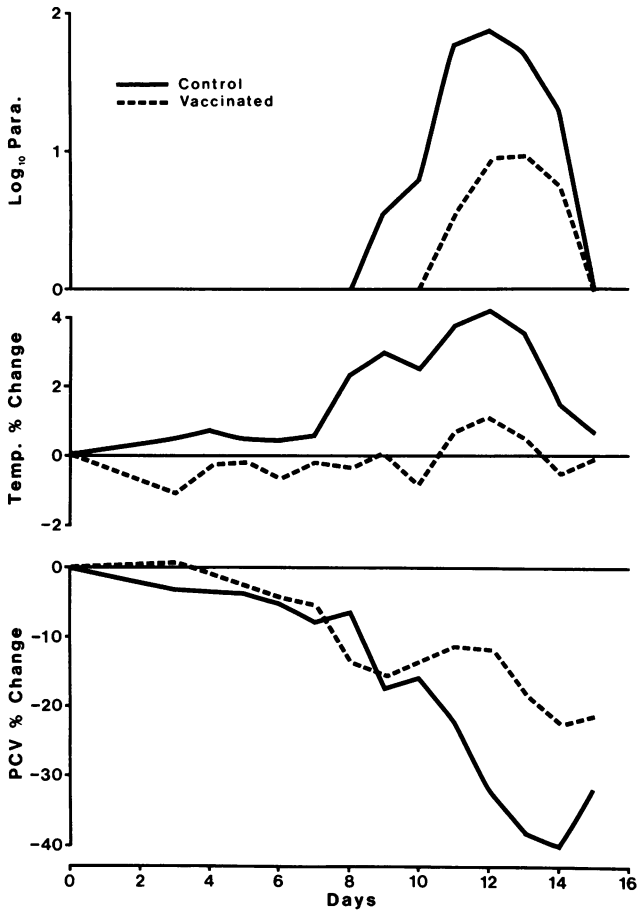

FIG. 3. Mean log parasitemia (Para.) per cubic millimeter, mean percent PCV fall, and mean percent temperature (Temp.) rise (degrees Centigrade) in five cattle immune to and five cattle susceptible to *B. bigemina* upon subsequent challenge with *B. bovis*.

25 to 50% of animals in these two groups died, this mortality rate is similar to that observed in splenectomized calves immunized with *B. bovis* antigens (12, 16).

The results of IFA studies (Fig. 1) essentially agree with those of previous studies with hemagglutination (7, 10) and with those of IFA studies with antisera from cattle used in vaccination trials (19). The *B. bovis* antisera cross-reacted avidly with some antigens of *B. bigemina*, but the reverse did not occur. In contrast, immunoblotting results revealed a two-way cross-reactivity; apart from the greater sensitivity of immunoblotting, this anomaly could indicate that (i) antigens are lost during acetone fixation and subsequent lysis in IFA procedures and (ii) some antigens do not bind to tannic acid in hemagglutination. Likewise, the absence (as detected by immunoblotting) of dominant species-specific antigens of *B. bigemina* could be due to an inability of these antigens to bind to nitrocellulose. Nevertheless, common antigens exist between the species, and these elicit complex immune reactions whose mechanisms have been discussed previously (8). This complexity could be caused by subtle modifications of host proteins within infected erythrocytes. Such changes have been noted in erythrocytes of cattle infected with *B. bovis* and seem to be associated with infected erythrocytes rather than parasites (8, 11). The present studies indicate that similar changes occur in erythrocytes of cattle infected with *B. bigemina*, as bovine

TABLE 1. Data for homologous and heterologous challenges of immunized and control splenectomized calves (experiment 2)

Group	Treatment	Challenge organism	No. of surviving animals[a]	Day of death (*n*)
1	*B. bigemina* Celite antigen	*B. bigemina*	2	8 (1), 11 (1)
2	Control	*B. bigemina*	0	6 (4)
3	*B. bigemina* Celite antigen	*B. bovis*	3	12 (1)
4	*B. bigemina* drug-controlled infection	*B. bovis*	2	12 (1), 13 (1)
5	Control	*B. bovis*	0	9 (1), 12 (2), 13 (1)

[a] Four were tested in each group.

antisera to *B. bigemina* do not stain infected erythrocytes (C. G. Ludford, Ph.D. thesis, University of Queensland, Queensland, Australia, 1967), whereas rabbit antisera do. The inference must be that *B. bigemina*-infected erythrocytes contain antigens, either parasite metabolites or modified erythrocytic proteins, that are poorly immunogenic in bovines but avidly immunogenic in rabbits.

The nature of the factor in *B. bigemina* that induces protection against *B. bovis* has not been identified. However, if this material is a parasite protein, which is most likely, why doesn't *B. bovis* have a similar protein to induce protection against *B. bigemina*? The data obtained from the serological analyses in this study demonstrate that common antigens exist between the two species. Presumably, with *B. bigemina*, one or more of the common antigens is present in sufficient quantity to induce a protective response to the heterologous parasite. The serological evidence indicates that such an antigen(s) is present in only minor amounts in *B. bovis* and elicits only a weak immune response which is insufficient to protect against *B. bigemina*. Other common, but nonprotective antigens would account for the cross-reaction observed in IFA studies and detected by immunoblotting. The isolation of an antigen from *B. bigemina* that protects against both *B. bovis* and *B. bigemina* and that could be synthesized in vitro is important, as the antigen would be a prime candidate for use as a bivalent vaccine. Studies which may lead to the identification of such an antigen have been initiated in our laboratory.

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