STUDIES ON ANTIGENIC CHANGES AND STRAIN DIFFERENCES IN *BABESIA ARGENTINA* INFECTIONS

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Introduction

Parasites collected at relapses in chronic *Plasmodium knowlesi* infections were shown by Brown and Brown (1965) to be antigenically different. Other workers (Massaglia 1907; Lourie and O'Connor 1937; Broom and Brown 1940; Brown and Williamson 1962; Brown 1963; Gray 1961, 1962, 1965) described similar variants in chronic trypanosome infections. Gray (1965) found that parasites collected at relapses in an animal infected with *Trypanosoma brucei* were antigenically different. However, when the relapse variants were transmitted by the tsetse fly they reverted to a basic antigenic configuration.

Curnow (1968) reported the use of a parasitised erythrocyte agglutination test which demonstrated serologically distinct 'strains' of *Babesia argentina*. The present paper describes the use of this test in demonstrating antigenic differences between relapse parasites, the effect on them of transmission by the cattle tick *Boophilus microplus* and the occurrence of strains of *B. argentina* in herds of cattle in New South Wales.

Materials and Methods

Definitions

Isolate — Those organisms isolated from the wild population by transfer into artificial conditions of maintenance (Lumsden 1965).
Strain — The population derived from an isolate and made to reproduce continuously by serial passage in the host at the laboratory (Lumsden and Hardy 1965).
Stabilate — A population of organisms whose reproduction has been restricted by preservation in a viable state on a unique occasion (Lumsden and Hardy 1965).
Relapse — The recurrence of parasites at detectable levels in the peripheral circulation after the initial parasitaemia has declined.

Calves

Calves used in these studies were pure or crossbred Jerseys or Guernseys 6 to 12 months of age.

Isolates

Isolates were prepared from splenectomised calves at the laboratory after inoculating them with blood taken from animals infected by natural transmission in the field. They were preserved by freezing.

The isolates used were named after their district of origin, namely Wiangaree, Roseberry Creek, Rukenvale, Numinbah, Terrace Creek, Knockrow 1 and Knockrow 2. The Knockrow isolates were obtained from two subclinically infected animals on the same holding. Both were infected by the same batch of larvae ticks introduced from Queensland. The Rukenvale and Roseberry Creek isolates were obtained from adjoining holdings. The seven isolates were selected because tests showed that they were infective to *B. microplus*.

Stabilates

To prepare a stabilate, 500ml of blood was collected from the subclinically infected calf during a relapse and injected into a splenectomised calf. At the height of the initial parasitaemia in this calf blood was collected and frozen (see below).

Preservation of Parasites

Parasites were preserved by rapidly freezing whole blood, containing at least 1% of parasitised cells, to −80°C, after the addition of dimethyl sulphoxide to a final concentration of 8%. The blood was stored in 2 ml amounts in sealed glass ampoules under solid carbon dioxide. Parasites were retrieved from preservation by immersing the ampoules in a waterbath at 37°C. Using this technique, parasites were found to remain viable for over 2 years.

Detection of Parasitaemia in Non-Splenectomised Calves

Blood from each animal was examined daily for parasitaemia using the thick blood film technique of Mahoney and Saal (1961).

Transmission of Parasites Through Ticks

The Wollongbar laboratory strain of *B. microplus* was used throughout because on repeated examination it had been shown to be free of *Babesia*.

A splenectomised calf was infested with 10,000 larvae of *B. microplus*, and inoculated intravenously with sufficient parasites to ensure that the peak of the initial parasitaemia coincided with the final engorgement of the ticks. The ticks were collected over a three day period when at least 1% of the erythrocytes were parasitised.

