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PHARMACOLOGICALLY ACTIVE SUBSTANCES IN THE BLOOD, TISSUES AND URINE OF MICE INFECTED WITH *TRYPANOSOMA BRUCEI*

BY

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Goodwin & Richards (1960) showed that mice infected with *Babesia rodhaini* and other infective organisms excreted increased amounts of peptides in their urine. Goodwin, Jones, Richards & Kohn (1963) found that the urine of patients with severe burns contained increased quantities of active peptides and histamine. Previous workers have shown that during disease, injury and stress considerable quantities of kinin, histamine and other pharmacologically active substances are released from the tissues and plasma, and that the amount excreted in the urine is increased.

Beraldo (1950) found that in anaphylactic shock in dogs a kinin and histamine were formed; Brocklehurst (1960) showed the release of histamine and a slow reacting substance (SRS-A) during anaphylaxis in guinea-pigs, and Rocha e Silva & Rosenthal (1961) found histamine, bradykinin and probably 5-hydroxytryptamine present in perfusates from air pockets of experimentally burned rats.

Lewis (1927) showed that the first reaction to local tissue injury was the release of histamine or an " H-substance " and Krogh (1929) postulated the existence of a slowly eliminated " H-colloid." Hilton & Lewis (1957) suggested that this " colloid " may be plasma kinin or plasma-kinin forming enzyme which may be activated in the interstitial fluid. Edery & Lewis (1962) showed that when histamine increases capillary permeability it allows kinins or their precursors to escape from the blood to the interstitial spaces, and they suggested that the histamine released during injury may be responsible for the accumulation of plasma-kinin forming enzymes in the lymph.

Spector & Willoughby (1959) suggested that in thermal injury the initial capillary permeability caused by histamine is maintained by other endogenous mechanisms, such as activation of permeability increasing globulins.

Tella & Maegraith (1962) found that, in monkeys infected with *Plasmodium knowlesi*, the plasma bradykininogen fell to 10% of normal during the course of infection and suggested that a concomitant rise in the blood bradykinin probably occurs. Brocklehurst & Lahiri (1962, 1963) showed that perfusates from tissues during anaphylaxis *in vitro* contained bradykinin-forming enzyme and that in the blood of animals during anaphylaxis the bradykinin formation exceeded the rate of destruction.

The present work is an attempt to follow the changes in kininogen, kinin and histamine in the plasma, tissues and urine of mice infected with *Trypanosoma brucei*.

METHODS

Inoculation with Trypanosoma brucei. The strain was isolated in Pong Tamale (Ghana) in 1938 and has been maintained by blood passage in mice. Mice were infected by subcutaneous injection of 0.1 ml. of infected blood containing about 100,000 trypanosomes; the mice died 4 to 5 days after inoculation.

Control groups of mice were inoculated subcutaneously with normal mouse blood or washed rabbit erythrocytes. Some groups of mice were given washed mouse or rabbit blood cells intravenously.

Collection of urine. Groups of ten to fifty mice were housed in Perspex and stainless steel metabolism cages. The faeces were separated from the urine by a cotton mosquito-net screen or by means of a polyethylene funnel with a curved spout described by Brittain (1959). Urine was collected in a siliconed bottle containing 1 ml. of N-hydrochloric acid, and the *p*H was adjusted to 7.2 immediately before testing on isolated organs or to *p*H 6.0 before preparing extracts. Urine extracts were prepared by the methods described by Goodwin *et al.* (1963), ethanol extracts being eluted from an Amberlite C G 50 type I ion-exchange column to give separate histamine and kinin fractions.

Preparation of test samples from blood. Groups of ten mice were killed with ether and heart blood was pooled in chilled siliconed tubes containing heparin. Kinin and kininogen extracts were prepared by the methods of Brocklehurst & Lahiri (1962) and Lahiri (1962). The tubes were centrifuged at 2,900 revs/min for 10 min at 4° C. The plasma was pipetted off into three times its volume of cold redistilled ethanol, left for 10 min, recentrifuged at 3,500 revs/min for 5 min and the supernatant fluid removed, dried *in vacuo* below 50° C and reconstituted in de Jalon solution to the same volume as the original plasma and assayed for kinin. The precipitate from the ethanol was washed with distilled water, centrifuged and the supernatant fluid was discarded; the deposit was heated in a boiling-water bath for 10 min to destroy kininase. A solution of trypsin (B.D.H.) was added to give 0.4 mg of trypsin to every 1 ml. of original plasma and the mixture was incubated for 20 min at 37° C to release kinin from the precursors. The reaction was arrested with ethanol, the mixture centrifuged and the supernatant fluid was dried *in vacuo* below 50° C. The dry extract was reconstituted in de Jalon solution to the same volume as the original plasma and assayed for released kinins.

