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KININ, KININOGEN AND KININASE LEVELS DURING ACUTE *Babesia bovis (=B. argentina)* INFECTION OF CATTLE

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1 Kinin levels began rising on day 3 after infection of cattle with *Babesia bovis* (= *B. argentina*) and attained a maximum value of 98% above preinfection levels by day 7.

2 Kininogen levels began falling on day 3 and reached minimum levels of 83% below preinfection levels on day 8.

3 Changes in both kinin and kininogen levels on day 3 coincided with the detection of low levels of parasites, and with a fall in packed cell volume.

4 Plasma kininase levels rose significantly 6 to 9 days after infection. Preparations of lysed and sonicated uninfected and infected red cells contained kininase activity, the respective red cell preparations being 23.9 and 11.4 times more active per mg protein than uninfected red cell preparations. The effect of pH, and the inhibitors disodium edetate, 1,10 phenanthroline and aprotinin on normal and infected plasma and on the various red cell preparations suggested that the rise in plasma kininase levels during infection was probably at least partly due to parasite products.

5 These results are discussed in relation to previous data showing that both kallikrein activation and the onset of hypotension also occur on or about day 3.

Introduction

Babesia argentina, now thought to be identical with *B. bovis* (Hoyte, 1976), is an intraerythrocytic protozoan parasite of cattle. It produces an acute, often fatal syndrome characterized by generalized inflammation and vascular disturbances including vasodilatation, perivascular oedema and sluggish blood flow (Wright, 1972). The disease is similar in many respects to both *Plasmodium knowlesi* and *Trypanosoma brucei* infections in which various inflammatory agents, and in particular kinins and the kinin-forming enzyme kallikrein play a part (Tella & Maegraith, 1966; Boreham, 1968a,b; Onabanjo & Maegraith, 1970; 1971; Boreham & Goodwin, 1970). Activation of kallikrein has also been demonstrated in acute *B. argentina* infections (Wright & Mahoney, 1974). Kinin-inactivating enzymes (kininases) have been described in red blood cells, leucocytes, thrombocytes and plasma (Erdos & Yang, 1970). Plasma kininase levels were elevated in acute *Plasmodium knowlesi* infections of monkeys (Onabanjo, Bhabani & Maegraith, 1970), but not in rabbits with chronic *Trypanosoma brucei* infections (Boreham, 1968a) where plasma kinin levels were greatly elevated (Boreham, 1968b).

Hypotension has been observed in the three diseases (Skirrow, Chonsuphajaisiddhi & Maegraith, 1964; Boreham & Wright, 1976; Wright & Kerr, 1977). In *T. brucei* infections a close link between hypotension and kallikrein activation was found. As kinins produce hypotension, they may contribute to the vascular disturbance seen in babesiosis. This paper presents data on kinin, its precursor kininogen, and kininase during acute *B. argentina* infections, and discusses their possible involvement in the disease process.

Methods

Bos taurus calves, three months old, of mixed breeds and either sex were purchased from a zone free from *Boophilus microplus* (the vector of *B. bovis* in Australia), and maintained under tick-free conditions at the laboratory. Calves were splenectomized two weeks before infection.

B. bovis Lismore strain was freshly passaged by ticks to a donor calf from which inocula were then obtained. Calves were infected with 1×10^7 *B. bovis* intravenously on day 0.

Experimental procedures

Jugular blood was collected daily. Packed cell volume (PCV) was determined according to the technique of Dacie & Lewis (1968). Parasitaemia was assessed by the thick jugular blood film technique of Mahoney & Saal (1961). Kinins were extracted from 3 ml of whole blood collected in sterile plastic syringes without anticoagulant, and added to a tube containing 5 ml absolute ethanol at 3°C. The tube was shaken and allowed to stand for 10 min, then centrifuged at 2000 *g* for 10 min at 5°C. The supernatant was removed, and a further 5 ml chilled absolute ethanol added to the precipitate. The supernant was obtained as before and the combined supernatants evaporated to dryness in a Buchler Evapomix and resuspended in 2 ml of de Jalon solution (Brocklehurst & Lahiri, 1962). The extracts were stored at −70°C and were assayed within 30 days of collection. The recovery of synthetic bradykinin added to plasma (1 and 5 ng/ml) and extracted by the above method, ranged from 83–92%.

