

Age Resistance in Bovine Babesiosis: Role of Blood Factors in Resistance to *Babesia bovis*

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In vitro cultivation of *Babesia bovis* in erythrocyte cultures demonstrated that blood from young animals contains a factor(s) responsible for their resistance to severe babesiosis. This factor is independent of antibody, is present in the serum of all young animals tested, and is dialyzable. The presence of this factor results in inhibition of parasite multiplication and the eventual death of the parasite while inside the erythrocyte.

Babesiosis is one of the most important diseases affecting cattle in tropical and subtropical regions of the world. Whereas high morbidity and mortality values are observed when mature animals are infected for the first time, calves generally exhibit a milder form of infection. Animals infected at a young age (generally <9 months) are resistant to severe disease when rechallenged as adults (8). In endemic areas, the existence of cattle herds often depends upon this generally mild natural preimmunization. However, the reason these animals are resistant to severe infection is unclear. Speculation has focused on the role of colostral antibody in providing passive protection (4, 5), on biochemical characteristics of calf erythrocytes that may be unfavorable for parasitic development, and on the role of the spleens of young animals in better controlling the babesial infection by erythrophagocytosis (8).

Experiments designed to determine the mechanism of age resistance in calves are difficult to design and interpret. The myriad of biochemical, physiological, and immunological events occurring in living animals are impossible to fully measure. The separate roles of specific and nonspecific immunity, physiology, and nutrition cannot easily be determined. By using an in vitro technique which supports continuous growth of *Babesia bovis* at rates equal to those observed in cattle, we sought to determine the role of blood components in this phenomenon of age resistance.

MATERIALS AND METHODS

Culture and parasites. A Mexican isolate of *B. bovis* (syn. *B. argentina*) was used in all experiments (9). This isolate was stored in liquid nitrogen and reactivated by passage through one or more splenectomized bull calves (*Bos taurus*) before introducing it into culture, utilizing the microaerophilus stationary phase

(MASP) culture technique (7). Briefly, cultures consisted of 7 to 10% bovine erythrocytes in a medium of 40% bovine serum and 60% medium 199 (Hanks salts) supplemented with 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 100 U of penicillin G and 100 µg of streptomycin sulfate per ml. After adjusting the pH to 7.0 with 1 N NaOH or 1 N HCl, 0.2 ml of culture was added to each well of a 96-well flat-bottom tissue culture microtiter plate, and 8 of 16 replicate cultures were prepared for each treatment. This amount consisted of both uninfected culture and previously infected culture, effecting a subculture as described below. Cultures were incubated at 37 to 38°C in an atmosphere of 5% CO₂ and humidified air. At 24-h intervals, the overlaying supernatant was removed and replaced with fresh medium without erythrocytes. At intervals of 2 or 3 days, subculture was accomplished by suspending the infected erythrocytes in fresh medium and mixing one part infected culture with four to eight parts of new culture prepared with freshly collected uninfected bovine erythrocytes. Packed-cell volumes were determined by the microhematocrit method, and the percentage of infected erythrocytes (PE) was determined by microscopic examination of 500 to 1,000 erythrocytes on Giemsa-stained thin blood films from three randomly selected replicate cultures. The standard deviation in PE among the three replicates was always less than ±25% of the mean. The cumulative increase (CI) during a series of subcultures was calculated by dividing the final PE by the initial PE and multiplying by the cumulative dilution (CD), which was the factor by which erythrocytes present at the start of an experiment were diluted with fresh erythrocytes during the series of subcultures. Thus, $CI = (\text{final PE}/\text{initial PE}) \times CD$. For example, mixing one part infected culture with a packed-cell volume of 4.5 and a PE of 10 with four parts new culture with a packed-cell volume of 9 reduces the PE at subculture to 1, with a CD of 10. A series of three such subcultures would yield a CD of 1×10^3 and a CI of 1×10^4 . Because 30 to 60% of the erythrocytes are usually destroyed by infection during the course of each passage, a dilution of 10- to 25-fold was generally achieved at each subculture.