Alcoholic extracts were also passed through an Amberlite C G 50 type I ion-exchange column and fractions were collected.

Histamine was determined in plasma by Code's (1937) modification of Barsoum & Gaddum's (1935) method.

Kinin, kininogen and histamine extracts were also prepared from separated leucocytes, erythrocytes and trypanosomes.

Tissues. Ears, abdominal skin and feet were taken from mice killed with ether and prepared by Code's (1937) method for histamine assay. Samples of these tissues were chopped finely in chilled ethanol and the kinins were extracted by the methods described for plasma.

Cell counts. Total trypanosome, erythrocyte, leucocyte and differential counts were made on groups of mice throughout the course of the infection.

Enzymic destruction of active substances. Urine specimens and blood and tissue extracts were incubated for 1 to 24 hr at 37° C with chymotrypsin (Armour Pharmaceutical Co.), papain (Light & Co.) and cysteine, peptidase (Light & Co.), pepsin (Armour Pharmaceutical Co.), trypsin (B.D.H.) or histaminase (prepared from fresh pig kidney) and tested for activity. Control tubes containing no enzyme, enzyme and distilled water, and samples known to contain histamine or kinin, were prepared for every test.

Urine, blood and tissue samples were also heated with an equal volume of 0.1 N-hydrochloric acid or 0.1 N-sodium hydroxide solution in a boiling-water bath for 10 min, neutralized and compared with untreated samples.

Assay methods. Guinea-pig ileum was suspended in a 3-ml. organ-bath of oxygenated Tyrode solution at 37° C. Rat duodenum was prepared by the method of Horton (1959) in a 3-ml. organ-bath of oxygenated de Jalon solution at 31° C. Virgin rats were given 100 mg/kg of stilboestrol subcutaneously 24 hr before being killed, the uterus was removed and one horn was suspended in a 3-ml. organ-bath of de Jalon solution at 31° C.

Atropine (10^{-6}) was used in all preparations to depress spontaneous movements. Atropine, mepyramine, chlorcyclizine, triprolidine, iproniazid and lysergic acid diethylamide were used as antagonists, usually in concentrations of 10^{-4} in the organ-bath. Some extracts were injected intravenously into rats anaesthetized with pentobarbitone sodium and the blood pressure was recorded from a carotid artery with a Condon capillary manometer.

RESULTS

The course of the infection varied little from test to test. The trypanosomes first appeared in fresh coverslip preparations of peripheral blood 36 to 48 hr after inoculation; parasitaemia increased daily until on the fifth day the trypanosomes were as numerous as the erythrocytes. The total erythrocyte count remained uniform throughout the infection. The total leucocyte count rose steadily during the infection until on the fifth day it was about twice its original level. Differential counts made on normal mice always show a high proportion (65 to 70%) of lymphocytes. From the onset of the infection there was little change in the absolute numbers of lymphocytes but the number of neutrophils increased steadily (Fig. 1).

