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IMMUNE REACTIONS AND KININ FORMATION IN CHRONIC TRYPANOSOMIASIS

BY

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Richards (1965) has shown that the concentration of kinins in the plasma and tissues of mice acutely infected with trypanosomes progressively increases until death occurs after 5 days. Kinins are also present in the urine of mice, rats and rabbits infected with *Trypanosoma* (*Trypanozoon*) *brucei* subgroup organisms (Goodwin & Richards, 1960; Boreham, 1966). It is possible that the kinin detected in the urine of infected animals may be derived from plasma proteins that have leaked through the kidneys because urinary kallikrein will release kinin from its precursor in plasma protein (Horton, 1959). If this is the case it is likely that urinary kinin is not important in the pathology of trypanosomiasis.

Trypanosomiasis in rabbits, cattle and man follows a different course from that seen in laboratory rodents. Waves of parasitaemia occur at intervals of approximately 7–10 days and at no stage of the disease is the number of parasites in the blood large. In the rabbit this will continue for 5–6 weeks before death occurs but in cattle and man the disease process may continue for 1 or 2 years. Infected blood may be taken at any stage of the infection and the trypanosomes preserved by freezing. Such a preparation is called a stabilate. This term has been defined by Lumsden & Hardy (1965) as "a population of trypanosomes preserved in a viable condition on a unique occasion, and maintained at approximately $-80°$".

Trypanosomes in successive parasitaemic waves contain antigens which differ in their properties as shown by neutralization and agglutination reactions. These antigens are called the variant antigens (Lumsden, 1965). In addition other antigens are found which are common to all parasitaemic waves. These are called the common antigens and may be detected by the precipitin, fluorescence and complement fixation reactions.

In order to understand more fully the disease processes which occur in chronic trypanosomiasis, work has been undertaken to investigate the level of kinins in the blood of infected rabbits and cattle, and to correlate their production with parasitaemia and the immunological response.

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METHODS

Kinin extraction

Whole blood was collected, without anticoagulant, from the marginal ear vein of a rabbit or from the jugular vein of cattle and 3 ml. of it added to a tube containing 5 ml. of chilled absolute alcohol. The tube was shaken, allowed to stand for 10 min and centrifuged at 1,500 *g* for 10 min at 4° C. The supernatent was taken off, evaporated to dryness at temperatures below 40° C using a Rotary Evapo-mix (Buchler Instruments, New York). The dried material was suspended in 2 ml. of de Jalon solution immediately before assay (Brocklehurst & Lahiri, 1962). Siliconed glassware or polythene apparatus was used throughout.

Kininogen extraction

The method described by Diniz & Carvalho (1963) was used. Samples of 0.2 ml. of plasma were denatured by heating with 0.2% acetic acid in a boiling water bath for 30 min, and kinin was liberated from it by adding trypsin.

Assay of kinins

Free kinin in the blood and kinin liberated from its precursor were assayed on the isolated rat uterus preparation. Virgin rats weighing 150–200 g were injected intraperitoneally with stilboestrol 100 mg/kg body weight, 16 hr before sacrifice. One horn of the uterus was suspended in a 3 ml. bath containing de Jalon solution at 31° C. The de Jalon solution contained atropine sulphate (10^{-6} g/ml.), mepyramine maleate (10^{-7} g/ml.) and 2-bromolysergic acid diethylamide (5×10^{-6} g/ml.). The contact time of each sample was 45 sec and the interval between tests 5 min. Maximal contractions were usually obtained with 0.8 ng of synthetic bradykinin in the bath. Packed cell volumes were determined concurrently with kinin extractions so that results could be expressed as ng of bradykinin/ml. of plasma.

Trypanosome stabilates

The classification of trypanosomes by Hoare (1966) was adopted. Two trypanosome stabilates were used:

1. *Trypanosoma (Trypanozoon) brucei* 427 subgroup obtained from Dr. B. Weitz, Lister Institute of Preventive Medicine, Elstree, Hertfordshire.