Kininogen was measured after denaturation by the method of Diniz & Carvalho (1963); 0.2 ml of the sample in 1.8 ml 0.2% acetic acid, 0.06 ml NaOH and 0.5 ml 0.2 M Tris/HCl pH 7.8 was incubated with 2 mg trypsin in 1 ml 0.2 M Tris/HCl, pH 7.8 at 37°C for 30 minutes. The reaction was stopped with 8 ml absolute ethanol at 70°C and the mixture maintained at 70°C for 30 minutes. The mixture was centrifuged at 2000 *g* for 10 min, the supernatant removed and a further 8 ml absolute ethanol at 70°C was added. The supernatant was evaporated to dryness, resuspended in 2 ml de Jalon solution and stored as described for kinin extraction.

Kinin was assayed on the rat isolated uterus as described by Boreham (1968a), except that rats were sensitized with stilboestrol (Calbiochem) 0.1 mg/kg body weight subcutaneously 22 to 24 h before use. Contractions were recorded with a Statham UC-4 universal transducer and a Varian Techtron model 135-A rectilinear pen recorder. Synthetic bradykinin (Sandoz) was used as a standard. A contact time of 1 min, with a washing time of 1 min and an interval of 5 min between tests was used. The de Jalon solution contained atropine sulphate (1 mg/l), mepyramine maleate (0.1 mg/l) and D-lysergic acid (1 mg/l).

Preparation of parasite and erythrocyte extracts

Crude lysate (Nor L) and sonicate extracts (Nor S) of uninfected red cells were prepared from a calf as described by Goodger (1971). This calf was then infected with 1×10^8 *B. bovis* Lismore strain intravenously. Infected red cells were concentrated by the differential lysis method (Mahoney, 1967) and crude lysate (Arg L) and sonicate (Arg S) extracts (Goodger, 1971) prepared from them. These extracts were stored at −70°C until required. Protein concentrations of the extracts were estimated by the Biuret method (Layne, 1957).

Kininase assay

Plasma (40 µl) in 0.460 ml 0.02 M Tris/HCl buffer pH 7.4 was incubated at 37°C with 500 ng synthetic bradykinin (Bk) in 0.5 ml 0.02 M Tris/HCl pH 7.4. At 0, 5, 10, 15, 20 and 25 min, 10 µl samples were withdrawn and assayed on the rat isolated uterus in oestrus.

Parasite and erythrocyte extracts were incubated with 500 ng Bk and assayed as above. Generally 10 mg of erythrocyte protein and 3 mg parasite protein was used in the same total volume.

Inhibition of kininase activity

A sample (0.1 ml) of Nor L, Arg L and Nor S and Arg S extracts, and infected plasma were incubated with 0.3 ml inhibitor and 0.1 ml 0.02 M Tris/HCl buffer pH 7.4 at 37°C for 15 minutes. Bk (500 ng) in 0.5 ml of 0.02 M Tris/HCl pH 7.4 was then added and samples were assayed at 0, 5, 10, 15, 20 and 25 minutes. Samples without inhibitor were assayed in the same way. All samples exhibited linear rates of Bk inactivation for at least 20 min; generally 80% or more was inactivated by 20 minutes. Bk incubated at 37°C in buffer and alone showed no decrease in activity during 25 minutes. Kininase alone was also assayed. Inhibitors incubated with 0.1 ml of the enzyme source were aprotinin (Bayer) 3000 iu, 1,10 phenanthroline hydrate 60 µg or disodium edetate 100 µg.

The level of kininase activity was defined as the amount of Bk inactivated by 1 mg protein/s or by 1 ml plasma/second. This was calculated from the amount inactivated in 10 or 15 min by a known amount of enzyme added to a solution containing 500 ng Bk.

