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Bovine Babesiosis: Protection of Cattle with Culture-Derived Soluble *Babesia bovis* Antigen

Abstract. *Adult Hereford* (Bos taurus) *cattle were protected from severe reactions and death caused by the tick-borne protozoan hemoparasite* Babesia bovis, *3 months after vaccination with a cell culture–derived immunogen. The immunogen consisted of filtered, freeze-dried supernatant fluid collected from long-term cultures of* Babesia bovis *in vitro. It was reconstituted with saponin adjuvant and injected twice subcutaneously at 2-week intervals. Serum collected from vaccinated cattle caused thickening of the merozoite surface coat, aggregation, and lysis of merozoites in vitro. This reaction was identical to that caused by serum from immune carrier cattle suggesting that the protective antigen present in culture fluids is merozoite surface coat antigen. No mortality occurred among vaccinated cattle, whereas mortality among unvaccinated cattle and* Babesia bigemina*–immune cattle was 62.5 percent indicating that immunity to bovine babesiosis is species-specific.*

Bovine babesiosis, caused by the intraerythrocytic protozoan parasite *Babesia bovis (= argentina)*, is an acute, tick-borne hemolytic disease that affects cattle throughout the world (*1*). The severity of the disease is attributed to erythrocyte destruction and plugging of capillaries with parasitized erythrocytes leading to impaired organ function (*1*). Mortality is high, often exceeding 50 percent of affected cattle (*2*). Chemotherapeutic agents effective against the parasite are commercially available and recovered animals are immune to disease from homologous or heterologous strains of *B. bovis* (*3*). Therefore, immunoprophylaxis is directed toward prevention of the disease rather than infection and has traditionally been achieved through "premunition" (*4*), that is, by infecting cattle with parasites and treating them with drugs as necessary. Immunity to babesiosis persists for at least 6 months after cattle have been freed of infection through drug treatment or their own immune system (*5*). The development of immunity as a result of natural infection provides the rationale for attempting to develop an effective inactivated vaccine for bovine babesiosis. We report here that cattle can be protected against babesiosis for an extended period of time by vaccination with soluble antigen extracted from the medium of long-term cultures of *B. bovis*–infected erythrocytes. We present evidence that merozoite surface coat antigen is present in the culture medium, and that this antigen may be responsible for the induction of the immunity.

Bovine babesiosis is similar in many ways to human malaria caused by *Plasmodium* spp. (*6*). Furthermore, several species of *Babesia* infect man, producing a syndrome that has often been misdiagnosed as malaria (*6, 7*). Recent progress toward the development of an inactivated vaccine for babesiosis has closely paralleled similar studies in malaria research (*8*). Both parasites have been grown in vitro and their respective culture-derived antigens have been partially purified and characterized (*9, 9a*). *Babesia bovis* merozoites are abundant in culture supernatant fluids and possess a loosely adhering surface coat visible under the electron microscope. The infectivity of culture-derived merozoites is destroyed by freeze-thawing. The success of recent immunization and challenge experiments with killed, culture-derived *Plasmodium* (*10*) and *Babesia* (*11*) is attributed to the antigenic composition of the extraerythrocytic invasive stage of these parasites, that is, the merozoite. Specifically, the merozoite surface coat elicits the production of antibodies that prevent erythrocyte invasion by parasites and facilitates their immune destruction by phagocytic cells (*9a, 12*). However, preparations of malaria and babesia merozoites are contaminated with erythrocyte stroma, and this limits their usefulness as a vaccine. Soluble *B. bovis* antigens prepared from medium of primary cultures by salt precipitation or pervaporation induce short-term protection from tick-borne babesiosis when combined with Freund's complete or incomplete adjuvant (*11*).