2. *Trypanosoma (Trypanozoon) brucei E.A.T.R.O.* 2/1/4 isolated from *Glossina pallidipes* in South Busoga, Uganda, in 1960.

Infections of the animals

New Zealand white rabbits weighing 2.5–3.5 kg were infected with 10^7 *T.(T.) brucei* 427. The inoculum was prepared by diluting mouse or rat blood, heavily infected with trypanosomes, with phosphate buffered saline (pH 7.8).

While visiting Uganda, two East African Zebu cattle, numbers 496 and 539, each weighing approximately 150 kg, were infected with 10 ml. of a 10^{-2} dilution of the stabilate *T.(T.) brucei E.A.T.R.O.* 2/1/4. This suspension was shown to contain 10^7 ID63s/ml. as assessed by infectivity titration (see below).

Collection of blood samples

Blood samples were collected daily from the rabbits and cattle. Rabbits were bled directly from a small cut in an ear vein into chilled alcohol or into a heparinized tube. Bovine blood was withdrawn in 10 ml. volumes from the jugular vein using a disposable plastic syringe. From each sample 3 ml. was transferred immediately to chilled alcohol and the remainder to a heparinized tube for kininogen assays and infectivity titrations. A further 20 ml. was removed from the jugular vein with a glass syringe; serum obtained from it was stored in 0.7 ml. aliquots at −20° C and used for immunological tests.

Estimation of parasitaemia

Parasitaemia was determined daily on both cattle using the infectivity titration technique of Lumsden, Cunningham, Webber, van Hoeve & Walker (1963). Serial ten-fold dilutions of the blood were prepared in phosphate buffered saline (pH 8.0) (Lumsden, Cunningham, Webber, van Hoeve, Knight & Simmons, 1965) at 0° C, immediately after collection. A volume of 0.1 ml. of each dilution was injected intraperitoneally into each of six mice. Tail blood of the mice was examined daily for 21 days to see whether or not they became parasitaemic. Results were calculated as described by Lumsden *et al.* (1963) in terms of the number of infective doses (ID63)/ml. of blood. The concept of the ID63 supposes that if a large group of mice are injected with an average of one infective trypanosome each, 37% will not become infected.

Preparation of stabilates

Stabilates were prepared daily from bovine blood. Glycerol was added to heparinized blood to give a final concentration of 7.5%. The blood was then placed in capillary lymph tubes and sealed as described by Cunningham, Lumsden & Webber (1963). The tubes were stored at −79° C in solid carbon dioxide.

Agglutination test

The agglutination test was carried out as described by Cunningham & Vickerman (1962), using the daily serum samples and the stabilate *T.(T.) brucei E.A.T.R.O.* 2/1/4 as antigen. Serial twofold dilutions of the serum were prepared in phosphate buffered saline (pH 7.8) commencing at a 1 in 10 dilution. Approximately 0.1 ml. of each dilution was placed on a siliconed glass slide in a moist chamber. A tube of antigen was removed from the dry ice cabinet, the ends broken off and one end drawn out to a very fine point in a low Bunsen flame. This was then lightly touched onto each drop of dilute serum so that a small amount of antigen was drawn out. The slides were allow to stand for 1 hr and then examined microscopically for agglutination of the trypanosomes.

Neutralization test

The neutralization test (Cunningham & van Hoeve, 1963) was carried out using the original stabilate and the stabilates prepared at the peaks of parasitaemia. A trypanosome suspension containing 10^4 ID63s/ml. was prepared and 0.2 ml. allowed to react with 1.8 ml. of serum at 0° C for 15 min. A volume of 0.1 ml. (100 ID63s) was injected intraperitoneally into each of six mice. The mice were examined daily for 21 days to see whether they became parasitaemic. Neutralizing antibody was assumed to be present when none of the mice became parasitaemic.