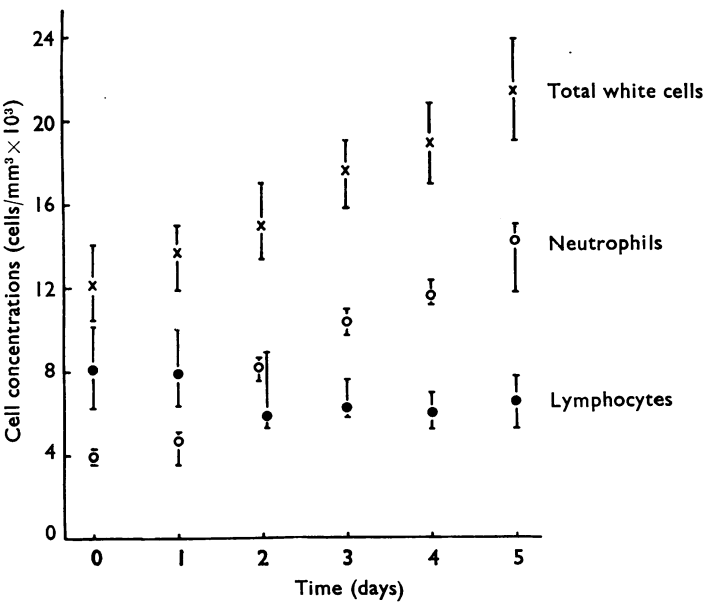

Fig. 1. The effect of *T. brucei* infection on blood white cell concentrations (ordinate, cells/mm^3 × 10^3) in mice. Abscissa, days after infection. The figures are pooled results of 250 mice.

During the first three days of the infection the plasma histamine activity progressively increased; on the fourth and fifth days it decreased somewhat although it still remained above the normal level (Fig. 2, Table 1). The increase in histamine activity preceded the parasitaemia. The activity was destroyed by histaminase and antagonized by antihistamine compounds; column chromatography showed the substance to have the same properties as histamine.

The kinin activity of plasma showed a progressive rise throughout the infection till death (Fig. 3, Table 1). Extracts caused relaxation of the rat duodenum and contraction of the

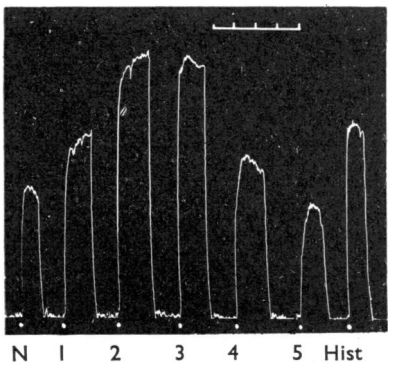

Fig. 2. The effect of histamine extracts of plasma from mice infected with *T. brucei* on the response of the guinea-pig isolated ileum. Tyrode solution with atropine (10^{-6}) was used in a 3-ml. organ-bath. At N, the equivalent to 0.1 ml. of plasma from normal mice; at 1, 2, 3, 4 and 5, the equivalent to 0.1 ml. of plasma from mice on the 1st, 2nd, 3rd, 4th and 5th day of infection; at Hist, 15 ng of histamine base in 0.1 ml. Time scale in minutes.

TABLE 1

HISTAMINE AND KININ CONCENTRATIONS IN TISSUES FROM NORMAL AND INFECTED MICE

Of 100 mice infected on the same day with a standard number of trypanosomes, two groups of ten were killed each day and the tissues were pooled for extraction. Controls were not infected

Time from infection	Histamine in				Kinin in			
	Ears (μg/g)	Skin (μg/g)	Feet (μg/g)	Plasma (ng/ml.)	Ears (ng/g)	Skin (ng/g)	Feet (ng/g)	Plasma (ng/ml.)
Control	50	20	15	130	80	40	30	20
Day 1	70	30	20	150	100	65	50	30
Day 2	110	50	35	180	125	80	70	50
Day 3	100	60	45	190	160	110	85	70
Day 4	100	30	35	160	190	130	100	80
Day 5	90	25	25	150	230	155	125	100

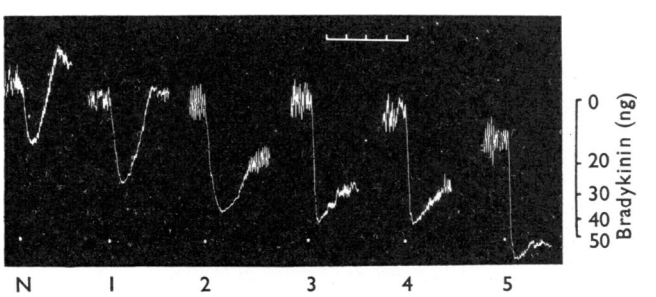

Fig. 3. The effect of kinin extracts of plasma from mice infected with *T. brucei* on the response of the rat isolated duodenum. De Jalon solution with atropine (10^{-6}) was used in a 3-ml. organ-bath. At N, the equivalent of 0.2 ml. of plasma from normal mice, at 1, 2, 3, 4 and 5, the equivalent of 0.2 ml. of plasma from mice on the 1st, 2nd, 3rd, 4th and 5th day of infection. The vertical scale gives tne doses of bradykinin (ng) which caused relaxations of the isolated duodenum from the zero line to the marks shown. Time marks in minutes.