In the present study the immunogen was extracted from the culture medium of actively growing cultures of *B. bovis*–infected bovine erythrocytes (*13*) that had been maintained in vitro for 196 days (approximately 98 generations) (*14*). Culture medium was collected over a 24-hour growth period during which parasitemia increased from 1.1 to 5.1 percent. After centrifugation at 11,877*g* for 15 minutes at 4°C, the cell-free supernatant fluid was filtered through a membrane with 0.22-μm pore size, freeze-dried in 28-ml portions (0.66 g of total protein), and stored at 4°C. Immediately before being used each portion was solubilized with 5 ml of sterile distilled water, mixed with 1 mg of saponin adjuvant (*15*), and inoculated subcutaneously into each of four Hereford *(Bos taurus)* cattle, each aged 18 months. A booster injection was given 2 weeks later. Four control cattle were each inoculated as above with lyophilized cell-free supernatant fluid from uninfected erythrocyte cultures. The only adverse effects of immunization were mild local swelling and increased body temperature for 48 to 72 hours.

The four immunized cattle and the four controls were challenged by exposing each of them to 1000 *Boophilus microplus* larvae from a *B. bovis*–infected

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ed colony (*2*) after the antibody titers of the immunized cattle had declined to minimum levels, that is, approximately 3 months after the last antigen injection (Fig. 1). Another group of unvaccinated cattle was also included in the experiment; this group had recovered from babesiosis $3\frac{1}{2}$ months previously caused by tick-borne infection with a related species, *B. bigemina* (*16*). *Babesia bigemina* shares certain antigens with *B. bovis* (*17*) and infections with this organism have been used in the past to protect cattle from the more virulent *B. bovis* (*18*). The *B. bigemina*–immune cattle were challenged in the same way as the other two groups.

Table 1. Pattern of *Babesia bovis* infection in immunized, control, and *B. bigemina*–carrier cattle. Cattle in the vaccinated group were inoculated subcutaneously twice at 2-week intervals starting 14 weeks earlier with lyophilized antigen derived from the cell-free culture medium of actively growing *B. bovis*–infected erythrocyte cultures. Each inoculation consisted of 0.66 g of protein, equivalent to 28 ml of culture supernatant, and was combined with 1 mg of saponin. Control cattle were inoculated with an equivalent amount of lyophilized supernatant from *Babesia*-free erythrocyte cultures plus saponin adjuvant. *Babesia bigemina* carriers had recovered from tick-borne infections that occurred 17 weeks earlier. All cattle were challenged with 1000 *Boophilus microplus* larvae from a *B. bovis*–infected colony.

Calf number	Parasites per 1000 erythrocytes after challenge on day									
	9	10	11	12	13	14	15	16	17	18
Immunized animals										
185	< 1	–	< 1	–	< 1	–	–	–	–	< 1
788	–	–	–	–	< 1	< 1	< 1	–	< 1	–
44	–	–	–	< 1	< 1	< 1	< 1	–	–	–
790	–	–	< 1	< 1	< 1	< 1	< 1	< 1	< 1	–
Control animals										
42	–	–	< 1	< 1	< 1	–	–	–	< 1	–
189	–	–	–	< 1	< 1	< 1	< 1	< 1	< 1	–
779	–	–	–	< 1	< 1	< 1	3	2	Died	
484	–	–	–	< 1	< 1	3	Died			
Animals previously infected with B. bigemina										
196	–	–	–	–	–	< 1	–	< 1	< 1	–
32	–	< 1	< 1	–	< 1	< 1	4	Died		
27	–	–	< 1	–	< 1	< 1	Died			
38	–	–	< 1	< 1	1	Died				