The cultures of *B. microplus* were maintained in humidified incubators at 28°C. The eggs of all females collected from each calf were mixed. The larvae hatching from the eggs were applied in batches of 20,000 to non-splenectomised calves. Thick blood films were examined daily to monitor parasitaemias in these infested calves. As parasitaemias in the non-splenectomised animals seldom reached the 1% level needed to prepare an antigen of the tick transmitted (TT) parasites, blood was taken on the third day of the initial parasitaemia and subinoculated into splenectomised calves. When one of these calves reached the desired parasitaemia, blood was harvested for antigen production. To preserve the TT parasites some of the blood used to prepare the antigen was frozen.

Preparation of Antiserums

Antiserums to particular isolates and stabilates were prepared by injecting non-splenectomised calves with 100

ml of blood containing the parasites and collecting serum seven days after the peak of the initial parasitaemia and before the first relapse. Antiserums to TT parasites were collected from the tick infested calves in the same way. Serums were stored at —15°C without the addition of preservative.

Measurement of Agglutinating Antibody

The original technique for preparing antigen and carrying out the parasitised erythrocyte agglutination test (Curnow 1968) was used throughout.

Antigens were prepared by injecting several splenectomised calves with blood containing the partciular parasites. Blood was collected from any calf in which more than 1% of erythrocytes were parasitised. The plasma was removed by centrifugation and the cells washed twice in isotonic sodium chloride solution. A parasitised erythrocyte concentrate was prepared using hypotonic saline by the method described by Mahoney (1967), and a 1% suspension of this in isotonic sodium chloride solution was used as antigen. At least 80% of erythrocytes in the antigen were parasitised.

Serums were diluted 1/5 in isotonic sodium chloride solution contained 1% normal calf serum. Doubling dilutions of serum were prepared in this fluid and 0.2 ml of each dilution was mixed with an equal volume of the antigen suspension and allowed to settle. The reactions were read macroscopically after 1 hour and were graded ++++ (an even mat of cells) to — (a discrete button or thick ring of cells). The titre was taken as the highest dilution of serum showing a ++ reaction (an even mat of cells with a distinct ring of cells near its periphery).

A titre of 1/40 was considered to be significant because this was the lowest titre at which all of the 1,200 uninfected cattle so far tested have been negative.

Experimental Procedures and Results

The Effect of Calf Passage and Preservation on a Stabilate

Stabilate C (see below) was thawed, injected into a splenectomised calf and preserved again by collecting blood at the height of the initial parasitaemia in the calf and freezing it. This cycle was repeated four times and an antigen was then prepared. An antigen was also made from stabilate C before undergoing this treatment. Both antigens were tested with antiserums to stabilates A, B, C and D (see below). The results (Table 1) show that the antigenicity of the parasites was not altered by these procedures.

Studies on the Antigens of Parasites Collected at Relapses

Calf 1 was inoculated with the Wiangaree isolate and relapses were detected by daily thick blood film examination. Six relapses were observed during the first 180 days of the infection. Stabilate A was collected from the initial parasitaemia and stabilates B, C and D from the