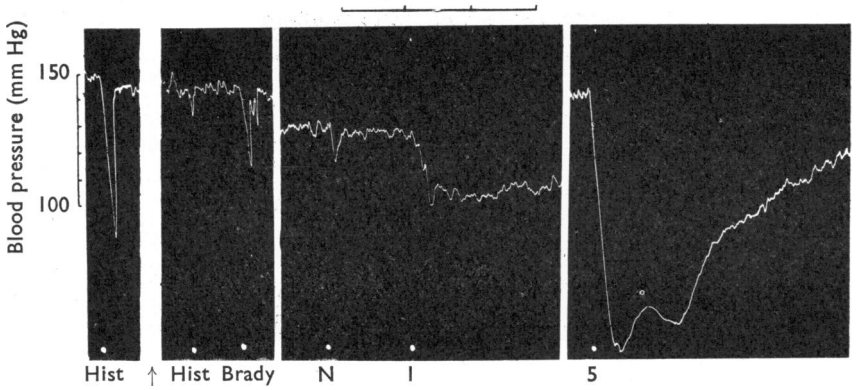

Fig. 4. Rat blood pressure, pentobarbitone sodium anaesthesia. Responses of the rat blood pressure to intravenous injections of histamine (Hist, 500 ng), bradykinin (Brady, 10 ng) and, at N, 0.2 ml. of plasma kinin-extract from normal mice and at 1 and 5, 0.2 ml. of plasma kinin-extract from mice on the 1st and 5th day of infection with *T. brucei*. At the arrow, chlorcyclizine (0.2 mg) was given to inhibit the response to histamine.

rat uterus and guinea-pig ileum; the reactions were unaffected by atropine, mepyramine, triprolidine, iproniazid or lysergic acid diethylamide. Activity was unaffected by incubation with pepsin and trypsin but was destroyed by chymotrypsin, papain and peptidase. Extracts caused a prolonged fall of arterial pressure in the anaesthetized rat (Fig. 4).

The active material was unaffected by boiling with 0.1 N-hydrochloric acid for 10 min but the activity was reduced to less than half by boiling with 0.1 N-sodium hydroxide solution for 10 min. It was readily dialysable through cellophane and was eluted from an amberlite C G 50 column in a similar way to bradykinin.

In nine experiments, kinin activity (means and standard errors) in the plasma of normal mice was equivalent to 15 ± 6.9 ng of bradykinin per ml. and on the fifth day of infection 84 ± 19.1 ng of bradykinin per ml. Sometimes there was a large increase on the second day followed by a lower activity on the third day but in all experiments a further increase occurred before death. The kininogen content of plasma showed a progressive decrease during the infection (Fig. 5), similar to that found in monkeys infected with *P. knowlesi*

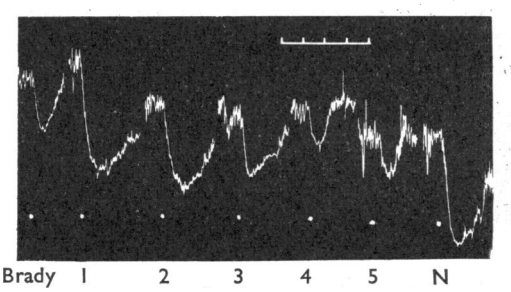

Fig. 5. The effect of kininogen extracts of plasma from mice infected with *T. brucei* on the response of the rat isolated duodenum. De Jalon solution with atropine (10^{-6}) was used in a 3-ml. organ-bath. At N, the equivalent of 0.2 ml. of plasma from normal mice; at 1, 2, 3, 4 and 5, the equivalent of 0.2 ml. of plasma from mice on the 1st, 2nd, 3rd, 4th and 5th day of infection; at Brady, 20 ng of synthetic bradykinin in 0.2 ml. Time marks in minutes.

by Tella & Maegraith (1962). Extracts of leucocytes followed a similar pattern to that of plasma. Trypanosomes separated from the blood of heavily infected mice contained small quantities of histamine; other pharmacologically active substances were present but insufficient material has so far been available for their identification.