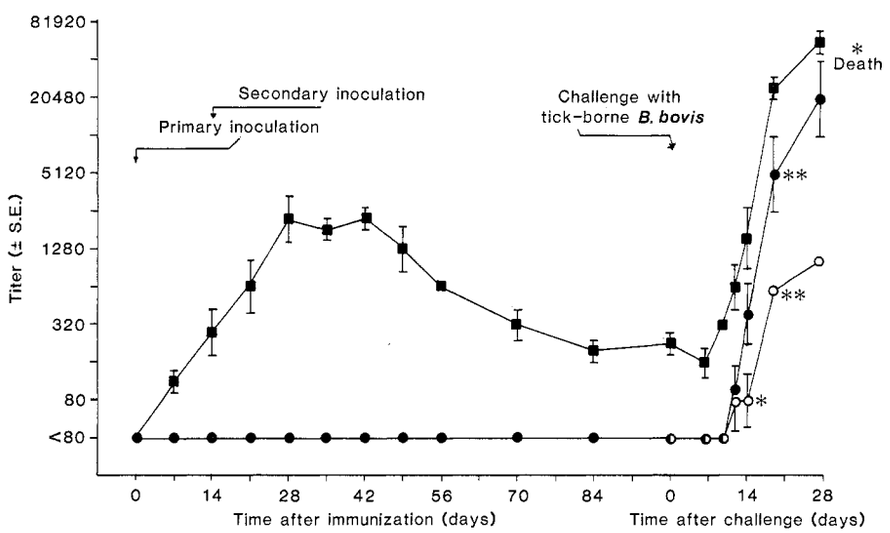

Fig. 1. Indirect fluorescent antibody response of experimental cattle to immunization with culture-derived *B. bovis* surface coat antigen and challenge with tick-borne infection. Serum antibody to *B. bovis* was first detected 7 days after immunization in cattle immunized with surface coat antigen (■). Antibody titers continued to rise through 2 weeks after booster injections at which time a plateau was reached at mean titers between 1810 and 2152. Serum antibody titers began to decline 3 weeks later and stabilized at mean values of 190 to 226, 12 weeks after the booster injection. Cattle inoculated with the supernatant of uninfected erythrocyte cultures (●) failed to develop detectable antibody response to *B. bovis*. Cattle that had recovered from a tick-borne *B. bigemina* infection $3\frac{1}{2}$ months previously (○) developed mean antibody titers of up to 113 to *B. bovis* which became undetectable 3 months prior to challenge. The mean antibody titer of this group to *B. bigemina* antigen was 453 at the time of challenge with tick-borne *B. bovis*.

All 12 cattle became infected after challenge (Table 1). The rectal temperature of the vaccinated cattle began to rise 6 days after the challenge, 3 days before the control and *B. bigemina*–immune cattle. This early febrile response was unaccompanied by packed cell volume (PCV) reductions, suggesting that early fever was part of a hypersensitivity response by vaccinated cattle triggered by early recognition of *B. bovis* antigen by the immune system. *Babesia bovis* parasites are injected by infected ticks from 2 to 4 days after tick attachment.

The PCV of all cattle began to decline between 9 and 10 days after challenge when parasites first became detectable in Giemsa-stained smears of blood from ear veins (Table 1). Two of the four control cattle and three of four *B. bigemina*–immune cattle died between 14 and 17 days after challenge. None of the vaccinated cattle died. The death of five out of eight unvaccinated cattle midway through the course of the disease (Table 1) prevented a meaningful statistical comparison of clinicopathologic changes with vaccinated animals. The high mortality among the unvaccinated cattle was not associated with greater PCV reductions or longer durations of parasitemia compared to the vaccinated animals. For the five animals that died the mean PCV reduction before death was 37 percent. However, cattle that succumbed to infection generally had higher parasitemias than survivors (Table 1). Postmortem studies revealed that blood capillaries in the central nervous system and kidneys were plugged with parasitized erythrocytes (Fig. 2). These microscopic lesions probably contributed to the signs of central nervous system disturbance (depression, ataxia, incoordination, paddling, opisthotonus) and kidney failure (increased urea nitrogen in the blood) immediately before death. Although the vaccinated cattle experienced variable degrees of parasitemia accompanied by fever and a mean PCV reduction of 47 percent, they continued to eat and showed relatively mild clinical signs and symptoms of babesiosis.