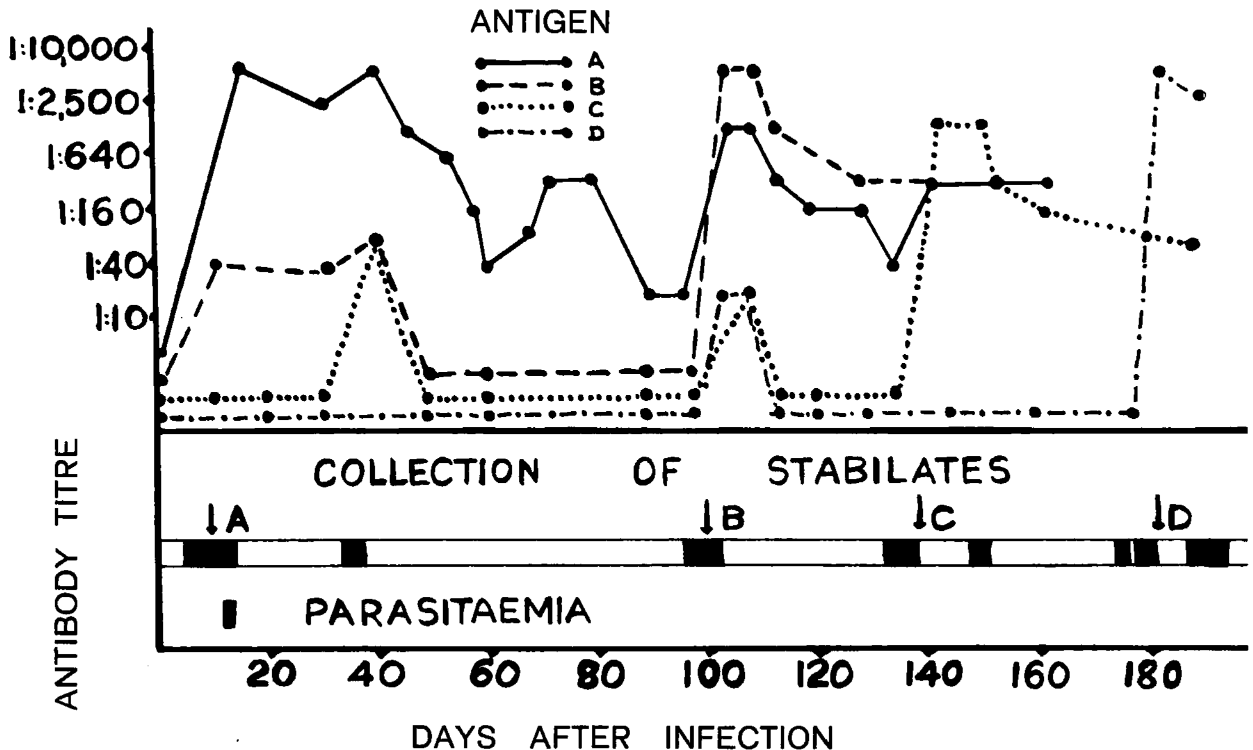

Figure 1. Reciprocal *Babesia argentina* agglutination test titres obtained by testing antigens of four stabilates, taken from calf 1 with serums from the same calf.

TABLE 1

The Effect of Five Cycles of Thawing, Calf Passage and Preservation on the Antigens of a B. argentina *Stabilate*

Antiserum to Stabilate	Agglutination Test Titres	
	With Original Stabilate C Antigen	With Antigen From Stabilate C After 5 Cycles of Thawing, Calf Passage and Preservation
A	—	10
B	10	20
C	640	1,250
D	—	—

— Indicates a titre less than 10.

second, third and sixth relapses, respectively. Stabilate A was probably the same antigenically as the Wiangaree isolate.

Antigens for the agglutination test were prepared from splenectomised calves infected with stabilates A, B, C and D and were tested with serums obtained at least three times a week from calf 1 during the first 190 days of its infection. The results are shown in Figure 1.

It can be seen that the presence of antibodies to the infected cells of a particular stabilate were not demonstrated in high titre until just after the relapse from which the stabilate was taken.

Two other stabilates (E and F) were established from relapases in calf 1, 50 and 82 weeks respectively after infection. Antigens were prepared from these stabilates and similar results were obtained when they were tested with serums from calf 1 over a period of 84 weeks.

The Effect of Tick Transmission on the Antigens of Parasites Collected at Relapses

Antiserums to stabilates A, C and D from the above experiment were prepared by inoculating non-splenectomised calves 2, 3 and 4, respectively, with the stabilates. The same stabilates were transmitted through ticks to non-splenectomised calves 5, 6 and 7 and antiserums to the TT parasites so obtained were collected from these calves.

Stabilate antigens A, C and D were prepared from splenectomised calves infected with these stabilates and TT antigens A, C and D were prepared from splenectomised calves injected with blood taken from calves 5, 6 and 7 on the third day of the initial parasitaemia.

The results obtained by cross testing these antigens and antiserums are shown in Table 2.