The effect of pH on kininase actvity was assessed by incubating 0.1 ml of normal or infected plasma, Nor S, Nor L, Arg S or Arg L for 10 min at 37°C with 0.4 ml buffer of pH 4.0, 5.0, 5.5, 6.0, 6.5, 7.0 (0.1 M citrate phosphate buffer), 7.4, 8.0 (0.1 M Tris/HCl), and with 500 ng synthetic Bk in 0.5 ml of the appropriate buffer. Aliquots were assayed on the rat isolated uterus at 0 and 10 minutes. The activity at any pH was defined as a percentage of the activity at pH 7.4. All enzymes had an optimum activity around pH 7.4. The pH of aliquots was not readjusted to pH 7.4 before addition to the organ bath as the amount used (10 µl in 3 ml de Jalon) did not alter the pH in the organ bath.

Statistical analysis

Data were analysed by the unpaired *t* test. The mean percentage change on a given day was compared with the mean of pre-infection values.

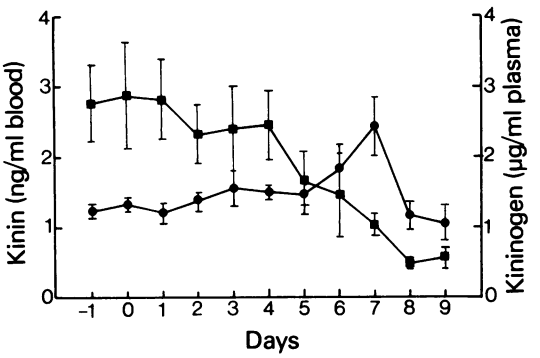

Figure 1 Kinin concentration (ng synthetic bradykinin equivalents/ml blood, ●) and kininogen concentration (µg synthetic bradykinin equivalents/ml plasma, ■) from 6 calves infected with *B. bovis* on day 0. Values are means; vertical lines show s.e. means.

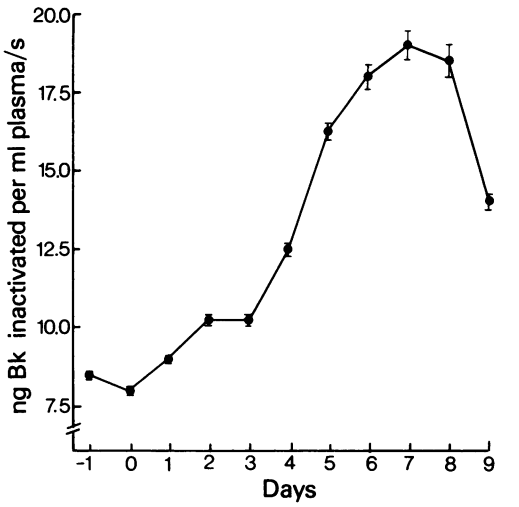

Figure 2 Plasma kininase activity during infectioh of 6 calves with *B. bovis*. Calves were infected on day 0. Levels expressed as ng bradykinin (Bk) inactivated per ml plasma/s and plotted as means; vertical lines show s.e. means.

Results

The PCV fell continuously from day 2 onwards and had fallen by 64% terminally. The PCV fall was significant from day 5 onwards ($P < 0.01$).

Parasites were first detected on day 3 (approximately 10/mm³ blood) and reached maximum counts by days 7–8 (approximately $2–4 \times 10^5$/mm³ blood).

Kinin levels (Figure 1) started rising on day 3 and reached maximum levels on day 7 ($P < 0.0025$).

Kininogen levels started falling on day 3 and reached minimum levels on day 8 ($P < 0.001$).

Plasma kininase activity

The plasma kininase levels (Figure 2) were significantly higher from day 6 onwards ($P < 0.01$). Plasma alone had no effect on the isolated uterus preparation of the rat.

Parasite and erythrocyte kininases

Extracts of lysed infected erythrocytes contained 23.9 times more kininase activity per mg protein than did extracts of the lysed erythrocytes prior to infection. Extracts from infected erythrocytes which had been lysed and sonicated contained 11.4 times more kininase than similar extracts made from erythrocytes prior to infection. None of these extracts had any effect on the isolated uterus of the rat.