The mechanism of protection and relative importance of humoral as opposed to cellular immune systems in babesiosis is not clear. The mean antibody titer of vaccinated animals increased significantly 10 days after tick exposure, 2 days earlier than either group of unvaccinated animals. This finding is consistent with the hypothesis that vaccinated animals

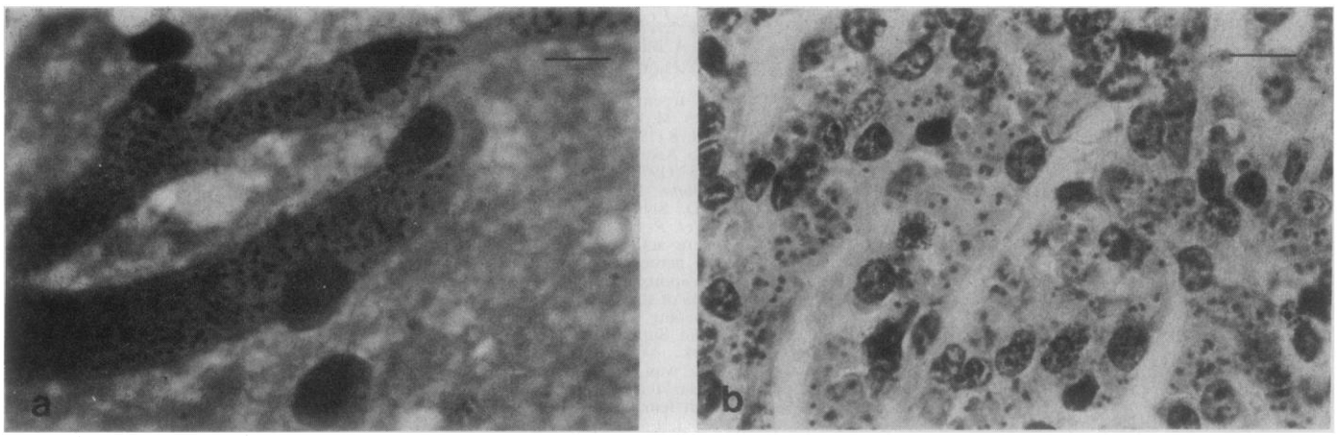

Fig. 2. Plugging of blood capillaries of (a) brain and (b) kidney glomerulus with *B. bovis*–infected erythrocytes in an animal that died of babesiosis. *Babesia* parasites appear as dots, singly or in pairs, within the erythrocyte mass. Parasitemia is greater than 90 percent in these organs as opposed to approximately 5 percent in jugular vein blood. Scale bar, 10 μm.

recognized parasite antigens in the challenge exposure earlier than did unvaccinated animals (*11*).

Evidence that the soluble immunogen is, in fact, merozoite surface coat antigen was documented by use of electron microscopy. Culture-derived parasites were purified by differential centrifugation (*19*) and 0.1 ml of the merozoite pellet was incubated at 37°C for 30 minutes with 0.1 ml of serum collected from an animal (i) on the day of vaccination. (ii) during the period of maximum serum antibody titer (+ 42 days), and (iii) on the day of tick-borne challenge exposure (+ 101 days). Serum was also collected from a second animal that had recovered from babesiosis induced by tick-borne *B. bovis* 49 days earlier; this serum was incubated with culture-derived merozoites as above. At the end of the incubation period each mixture was fixed with 2.5 percent glutaraldehyde and 4 percent sucrose in 0.1*M* cacodylate buffer, *p*H 7.3. Samples were then processed for electron microscopy in the usual manner. Sections were examined with a JEOL 100 CX electron microscope.

Serum from the *B. bovis*–recovered animal reacted with the merozoite surface coat, causing thickening of the surface coat (Fig. 3a), aggregation, and lysis of merozoites. The induction of a similar immune response in cattle inoculated with soluble antigen (Fig. 3, b to d) demonstrates that surface coat antigen was present in culture supernatant fluid. The reaction of immune serum with the babesial merozoite surface coat is identical to that seen when merozoites of *Plasmodium* spp. are incubated with malaria immune serum. Antibody to surface coat interferes with erythrocyte invasion by malaria parasites and promotes phagocytosis by macrophages (*9a*, *12*). The continued presence of antibody to this parasite antigen on the day of challenge may explain the mechanism of protection from severe babesiosis among vaccinated animals. Two other soluble parasite antigens are present in the supernatant fluid of *B. bovis* cultures (*20*). However, their role in the parasite's life cycle and in the induction of protective immunity is not clear. Serum from immunized animals does not react with normal erythrocytes in either indirect fluorescent antibody or agglutination tests, indicating that erythrocyte antigens are either absent or present in negligible amounts in the soluble antigen fraction.