Precipitin test

One per cent. agar plates were prepared using glycine buffer (pH 7.4) containing 0.5% sodium azide as diluent. A central well 1 cm in diameter was cut for the antigen. This was surrounded by six similar wells, 4 mm apart. The antigen consisted of trypanosomes separated by differential centrifugation and lysed with distilled water. The daily serum samples were placed in the surrounding wells and the plates kept at room temperature for 48–72 hr. The lines of precipitation were observed after this period.

Immunofluorescence test

The test was carried out as described by Kimber (1966). The antigen for this test consisted of washed, separated trypanosomes of the *brucei* subgroup fixed on a glass microscope slide by gentle heat. Serial dilutions of the sera collected from the infected cattle were added to the antigen and after allowing them to react for 20 min a fluorescent conjugate was added. The conjugate consisted of a rabbit antibovine globulin conjugated with fluorescein isothiocyanate (Microbiological Associates, Bethesda, Maryland). The degree of fluorescence after removal of surplus conjugate was assessed microscopically.

Control tests on cattle

Control tests were carried out on the cattle to show that they were free from pathogenic trypanosomes. A volume of 0.5 ml. of bovine blood was injected into each of six mice on two separate occasions and samples of tail blood from these mice were collected at intervals for 60 days and examined microscopically for the presence of parasites.

Serological tests to detect agglutinating antibodies, precipitating antibodies and fluorescent antibodies were also carried out.

A volume of 5 ml. of whole blood was cultured in Weinman's medium (Weinman, 1960) to determine the presence of other flagellates in the blood of the cattle. These cultures were examined microscopically twice weekly for the presence of *Trypanosomatidae.*

RESULTS

Control experiments in cattle

Mouse inoculations and serological tests showed that the animals were not infected with pathogenic trypanosomes. Blood cultures showed the presence of *Trypanosoma (Megatrypanum) theileri* in the blood of both cattle.

Kinin formation in rabbits

The kinin activity in the blood of rabbits infected with *T.(T.) brucei* subgroup organisms increased after infection from less than 10 ng/ml. of plasma to almost 200 ng/ml. on the eighth day (Fig. 1). The kinin concentration then fell but, until death occurred after about 5 or 6 weeks, remained above preinfection levels. There was some evidence that subsequent smaller peaks of kinin occurred on days 14 and 20 but the results were not conclusive.

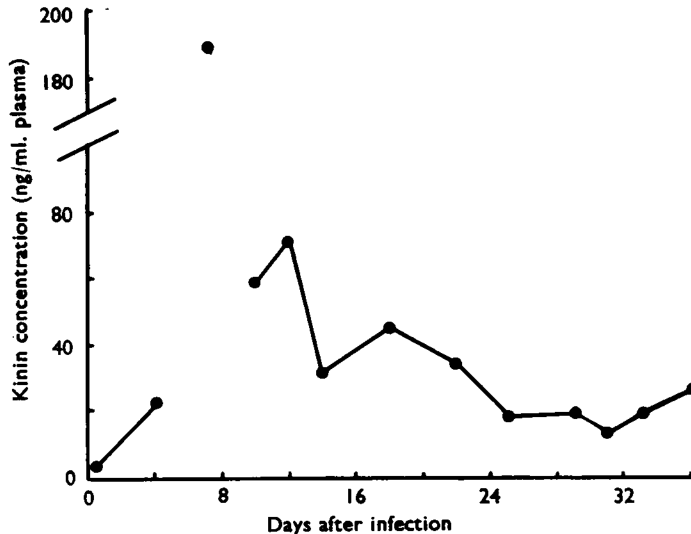

Fig. 1. Kinin concentration in the plasma of a rabbit chronically infected with *T.(T.) brucei 427*, expressed as ng of synthetic bradykinin/ml. plasma.

Kinin formation in cattle

The results for both animals were similar and are illustrated in Figs. 2 and 3. Increased kinin activity was detected in the blood on the twelfth day after infection and by the eighteenth day it had returned almost to the preinfection level (Fig. 2). A smaller second peak of kinin activity was detected 22 days after inoculation. Each kinin peak in the blood occurred 2–3 days after a peak in parasitaemia. After treatment of the cattle with diaminazene aceturate (Berenil, Hoechst), 7 mg/kg intramuscularly, the kinin concentration returned to low levels and no more parasites were detected in the blood by subinoculation into mice.