Blood sources. The United States is free of bovine

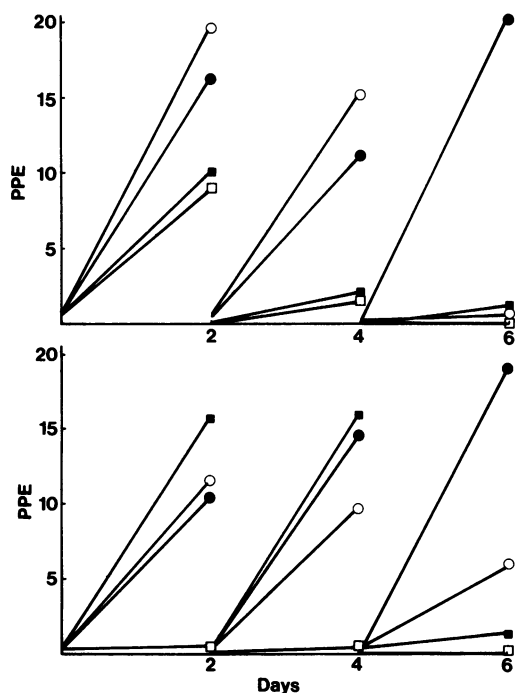


FIG. 1. Growth of *Babesia bovis* in MASP cultures, utilizing various combinations of adult bovine and calf blood elements. The percent parasitized erythrocytes (PE) was reduced by subculture at 2-day intervals. Symbols: ●, adult erythrocytes and adult serum; ○, adult erythrocytes and calf serum; □, calf erythrocytes and calf serum; ■, calf erythrocytes and adult serum.

babesiosis, and routine testing of experimental animals by an indirect fluorescent antibody test indicated an absence of antibabesia antibodies. Bull calves of mixed dairy breeds and born of the University of Illinois dairy herd were used as a source of calf blood. After collection by venipuncture, the blood was immediately defibrinated by shaking with glass beads. Serum and erythrocytes were separated by centrifugation at $1,500 \times g$ for 15 min at 4°C . Erythrocytes were washed three times in 10 to 20 volumes of medium 199 (pH 7.0) to remove contaminating serum components. However, preliminary experiments indicated that the residual serum from unwashed sedimented erythrocytes did not significantly affect the outcome of an experiment. Adult bovine blood was obtained from healthy mature dairy cows and processed as described above. Unless otherwise noted, cultures were prepared within 3 h of blood collection, and fresh erythrocytes and serum were collected on the day of each subculture. For experiments involving examination of serum factors, only adult erythrocytes were used in the preparation of new culture, which was used immediately after preparation or stored at 4°C for up to 4 days. In these instances, the pH was readjusted to 7.0 immediately before use, and the ability to support *B. bovis* in vitro was not affected.

Parasite inoculum was obtained from cultures which had been propagated continuously in vitro for 1 to 6

TABLE 1. Growth of *Babesia bovis* in MASP cultures with various mixtures of adult bovine and calf blood elements

Erythrocytes	Serum	Cumulative increase ^a (-fold)
Adult	Adult	5,893
Calf	Adult	1,408
Adult	Calf	324
Calf	Calf	18

^a Mean of seven experiments.

months with adult blood elements only. Before mixing infected culture with new culture, the overlaying medium of the infected culture was replaced with the homologous test medium containing the serum which was to be tested. Infected erythrocytes were never subjected to centrifugation, as this is known to affect babesial growth (7). During more than 3 years of culturing *B. bovis* by the MASP method, cultures were prepared utilizing blood elements from more than 30 animals, and mixing erythrocytes and sera from many animals did not affect the quality of the cultures (M. G. Levy, unpublished data).

Commercially prepared fetal calf serum (FCS), newborn calf serum (<10 days old), and bobby calf serum (10 days to 3 months old) were obtained from GIBCO and stored at -20°C until needed.

RESULTS

Blood was collected from calves 1 to 10 days of age at the start of each experiment. In experiments designed to determine the relative importance of each component (erythrocytes and serum), this schedule yielded a maximum age for any calf during the final collection of 15 days. All four combinations of adult and calf blood elements were prepared, and parasitemias were determined at the initiation of each experiment and at the end of each culture period. Although various growth patterns were observed during the three consecutive passages with the blood of seven calves, they all fit into general patterns (Fig. 1). Culture data for all seven animals has been summarized (Table 1). It is important to note that detectable parasitemias were observed in only two instances when homologous calf blood elements were used during three consecutive subcultures.

Calves are reported to become increasingly susceptible to severe disease as they age (2, 4, 5, 8, 9). We were able to collect blood from one animal at two different ages. The results (Table 2) indicate that both serum and erythrocytes collected between 8 and 14 days of age supported minimal parasite multiplication. Between 42 and 48 days of age, however, the serum component remained inhibitory, whereas calf erythrocytes supported luxuriant growth when adult serum was supplied.