Inhibition of kininases

Table 1 shows that the kininases in Nor L, Arg L, Nor S, Arg S were distinct. The efficacy of inhibitors in infected and uninfected plasma also differed. Excess of inhibitors did not increase the level of inhibition of any extract that was only partially inhibited. Inhibitors added to the rat isolated uterus before the addition of Bk did not diminish the response. The rat uterus gave maximum responses to 7–10 ng Bk in a 3 ml bath. All kininases were inactivated in an acid pH. Total inactivation occurred at pH 5.0 with all extracts but the normal erythrocytic extracts Nor L and Nor S were already inactivated at pH 5.5 and pH 6.0 respectively (Figure 3). Readjustment of the pH from 5.0 to 7.4 restored the enzymatic activity.

Discussion

The data indicate that during acute *B. bovis* infections a large turnover in kinins occurs. This turnover coincides with the activation of increasing amounts of kallikrein (Wright & Mahoney, 1974) even though the parasitaemia is relatively low. The kinin level was raised by as much as 98% during the infection, but the fall in the kininogen concentration was even more pronounced. At least 2.39 µg bradykinin equivalents per ml plasma were liberated during the infection (Figure 1). In severe *P. knowlesi* and *P. berghei* infections (Tella & Maegraith, 1966; Ohtomo & Katori, 1972) marked consumption of kininogen and elevated terminal levels of kinin (Onabanjo & Maegraith, 1971; Ohtomo & Katori, 1972) were found and considered responsible for the peripheral vascular stasis and terminal collapse. Activated

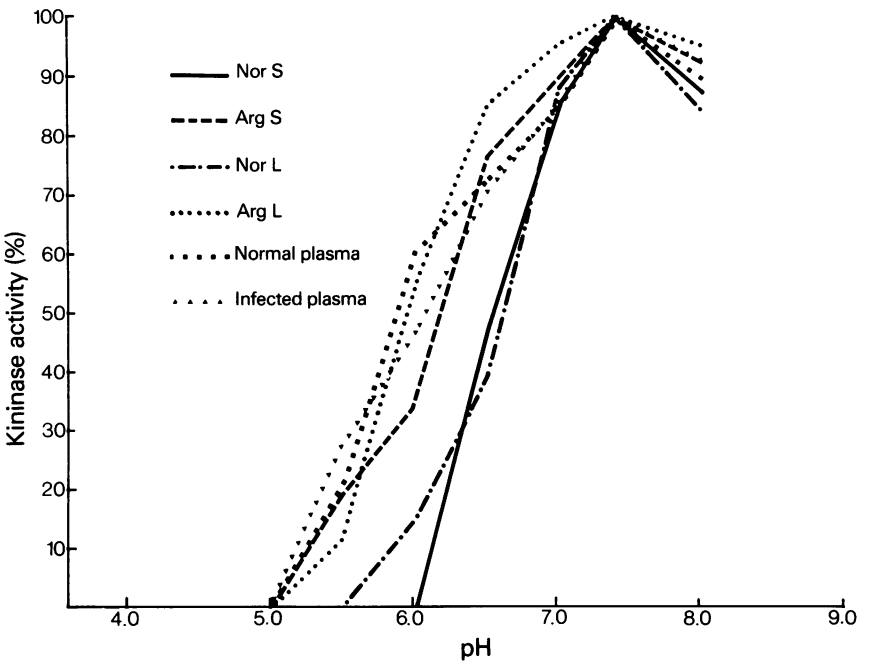

Figure 3 Inhibition of normal and infected plasma kininase and of Nor L (crude lysate extract of uninfected red cells), Nor S (sonicate extract of uninfected red cells), Arg L (crude lysate of infected red cells) and Arg S (sonicate extract of infected red cells) kininase activity at various pH.

kallikrein has been implicated in peripheral vascular changes in both *P. knowlesi* and *B. bovis* infections (Onabanjo & Maegraith, 1970; Wright, 1973). Eyre & Lewis (1972) and Eyre, Lewis & Wells (1973) suggested that kinins contribute significantly to the profound hypotension observed during bovine anaphylactic shock. It is likely that the increased liberation of kinins plays a significant part in the hypotension during *B. bovis* infections. The role of other inflammatory compounds is not yet known.