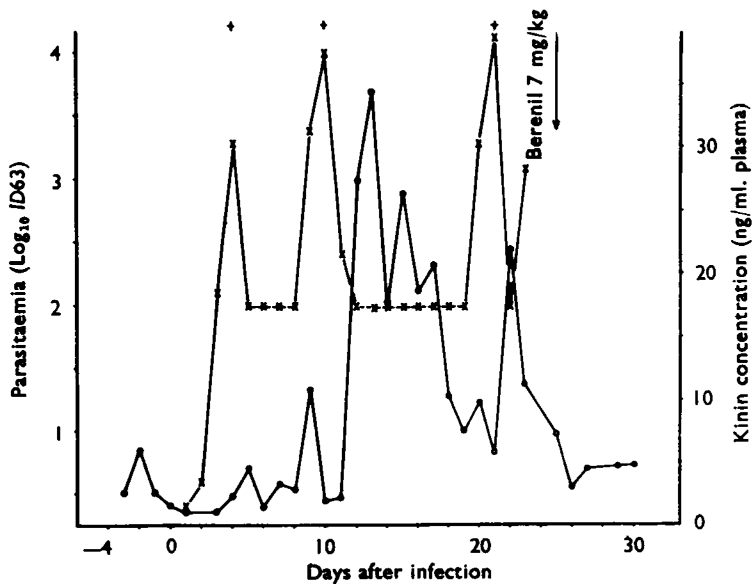

Fig. 2. Kinin concentration and parasitaemia in the blood of cow number 539, infected with *T.(T.) brucei E.A.T.R.O. 2/1/4* during the first 23 days of the infection and after treatment with berenil 7 mg/kg intramuscularly. ×——×, Parasitaemia estimated by the infectivity titration technique and expressed as mouse ID63s/ml. of blood ; ○——○, kinin concentration expressed as ng/ml. of synthetic bradykinin/ml. of plasma.

Figure 3 shows the results of kininogen determinations. Six days after infection the level of kininogen in the blood fell and remained low until about the fifteenth day. There was then a rise followed by another fall which coincided with the second peak in kinin activity. During these falls in plasma kininogen the amount of releasable kinin fell from about 6 μg/ml. plasma to 3 μg/ml. plasma. After treatment with diaminazene aceturate the kininogen concentration in the blood rose rapidly to 12 μg/ml. plasma suggesting that throughout the infection kininogen was being produced rapidly.

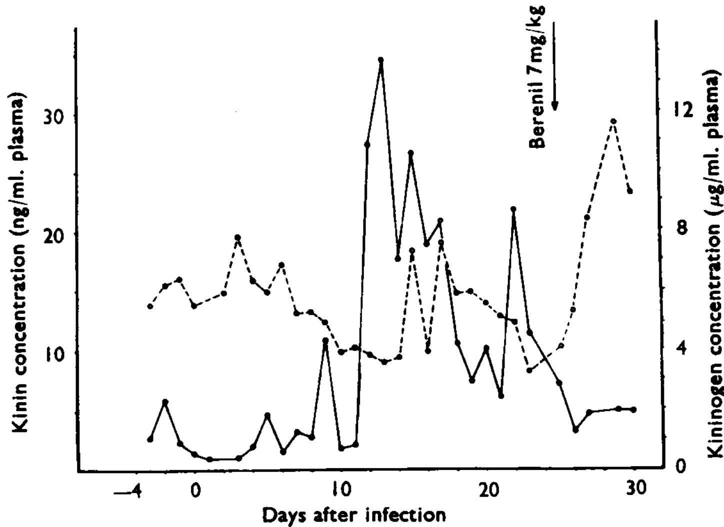

Fig. 3. Kinin and kininogen concentrations in the blood of cow number 539, infected with *T.(T.) brucei E.A.T.R.O.* 2/1/4. O——O, Kinin concentration expressed as ng/ml. of synthetic bradykinin/ml. of plasma; O– – –O, kininogen concentration expressed as μg of synthetic bradykinin released by trypsin/ml. of plasma.