TABLE 2. Effect of aging on the ability of calf blood to support babesial growth in MASP culture

Erythrocytes	Serum	PE in subculture:			Cumulative increase (-fold)
		1	2	3	
Adult	Adult	17.3	12.0	20.5	3,562
Adult	Calf ^a	19.4	15.1	0.5	98
Calf ^a	Calf ^a	8.8	2.7	0.0	0
Calf ^a	Adult	9.9	3.1	1.5	118
Adult	Adult	20.8	15.5	15.1	10,902
Adult	Calf ^b	16.9	3.4	0.1	57
Calf ^b	Calf ^b	15.7	1.0	0.1	65
Calf ^b	Adult	16.4	11.4	15.0	5,100

^a This animal was an 8-day-old Holstein-Hereford calf at start of experiment.

^b This was the same calf as in footnote *a*, 36 days later.

Because growth of *B. bovis* in adult bovine erythrocytes was inhibited by the presence of calf serum (Fig. 1, Tables 1 and 2), serum from another 10-day-old calf was used to prepare various ratios of calf and adult serum, and parasites were cultured in adult erythrocytes for five consecutive passages (Fig. 2). Although serum from this calf supported satisfactory growth during the first three passages, cultures containing greater than 20% calf serum failed to support growth beyond four passages. Commercially obtained FCS calf serum, and bobby calf serum supported only limited growth of the organisms when adult erythrocytes were used (Table 3). A titration of FCS (Table 4) indicated that concentrations as low as 10% were severely inhibitory.

The experiment described above indicated the severe inhibitory effect of FCS. In an attempt to determine the approximate size of the inhibition component, FCS was dialyzed against 5 volumes of adult bovine serum (ABS), and three changes of dialysate were accomplished over a 5-day period at 4°C. After dialysis, the FCS was incorporated into MASP cultures, and its effect on parasite multiplication and morphology were determined (Table 5). Whereas nondialyzed FCS remained inhibitory, normal levels of growth were observed when dialyzed FCS was used. This indicated the dialyzable nature of the inhibitory component. However, it did not determine whether a dialyzable component inhibitory for babesial growth was lost from the FCS or a necessary component for growth was ac-

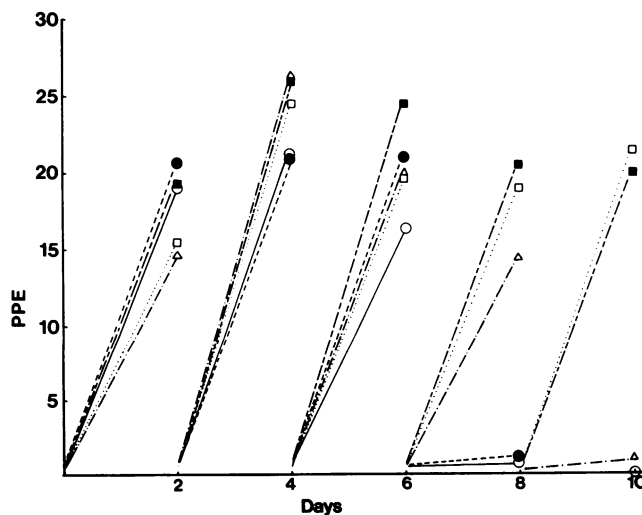


FIG. 2. Growth of *Babesia bovis* in MASP cultures, utilizing various ratios of adult bovine and calf serum. Adult erythrocytes were used in all treatments and the percent parasitized erythrocytes (PPE) was reduced by subculture at 2-day intervals. The serum concentration in the medium was 40% (vol/vol). The ratios of adult serum to calf serum were 1:0 (■), 1:1 (□), 1:3 (△), 1:9 (●), and 0:1 (○).

TABLE 3. Growth of *Babesia bovis* in MASP cultures containing commercial calf sera

Serum	PE	
	Initial	Final (48 h later)
Adult bovine	0.9	13.8
Calf	0.9	4.0
Newborn calf	0.9	2.3
FCS	0.9	0.8 ^a

^a Most parasites exhibited abnormal morphology.

quired from the ABS. The experiment described above was reversed, and ABS was dialyzed against FCS and the ABS incorporated into MASP cultures. These results (Table 6) suggest that the inhibitory activity is contained in FCS and may be recovered in ABS after dialysis.

DISCUSSION

It has been reported (4, 5) that resistance of calves to babesiosis caused by *B. bovis* is dependent upon receipt of adequate amounts of immune colostrum, as calves from susceptible cows are reported to be highly susceptible to virulent infection. However, attempts to repeat the latter finding were unsuccessful (8). In these experiments (8), it was reported that natural resistance was partially independent of immune colostrum and calves 6 to 7 months old were resistant to tick-transmitted *B. bovis*, regardless of the immune status of the cow. Later work (9) utilizing a severe tick challenge indicated that mortality among 6- to 9-month-old calves obtained from a *Babesia*-free area of Mexico was only one-fifth that of a comparable group of animals 12 to 15 months old. This natural resistance of young animals is reported to last for even longer periods (2). While there appears to be agreement that calves exhibit some degree of protection against severe disease caused by *B. bovis*, the mechanism of this protection is disputed.