It can be seen that antigens from the original stabilates did not react with antiserums to the TT antigens. However, antiserums to the original stabilates contained agglutinins in significant but low titre for the TT antigens. High titres were obtained between all antigens and antiserums prepared after tick transmission of the stabilates, whereas original stabilate antigens only showed reactions to a high titre with homologous antiserums.

The experiment was extended to determine whether parasites taken from relapses in other cattle infected with the Wiangaree strain and transmitted through ticks were the same antigenically as the TT antigens A, C and D.

TABLE 2

B. argentina *Agglutination Test Titres Obtained by Cross Testing Antigens Prepared From Three Stabilates With Antiserums to These Stabilates Before and After Tick Transmission*

Antiserum To	From Calf No	Antigen From					
		Stabilate A	Tick Transmitted A	Stabilate C	Tick Transmitted C	Stabilate D	Tick Transmitted D
Stabilate A	2	640	—	—	—	—	—
Tick transmitted A	5	—	5,000	—	640	—	2,500
Stabilate C	3	10	320	640	40	80	640
Tick transmitted C	6	—	2,500	—	1,250	—	10,000
Stabilate D	4	—	40	—	80	1,250	640
Tick transmitted D	7	—	10,000	—	5,000	—	5,000

— Indicates a titre less than 10.

A stabilate was collected from a relapse in calf 5, four months after infection and another six months later. Both stabilates were transmitted by ticks to non-splenectomised calves. The first of these stabilates was passaged by ticks through two more calves in a similar manner. Infections in the calves in the series were allowed to continue for four months before passaging to the next calf.

Antigens were prepared from the TT parasites taken at the initial parasitaemia in each calf. Antiserums were collected from them also. Cross testing of these antigens and antiserums and cross testing with TT antigens A, C and D and their antiserums produced titres above 1/80 in all tests and above 1/1250 in 44 of the 49 tests.

Cross Testing of Tick Transmitted Isolates

Each of the remaining 6 isolates was transmitted through ticks to a non-splenectomised calf, namely Roseberry Creek to calf 8, Rukenvale to calf 9, Numinbah to calf 10, Terrace Creek to calf 11, Knockrow 1 to calf 12 and Knockrow 2 to calf 13. Antigens for the agglutination test were prepared from splenectomised calves injected with blood taken from each of the infested animals (TT antigens). Antiserums to the TT parasites were collected from calves 8 to 13.

Cross testing was carried out between antigens and antiserums of these TT parasites and with antigens and antiserums derived from the Wiangaree isolate after tick transmission. The results are shown in Table 3.

In each homologous test a high titre was obtained while most heterologous titres were low. A titre of 1/160 was obtained between the Roseberry Creek TT antigen and antiserum to the TT Terrace Creek antigen. However, titres in homologous tests between these two TT antigens were much higher (1/5,000 and 1/2,500). Cross reactions between the two Knockrow TT antigens were not significant nor were the reactions between the Roseberry Creek and Rukenvale TT antigens.

Discussion

Stabilates appeared to be unaltered antigenically by repeated thawing, multiplication in a splenectomised calf and re-preservation as no change was found after five of these cycles. Stabilates and isolates used in experiments described in this paper were not subjected to more than two multiplications in splenectomised calves, nor to more than one thawing and one preservation before an antigen was prepared or ticks infected.

The six stabilate antigens (A, B, C, D, E and F) collected from calf 1 were found to be antigenically different and antibodies to them were not present in calf 1 until just after the relapse from which they were taken. This indicates that relapses might be visible evidence that parasites have undergone an antigenic change and multiplied because of the absence of specific antibodies. Relapse parasites might therefore be variants of a particular strain.

Low but significant titres with relapse antigens B and C were found in serum collected after stabilate A and before stabilates B and C (see Figure 1) were collected. These reactions may have resulted from small numbers of parasites that persisted from the earlier relapse that were included with the parasites used to prepare stabilates B and C and their antigens.