Immunological tests

Variant antibodies

The results of the neutralization tests are illustrated for cow number 539 in Table 1 and Fig. 4, and they confirm the results of Cunningham, van Hoeve & Grainge (1965). The table shows that the three peaks in parasitaemia which occurred 4, 9 and 20 days after infection were caused by three different antigenic variants. The first peak at 4 days (539/4) was produced by organisms of the same antigenic nature as the inoculated stabilate. Neutralizing antibody to the inoculated stabilate was detected on the fifth day, to the ninth day stabilate on the eleventh day and to the twentieth day stabilate on the twenty-third day. The peak in parasitaemia on day 9 (539/9) represents the first variant and the 20-day peak (539/20) the second variant.

TABLE 1

NEUTRALIZATION TESTS CARRIED OUT WITH THE DAILY SERUM SAMPLES COLLECTED FROM COW NUMBER 539 AFTER INFECTION WITH *T. (T.) brucei E.A.T.R.O.* 2/1/4 AND THE STABILATES COLLECTED AT THE PEAKS OF PARASITAEMIA (539/4, 539/9 AND 539/20)

0, No neutralizing antibody detected ; +, neutralizing antibody detected.

		Serum																			
Stabilate *E.A.T.R.O.*	Control	1	2	3	4	5	6	7	8	9	10	11	12	13	14	18	19	20	21	22	23
2/1/4	0	0	0	0	0	+	+	+	+	+	+										
539/4	0	0	0	0	0	+	+	+		+									+		
539/9	0				0	0	0	0	0	0	0	+	+	+	+						
539/20	0				0					0						0	0	0	0	0	+

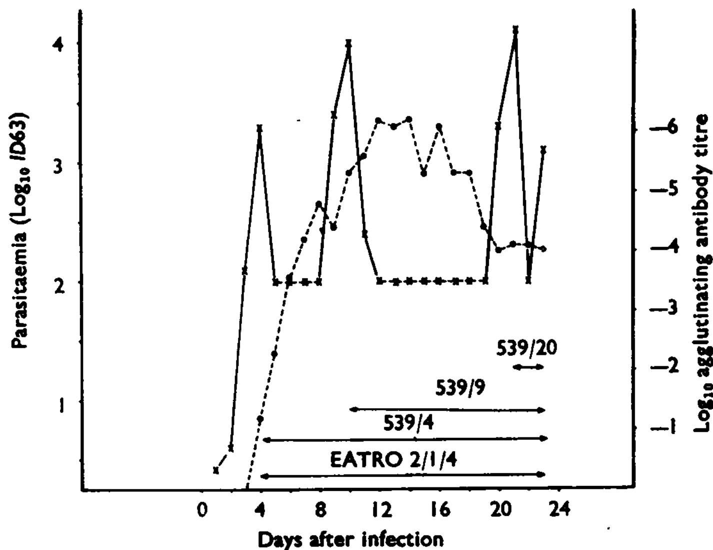

Fig. 4. The relationship between parasitaemia and titre of variant antibodies in cow number 539 aftel infection with *T.(T.) brucei E.A.T.R.O.* 2/1/4. ×——×, Parasitaemia expressed as $\log_{10}$ ID63/ml. blood; ○——○, $\log_{10}$ of agglutinating antibody titre; ←——→, period when neutralizing antibody could be detected for a particular stabilate (see Table 1).

Agglutinating antibodies to the inoculated stabilate were detected in the serum of both cattle on the fifth day of the infection (Fig. 4). Very high maximum titres were reached on the twelfth day just after the first variant had been produced. Titres remained high throughout the period of this investigation. It was not possible to carry out agglutination tests using the stabilates prepared at the peaks of parasitaemia as antigens because the numbers of parasites present were too small.

The agglutinating antibody titres for both cattle were extremely high and this may help to explain the low parasitaemia.