The kinin formation in *T. brucei* and *T. rhodesiense* infections is due to the activation of prekallikrein by antigen/antibody complexes (Boreham, 1970; Boreham & Goodwin, 1970). Early in the infection, activation of prekallikrein by the parasite may also occur (Boreham, 1970). Wright & Mahoney (1974) also found evidence for the activation of kallikrein early in infection. This activation was not due to tissue breakdown products such as red cell stroma, or to antibody. More recently Wright (1975) and Mahoney

Table 1 Effect of inhibitors on various kininase preparations

	No inhibitor		*EDTA*		*1,10 Phenanthroline hydrate*		*Aprotinin*	
	Rate	*% Inhibition*	*Rate*	*% Inhibition*	*Rate*	*% Inhibition*	*Rate*	*% Inhibition*
Nor L	0.023	0	0.019	18	0.00	100	0.00	100
Arg L	0.55	0	0.00	100	0.24	56	0.07	87
Nor S	0.028	0	0.00	100	0.00	100	0.00	100
Arg S	0.32	0	0.00	100	0.00	100	0.14	56
Normal plasma	0.109	0	0.00	100	0.014	87	0.096	12
Infected plasma	0.397	0	0.015	96	0.234	41	0.317	20

Rate is defined as the number of ng of bradykinin inactivated per mg protein/second. Nor L=crude lysate extract of uninfected red cells; Nor S=sonicate extract of uninfected red cells; Arg L=crude lysate of infected red cells; Arg S=sonicate extract of infected red cells.

& Wright (1976) have demonstrated both *in vitro* and *in vivo* activation of kallikrein with small amounts of parasite extracts. This suggests that the parasite directly activates kallikrein with the subsequent liberation of kinin. The role of endotoxin and lysosomal enzymes from polymorphonuclear leukocytes, both of which are known to produce kinins (Nies & Melmon, 1971; Movat, Steinberg, Habal & Rahadive, 1973) also should not be discounted.

Kinin-inactivating enzymes are contained in various constituents of the blood (Erdos & Yang, 1970). Strong kinin-inactivating activity has also been described in disintegrated but not in intact *T. brucei* (Boreham, 1968a). The non-specific proteases reported in the malarial parasites *P. knowlesi, P. berghei* and *P. falciparum* (Cook, Grant & Kermack, 1961; Levy & Chou, 1973; Levy, Siddiqui & Chou, 1974), may also act as exogenous kininases (Onabanjo *et al.*, 1970); however, the rise in plasma kininase levels in acute *P. knowlesi* infections was thought to be of endogenous origin. Non-specific proteases have also been reported in *B. argentina* and *B. bigemina* (Wright & Goodger, 1973).

In the present experiments plasma kininase levels rose from day 2 until days 6–7 at a time when the parasitaemia was relatively low, and red cell destruction was negligible; this suggests that the enzyme was not derived from red cells. However, during the last two days of infection when massive red cell destruction occurs, some of the plasma kininase may be derived from these cells.

The rise in kinins and kallikrein and the fall in kininogen, which accompany the progressive vascular stasis and hypotension observed in acute *B. bovis* infections (Wright & Mahoney, 1974; Wright & Kerr, 1977) suggest that the raised kininase levels play only a minor role. Recent evidence suggests that plasma kininase is significantly lowered in haemolytic states (Bielawiec, Lukjan, Kiershowska & Korfel, 1973). This further supports the view that the elevated plasma kininase levels observed in acute *B. bovis* infections are derived from red cells.

The finding that all observed kininases were inactivated at slightly acid pH was in agreement with work showing that this inactivation could result in the accumulation of kinins in inflamed tissue which may have a pH as low as 6 (Lurie, 1939; Edery & Lewis, 1962). The present results suggest that mobilization of the kinin system early in the disease plays an important role in the initiation and probably also in the maintenance of the acute vascular disturbances in acute *B. bovis* infections.

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