Histamine in the urine followed the same pattern as that in plasma; a sharp rise of activity occurred early in the infection and later decreased. The histamine concentration in the urine collected, prepared and diluted to a constant volume from normal mice was approximately 150 ng/ml. rising on the third day of infection to about 650 ng/ml. and falling to 120 ng/ml. on the fifth and final day. The kinin activity of urine increased progressively with the infection, as previously found by Goodwin & Richards (1960).

The histamine and kinin activity of ears, abdominal skin and feet followed the same pattern as plasma during the course of the infection but the absolute amounts of active material were much greater than in plasma and varied in different tissues (Table 1). On the day following the inoculation of trypanosomes, long before organisms were detectable by microscopical examination of the peripheral blood, a large release of active substances occurred in the tissues examined, particularly in the ears.

DISCUSSION

A monkey with an acute *P. knowlesi* infection becomes profoundly hypotensive; at the same time, plasma kininogen is depleted (Tella & Maegraith, 1962). It is tempting to think that the release of a kinin may contribute to the development of shock. Malarial parasites live in the erythrocytes and as they multiply cause rupture of the cells so that parasites, their metabolic residues, haemoglobin and empty red cell membranes are liberated into the circulating blood. It is possible that a part of this debris triggers the release of kinin from precursors in the plasma.

The present work has shown that during acute *T. brucei* infections in the mouse, a similar release of substances with kinin activity occurs. The kinin activity of the plasma increases and the precursors are depleted, but no haemolysis occurs and the erythrocyte count is undiminished. It is possible that the presence of trypanosomes in the blood, exposing as they do an extensive, unfamiliar surface to the plasma, might release kinin in much the same way as contact with a glass surface releases kinin from plasma. This explanation is unlikely. Plasma kinin activity increases early in the infection, when very few trypanosomes are present in the circulation, and mice infected with certain strains of *T. congolense* survive for long periods with large numbers of circulating trypanosomes.

It seems more probable that *T. brucei*, as a result of its multiplication and metabolism, produces substances that cause the release of kinin at such a rate that it is excreted by the kidney and that the plasma precursors are depleted. Moreover, during infection, substances with kinin-like activity and histamine are present in some of the tissues in concentrations which greatly exceed those in the plasma. On the fourth or fifth day, when the plasma kininogen is severely depleted, kinin activity is still present in the urine and is extractable from the ears, skin and feet in larger quantities than from normal animals. The larger part of the kinin activity found in the plasma and in the urine is probably derived from the tissues, especially towards the end of the infection. Much further analysis is required of the substances released from tissues during infection before they can with confidence be identified with kinins.

The ears, feet and abdominal skin of the normal mouse have a considerable histamine content; histamine has been associated with the release of kinin (Spector & Willoughby, 1959; Edery & Lewis, 1962). During infection with *T. brucei*, these tissues show a progressive rise in the amount of histamine which can be extracted from them. The source of this histamine has not yet been determined; histological or histochemical examinations have not yet been made on infected animals.

The cause of death in acute protozoal infections is obscure. The appearance of pharmacologically active substances of endogenous origin which have been demonstrated in the tissues of mice infected with *T. brucei* may well be linked with circulatory disturbances which lead to severe hypotension and death.

SUMMARY

1. Blood, tissues and urine were collected every day from mice infected with *Trypanosoma brucei*, precaution being taken to minimize artefacts, and assayed for histamine and kinins.

2. The mice excreted increasing amounts of pharmacologically active peptide and histamine in the urine as the infection progressed. The kinin in plasma and leucocytes increased during the infection, and the kininogen decreased. The kinin-like substance in the ears, feet and abdominal skin increased to a much greater extent than in the plasma, and the increase occurred before the trypanosomes became numerous in the blood. The total histamine in the tissues increased during the early days of the infection and, although it later fell, it still remained above the normal value.

3. It is suggested that tissues contribute histamine and pharmacologically active kinin-like substance to the blood stream during infection, and that these substances may contribute to physiological disturbance and death.

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