Common antibodies

The results for the common antibodies are shown in Figs. 5 and 6. Precipitins were first detected in cow number 496 on day 8 and for cow number 539 on day 10. Fluorescent antibodies appeared on day 9 in the serum of cow number 496 and day 13 in cow number 539.

DISCUSSION

Because the cattle studied were living just outside a tsetse fly belt it was necessary to show that the animals were free from pathogenic trypanosomes. *T.(M.) theileri* was present in the blood of the cattle but it is not thought to have affected the results because

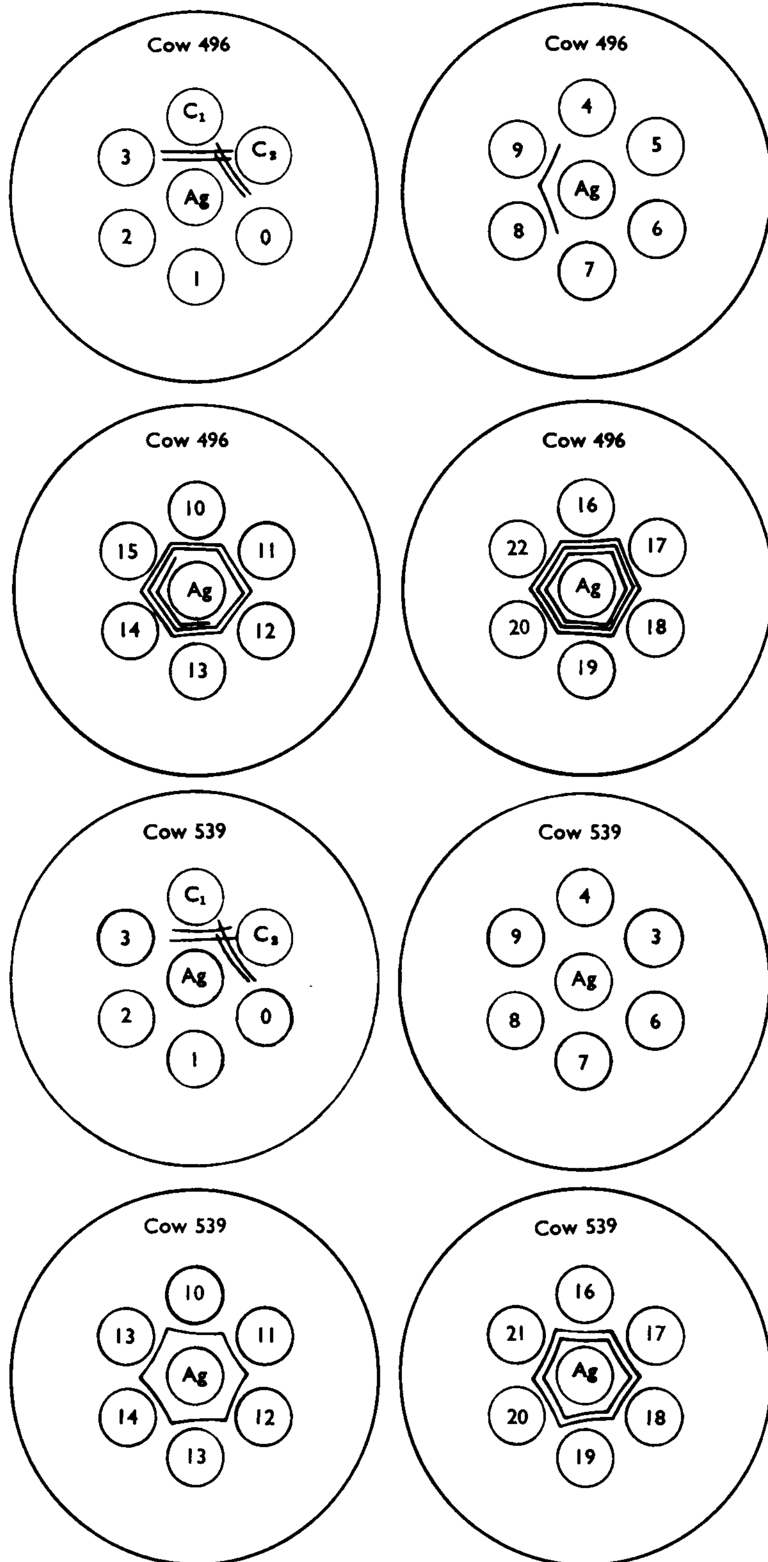

Fig. 5. The production of common precipitating antibodies in cattle number 496 and 539 after infection with *T.(T.) brucei E.A.T.R.O.* 2/1/4. Ag, Antigen consisting of separated washed trypanosomes lysed with distilled water ; C_1, serum from cattle, containing precipitins ; C_2, serum from a rabbit, containing precipitins ; O, preinfection bovine serum ; 1–22, serum collected on successive days after infection of the cattle.

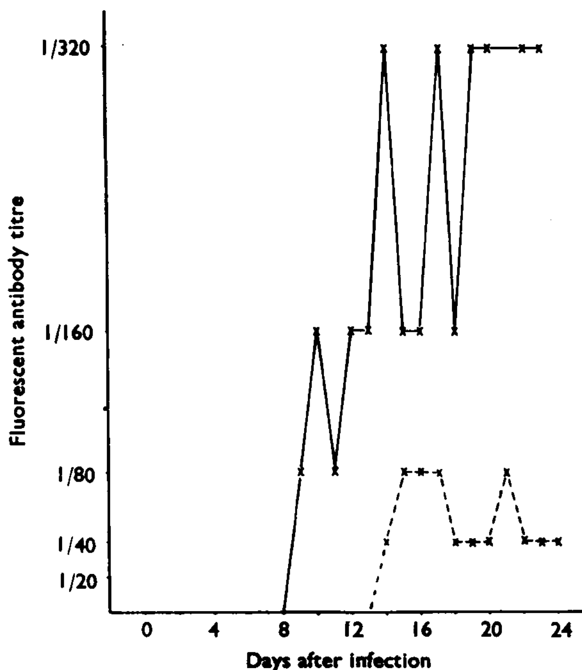

Fig. 6. Fluorescent antibody titre in the serum of cattle number 496 and 539 during the first 24 days after infection with *T.(T.) brucei E.A.T.R.O.* 2/1/4. ×——×, Cow number 496; ×——× cow number 539.

the organism is generally considered to be non-pathogenic and it has been shown that the non-pathogenic parasite *Trypanosoma* (*Herpetosoma*) *lewisi* in rats does not induce kinin formation even when present in large numbers (Boreham, 1966).

It has been shown by Lewis (1964) that kinins are produced in the inflammatory reaction and Corrado, Reis, Carvalho & Diniz (1966) have implicated kinins in the aetiology of some types of cardiovascular shock. Kinins have also been found in the blood on animals with allergic and immune conditions, especially anaphylactic shock (Brocklehurst & Lahiri, 1962, 1963; Dawson & West, 1965). The symptoms and signs of chronic trypanosomiasis in some ways resemble these states; and the liberation of kinins and other pharmacologically active substances may well play a part in the pathogenesis of this disease. The liberation of kinins seems to be a general phenomenon which accompanies trypanosomiasis and possibly other infections. Raised kinin activity has been found in trypanosome infections in mice (Goodwin & Richards, 1960), rats and rabbits (Goodwin & Boreham, 1966), guinea-pigs (Bhattacharya, Sen & Talwalkar, 1965) and now in cattle. Human patients with trypanosomiasis also have raised kinin levels in the blood (Boreham, unpublished work). Tella & Maegraith (1966) have demonstrated kinin release in acute malaria infections in the monkey and Goodwin & Richards (1960) found similar results with mice infected with *Babesia rodhaini*, *Streptococcus pyogenes* and Rift Valley fever virus.