The role of the early hypersensitivity-like reaction of vaccinated animals in the subsequent outcome of challenge infections is unclear. The saponin adjuvant Quil A appears to induce a limited homocytotropic antibody response to certain antigens (*21*). Similar responses were observed when Freund's complete adjuvant (FCA) was used (*11*). However, FCA is unacceptable for field use because it causes a severe local tissue reaction; saponin is acceptable for use in food-producing animals.

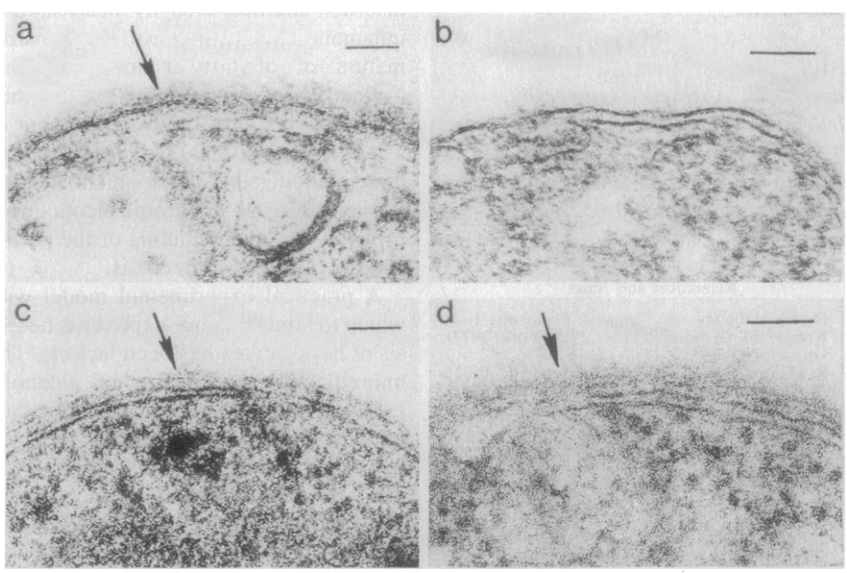

Fig. 3. Electron micrographs showing the effect of immune serum on *B. bovis* merozoites. (a) A merozoite that was incubated with immune serum collected from an unvaccinated animal that had been exposed to a tick-borne *B. bovis* infection 49 days earlier. A thickened surface coat (arrow) measuring about 26 nm covers the entire surface. (b) A merozoite that was incubated with bovine serum collected on the day of vaccination. The surface coat is normal. (c) A merozoite that was incubated with bovine serum collected at the point of peak antibody titer (42 days after immunization). The thickened coat (arrow) measuring about 30 nm covers the entire surface. (d) A merozoite that was incubated with bovine serum collected on the day of challenge with tick-borne *B. bovis*. The thickened coat (arrow) covers the entire surface. Scale bars, 100 nm.

Our study shows that long-term growth of the *Babesia* parasites in vitro did not appear to reduce their ability to produce protective antigens. The absence of erythrocyte stroma from the immunogen makes it useful for cattle of all ages. Vaccinated cattle were still immune to babesiosis when circulating antibody titers had stabilized at low levels, suggesting that protection persisted beyond the interval studied in this report. Subsequent exposure to tick-borne *Babesia* infection would probably confer species-specific protection for life (*1*). The finding that *B. bigemina*–immune cattle are susceptible to *B. bovis* suggests that protection of cattle from both species of parasite in the field will require vaccination with species-specific antigens.