In this report it was clearly demonstrated that normal blood alone, obtained from donor calves with spleens in situ, contains all of the factors needed to account for reduced parasite growth and virulence in these animals. Data from one animal (Table 2) suggests that the erythrocytic factor is lost fairly rapidly as the calf ages, whereas the serum component is fairly stable during this period. The erythrocytic factor is consistent with an inhibitory effect of fetal hemoglobin, as most of the fetal hemoglobin is replaced by adult types during this period (6). The serum component is dialyzable and as yet lacks further characterization. Based upon the dialysis experiments, it is difficult to determine whether a dialyzable inhibitory factor is con-

TABLE 4. Growth of *Babesia bovis* in MASP cultures containing various proportions of adult bovine serum and FCS

Adult serum/FCS (%/%)	PE		
	Initial	Final	
		Subculture 1	Subculture 2
40/0	2.3	15.0	9.5
30/10	2.3	11.6	0.7
20/20	2.3	5.6	0.0
10/30	2.3	2.0	0.0
0/40	2.3	3.3	0.0

tained in FCS or a necessary growth factor is obtained from the ABS. However, it is known that 30% ABS will support *B. bovis* in MASP cultures (7) whereas 30% ABS plus 10% FCS is severely inhibitory to *B. bovis* development in vitro (Table 4), which strongly suggests the presence of an inhibitory factor. Abnormal morphology in most, but not all, of the experiments utilizing calf serum suggests the intracellular inhibition or killing of parasites by this factor, which may be able to penetrate the infected erythrocyte because of the low molecular weight (<14,000) as determined by dialysis and the leakiness of infected erythrocytes (10).

All of the experiments reported here utilized blood from animals with spleens in situ. However, it is known that splenectomized calves are fully susceptible to virulent infection. In this laboratory, we have infected more than 30 sple-

TABLE 5. Growth of *Babesia bovis* in MASP culture containing dialyzed and nondialyzed FCS

Serum	PE	
	Initial	Final (72 h later)
Expt 1		
Adult bovine (fresh)	2.0	30.8
Adult bovine (stored 5 days at 4°C)	2.0	29.2
Fetal calf (stored 5 days at 4°C)	2.0	2.9 ^b
Fetal calf (dialyzed) ^a	2.0	17.7
Expt 2		
Adult bovine (fresh)	1.4	21.2
Adult bovine (stored 5 days at 4°C)	1.4	17.9
Fetal calf (stored 5 days at 4°C)	1.4	0.9 ^b
Fetal calf (dialyzed) ^a	1.4	17.9

^a FCS was dialyzed against adult bovine serum.

^b The majority of organisms exhibited abnormal morphology after cultivation with nondialyzed FCS.

TABLE 6. Effect of dialysis of adult bovine serum against FCS on the ability of the adult serum to support growth of *Babesia bovis* in MASP culture^a

Serum	PE		
	Initial	Final	
		Subculture 1	Subculture 2
Adult (control)	1.1	12.3	10.2
Dialyzed adult ^b	1.1	2.9	0.1

^a Three replicates of this experiment yielded similar results.

^b ABS (25 ml) was dialyzed against FCS (50 ml) for 3 days at 4°C. Control serum was stored at 4°C during this period.

nectomized calves 2 to 90 days after surgery with resulting virulent infection (M. Filipov, unpublished data). Such results prompt speculation on the role of the spleen in controlling parasitemia in vivo. It would be necessary to examine serum obtained from splenectomized calves to prove a splenic origin for the inhibitor. It is known that immune cells secrete soluble materials capable of causing intraerythrocytic death of both *Plasmodium* and *Babesia* (1).

The results obtained with commercial bovine sera were similar to those obtained with freshly collected sera, i.e., lack of satisfactory growth when sera from young animals was used. This suggests that the method of serum separation, which was clot extraction for the GIBCO sera and centrifugation of defibrinated blood for sera collected by us, did not appreciably affect the quality of the final product.

Some types of cattle, i.e., *Bos indicus* breeds, are reported to be more resistant to *B. bovis* than is *Bos taurus* (3). Application of the above described culture technology may more clearly

elucidate the reason for this resistance. Because all cattle would not be expected to be equally resistant to *B. bovis*, the screening of animals for susceptibility to babesiosis by an in vitro test as described in this report might allow selection of strains destined for tropical regions of the world.

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