When stabilates A, C and D were transmitted through ticks and the TT antigens and antiserums were prepared and cross tested, all reactions were of a high titre (Table 2). This indicates that during tick transmission an antigenic change occurred and the antigens reverted to a common specificity. These findings with *B. argentina* are similar to those of Gray (1965) with variants of *T. brucei* transmitted by the tsetse fly.

TABLE 3

Agglutination Test Titres Obtained by Cross Testing Antigens from Seven Tick Transmitted Isolates of B. argentina *With Antiserums to the Tick Transmitted Isolates*

Antiserum		Tick Transmitted Isolate Antigen						
Tick Transmitted Isolate	Calf No	Wiangaree	Roseberry Creek	Rukenvale	Numinbah	Terrace Creek	Knockrow 1	Knockrow 2
Wiangaree	5	5,000	10	—	20	—	—	—
Roseberry Creek	8	10	5,000	—	20	10	—	—
Rukenvale	9	10	—	5,000	—	—	—	—
Numinbah	10	—	20	—	10,000	—	—	—
Terrace Creek	11	20	160	20	10	2,500	—	—
Knockrow 1	12	—	—	—	10	—	1,250	—
Knockrow 2	13	—	—	10	—	—	20	1,250

— Indicates a titre less than 10.

The antigens of stabilates A, C and D were evidently not transmitted through the ticks as serums from calves 5, 6 and 7 did not react with the original stabilate antigens. Some significant reactions were obtained in cross tests between the TT antigens and antiserums to stabilates C and D from calves 3 and 4. Possibly the stabilates used to infect calves 3 and 4 contained contaminating parasites or minor antigens which were able to stimulate antibodies but which were not present in sufficient quantity in the stabilate antigens to react with the serums from calves 5, 6 and 7. During tick transmission a quantitative increase in these antigens or parasites may have occurred allowing reactions of the TT antigens with the antiserums to the original stabilates.

Four stabilates collected over a 12 month period from three calves infected with the Wiangaree strain reverted to a common specificity when transmitted through ticks indicating that the antigens concerned may be strain specific. However, the period of 12 months over which the experiment was conducted was short and the number of passages was small. Over a longer period and with frequent passages, as under field conditions, there may be a drift away from a reversion to a common specificity.

The incidence of cross reactions between the TT isolates was low (Table 3) and the one significant reaction that did occur (Roseberry Creek antigen and Terrace Creek antiserum) may have been due to the transmission of minor antigens that were not strain specific. The variety of antigenically different isolates obtained suggests that a wide variety of strain specific antigens exist in New South Wales.

The two Knockrow TT antigens were antigenically different even though they were obtained from cattle infected by the same batch of ticks. Information on the incidence of parasites carrying strain specific antigens, their distribution in the enzootic areas of Queensland and competition between strains in the tick and in the host would be needed before this occurrence could be explained.

While this preliminary work suggests that the parasitised erythrocyte agglutination test may be of value in distinguishing antigenic differences between *B. argentina* parasites, more work on the incidence and stability of strain specific antigens is needed before the results of its use in the field could be interpreted.

Summary

Using a parasitised erythrocyte agglutination test, it was shown that *Babesia argentina* parasites collected at relapses in the one animal were antigenically different from one another. When these relapse parasites were transmitted through the tick, *Boophilus microplus,* they reverted to a common antigenic type which was thought to be strain specific.

Seven isolates collected from field carriers of *B. argentina* in New South Wales were tick transmitted to uninfected calves, and antigens and antiserums of the resulting tick transmitted parasites were cross tested. High titres were obtained in all homologous tests while heterologous tests showed insignificant titres in all but one case. This indicated that there is a multiplicity of strain specific antigens present in the parasites found in New South Wales.

Two of the isolates that were antigenically different came from subclinically infected animals on the same property and which had been infected by the same group of ticks carried from Queensland.

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