The concentration of soluble antigen derived from the *B. bovis* culture system that we used is probably much lower than that attainable with the recently developed microaerophilus stationary phase (MASP) culture system (*19*). Studies are now needed on the duration of immunity after various doses and injection regimens with cell-free immunogens derived from the more efficient MASP culture system.

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14. The parasites were actively growing in vitro approximately 50 percent of the time with a generation time of approximately 24 hours alternating with periods of storage at 4°C.
15. The Quil A, containing 1.5 percent dry matter, was obtained from Superfos Export Co. a/s, Vedbaek, Denmark.
16. *Babesia bigemina* was isolated from pooled blood collected from 70 cattle in Tizmin, Yucatan, Mexico. Adult female ticks were infected by allowing them to feed on an animal during peak parasitemia, and their larval progeny were used to establish carrier infections in the cattle that were subsequently challenged with tick-borne *B. bovis* in this report.
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Alcohol-Dependent Liver Cell Necrosis in vitro: A New Model

Abstract. *In alcoholic liver injury, necrosis is involved in the progression from benign fatty liver to alcoholic hepatitis and cirrhosis. However, there is no practical model of alcohol-dependent liver cell necrosis. The calcium-dependent killing of cultured rat hepatocytes by two different membrane-active hepatotoxins, galactosamine and phalloidin, is potentiated by ethyl alcohol. This indicates that some general physical effect of alcohol on cellular membranes renders cells susceptible to otherwise nonlethal injuries. The in vitro model described in this report may thus be used to search for a general mechanism underlying alcohol-related tissue injury.*

Alcoholic liver disease is primarily restricted to the benign and asymptomatic fatty liver. After years of alcohol (ethanol) abuse, a minority of alcoholics suddenly develop the potentially lethal condition of alcoholic hepatitis, an acute disorder characterized by necrosis and inflammation (*1*). Similarly, the transformation of fatty liver into cirrhosis—whether or not mediated by alcoholic hepatitis—involves the establishment of a necrotizing process (*2*). Thus the key to understanding the development of serious liver disease in chronic alcoholics is probably an understanding of the pathogenesis of liver cell necrosis.

A practical experimental model with which to study alcohol-dependent necrosis of hepatocytes has been lacking. The unpredictable occurrence of alcoholic hepatitis and the small amount of tissue obtainable by needle biopsy preclude functional studies at the cellular level. The baboon model of alcoholic hepatitis and cirrhosis (*3*) is also of limited usefulness because of the small proportion of animals developing these disorders, the unpredictability of the time at which necrosis occurs, and the expense of maintaining these primates.

In attempting to develop a model of alcohol-dependent necrosis, we were guided by several considerations: (i) alcohol does not kill hepatocytes at the concentrations reached in chronic alcoholics (~ 100 m*M*) (*4*, *5*); (ii) alcohol can damage organs in which it is not metabolized, such as the heart (*6*) and pancreas (*7*); (iii) the presence of alcohol "fluidizes" or "disorders" various biological membranes in a dose-dependent fashion (*8*) and interferes with certain membrane functions (*5*, *9*); (iv) toxic liver necrosis generally involves damage to the plasma membrane, resulting in an influx of lethal amounts of calcium ions (*10*, *11*); and (v) alcohol enhances the liver cell necrosis produced by a wide variety of chemicals (*12*). In view of these points, we hypothesized that the physical effects of alcohol on biological membranes promote liver cell necrosis by potentiating the action of other membrane-active hepatotoxins.

We have used primary cultures of adult rat hepatocytes to explore the mechanisms underlying the liver cell death produced by various toxins (*10*, *11*). Two of these, galactosamine and phalloidin, act on the plasma membrane to cause reversible cell injury in the absence of extracellular Ca^{2+} (*11*, *13*) and lethal injury in its presence (*10*, *11*). In both cases there is probably disruption of the permeability barrier function of the plasma membrane. With each toxin, however, the mechanism of such damage is different. Phalloidin, a bicyclic heptapeptide isolated from the mushroom *Amanita phalloides* (*14*), polymerizes actin monomers intimately associat-

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