The concentration of kinin in the plasma of rabbits infected with *T.(T.) brucei* 427 was raised and followed a similar course to that previously described in the urine

(Boreham, 1966). The largest amounts were always found early in the infection around the tenth day, but throughout the infection the kinin concentration was above control levels. There was some evidence from the results obtained in rabbits that subsequent peaks of kinin activity occurred in the blood and it is possible that these might be associated with the waves of parasitaemia.

The rabbit is not a good experimental animal in which to undertake further studies of this nature because the immunological responses in this species are complex (Gray, 1962) and for this reason cattle were used to resolve this point.

The results obtained in cattle confirm the finding that the largest amounts of kinin are always present early in trypanosome infections and also show that kinin was released in the blood 2 or 3 days after a peak in parasitaemia. It is believed that at the peaks of parasitaemia an antigen-antibody reaction takes place, resulting in a reduction in the number of infective organisms followed by the destruction and removal of most of the trypanosomes from the blood. This is followed by the appearance of an immunologically different variant which is responsible for the subsequent peak in parasitaemia. It seems probable from these results that the antigen-antibody reaction is also implicated in the release of kinins.

The actual amount of kinin liberated in the infected cattle was probably much greater than was detected. The kininogen concentration in the blood fell to half the control value suggesting that at least 3 μg of releasable kinin/ml. plasma has been utilized. Kinins are very rapidly destroyed by kininase in the blood and this is probably the reason why much of the kinin released *in vivo* was not detected (Erdös, Renfrew, Sloane & Wohler, 1963).

The variant antibodies, as detected by the agglutination and neutralization reactions, were found in the sera of the cattle early in the infection before any increase in kinin concentration. The common antibodies, however, were demonstrated at about the time kinin release occurred. It is thus likely that it is the common antibodies which are participating in the reaction which results in the liberation of kinins. Possible mechanisms by which this might occur are at present under investigation but it seems possible that the antigen-antibody complex may activate prekallikrein in a similar way to glass activation.

Cunningham & van Hoeve (private communication) have suggested that precipitinogens are continually being released throughout trypanosome infections and that these are important in the development of the pathological changes which occur in trypanosomiasis. The results obtained provide the first experimental evidence in support of this hypothesis although the complement fixing antigen could equally well be involved.

A recent World Health Organization report (1965) states that when an antibody combines with an antigen which is part of, or closely attached to, a cell membrane, and complement is activated close to the membrane, the end result is the formation of a substance that can damage the membrane of a variety of cells. Eisen (1961) has made observations which suggest that the plasma kinin system is activated when blood or plasma comes into contact with damaged tissues. Goodwin, Jones, Richards & Kohn (1963) have shown that when extensive tissue damage occurs, as in severe burns, pharmacologically active peptides are released.

Two possible mechanisms of kinin release are thus suggested:

(1) The antigen-antibody reaction causes local tissue damage resulting in the release of kinins.

(2) The antigen-antibody reaction activates the plasma kinin system and the liberated kinin causes permeability changes in the vascular endothelium.

In either case a vicious circle would be set up resulting in the release of more kinins.

Increased vascular permeability has been shown to be one of the principal lesions in trypanosomiasis (Boreham & Goodwin, 1967; Goodwin & Hook, 1968). The cause of these vascular changes is at present uncertain but these results strongly suggest that kinins, even if they do not initiate these changes, will certainly help to maintain them and therefore play an important part in the disease processes which occur in chronic trypanosomiasis.

SUMMARY

1. Kinin activity in the blood of rabbits and cattle with chronic trypanosomiasis has been investigated.

2. Increased kinin activity occurred 2 or 3 days after peaks in parasitaemia ; at the same time the amount of precursor in the blood decreased.

3. Liberation of kinins in chronic trypanosomiasis probably results from an antigen-antibody reaction.

4. Variant antibodies can be readily detected 5 days after infecting cattle with *T.(T.) brucei* subgroup organisms and the common antibodies 8–13 days after infection. These results suggest that the common antibodies are implicated in the release of kinins.

5. The possible importance of kinins in the pathogenesis of chronic trypanosomiasis is discussed.

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