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In vitro studies on the mechanism of kinin formation by trypanosomes

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1. An investigation into the mechanism of kinin release in trypanosomiasis has been made *in vitro*.

2. Trypanosomes contain a low level of kininase but no kinin-forming enzyme nor substrate for salivary kallikrein.

3. The kininase activity in the plasma of infected rats does not differ from that of normal animals, and trypanosomes do not affect the breakdown of synthetic bradykinin by enzymes in the blood.

4. In trypanosomiasis the kininogenase precursor in plasma is activated. The hypothesis is put forward that the antigen-antibody complex may be involved in this.

It is now well established that raised kinin concentrations are found in the blood and tissues of a wide variety of hosts infected experimentally with pathogenic trypanosomes (Goodwin & Richards, 1960; Boreham, 1968). The mechanism of kinin release in trypanosomiasis is not known but it has been suggested that an antigen-antibody complex is implicated (Boreham, 1968). The physical presence of trypanosomes in the blood is unlikely to be sufficient in itself to cause kinin release because no free kinin could be detected in the blood of rats infected with the non-pathogenic parasite *Trypanosoma (Herpetosoma) lewisi* even when 10^8–10^9 parasites/ml. were present (Boreham, 1966).

This work has been undertaken to investigate how free kinin accumulates in the blood of animals infected with pathogenic trypanosomes.

Methods

Trypanosomes

Trypanosoma (Trypanozoon) brucei 427, a pathogenic strain of trypanosomes obtained from the Lister Institute of Preventive Medicine, has been used throughout this study. The trypanosomes were maintained in rats or mice by syringe passage of infected blood every 2 or 3 days.

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Separation of trypanosomes

Blood was collected from rats which had been infected 3–5 days previously. Trypanosomes were separated from the heavily infected blood by differential centrifugation (Boreham, 1968).

Ultrasonic treatment of trypanosomal suspensions

Disintegration of trypanosomes was carried out with an M.S.E. ultrasonic disintegrator at 20,000 c/s for 5 min.

Assay of kinins

Virgin rats weighing 150–200 g were injected intraperitoneally with stilboestrol 100 mg/kg 16 hr before they were killed. One horn of the uterus was suspended in a 3 ml. organ bath containing oxygenated 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 for each sample was 45 sec and the interval between tests 5 min. Maximal contractions were usually obtained by addition of 0.8 ng of synthetic bradykinin (BRS-640) to the bath.

Kinin activity of trypanosomes

Washed, separated trypanosomes (10^9 parasites in 0.2 ml.) or an equivalent number of disintegrated trypanosomes were added to an isolated rat uterus preparation which was sensitive to 0.2 ng/ml. of synthetic bradykinin.

Test for a substrate for salivary kallikrein in trypanosomes

A volume of 0.8 ml. of a suspension of washed separated trypanosomes or disintegrated trypanosomes (10^{10} parasites/ml.) was incubated at 37° C with 0.2 ml. of undiluted human saliva in which kininase had been inhibited by addition of 1,10-phenanthroline hydrate (100 μg/ml.). At intervals of 5 min up to 40 min 0.1 ml. samples were removed from the reaction mixture and tested for liberated kinins on the isolated rat uterus preparation.

Kininase activity in trypanosomes

A volume of 0.5 ml. of washed separated trypanosomes or disintegrated trypanosomes (10^{10} parasites/ml.) was incubated with 0.5 ml. of synthetic bradykinin solution (1 μg/ml.) at 37° C. A sample of 0.01 ml. was tested on the isolated rat uterus preparation at 5 min intervals for kinin activity.

Kininase activity of plasma

Plasma kininase assays were performed on the plasma separated from ten rats which had been infected with *T. (T.) brucei* 427 for 4–6 days and were showing a heavy parasitaemia of about 10^9 parasites/ml. of blood. Synthetic bradykinin (500 ng contained in a volume of 0.05 ml.) was added to 1 ml. of a 1 in 40 dilution of rat plasma in phosphate buffered saline (pH 7.2) at 37° C. Samples of 0.1 ml. from this mixture were tested on the isolated rat uterus preparation at 5 min inter-

vals and the time when the bradykinin content of the incubation mixture had fallen to 1% was determined. Similar experiments were performed on the plasma of ten normal control rats.

Kinin-forming activity of trypanosomes

A kininogen substrate was prepared from rabbit blood as described by Amundsen, Nustad & Waaler (1963). A volume of 0.5 ml. of substrate was incubated at 37° C with either 0.5 ml. of washed separated trypanosomes (10^{10} parasites/ml.) or an equivalent number of disintegrated trypanosomes. Samples of 0.1 ml. were removed at 5 min intervals from the mixture and tested on the isolated rat uterus preparation for activity.

Kinin-forming activity of plasma

The kinin-forming activity of the plasma of ten rats infected for 4–7 days with *T. (T.) brucei* 427 and showing a parasitaemia of 10^9 parasites/ml. was determined. A volume of 0.5 ml. of rabbit kininogen substrate (Amundsen *et al.*, 1963) was incubated at 37° C for 30 min with 0.5 ml. of plasma from an infected rat containing 1,10-phenanthroline hydrate (100 μg/ml.) in the presence of 1 ml. of Tris buffer (pH 7.8). The reaction was terminated by addition of 0.2 ml. soya bean trypsin inhibitor (1 mg/ml.) (Sigma Chemicals). The samples were assayed on the isolated rat uterus preparation as described by Davies, Holman & Lowe (1967), to determine the amount of kinin released. Similar experiments were performed with infected rat plasma which had been acidified to pH 2 for 10 min, a procedure which activates kinin-forming enzyme precursors (Horton, 1959). As controls, the kinin-forming activity of plasma of ten normal rats was determined before and after acidification. Each determination was carried out in triplicate and the mean of the results taken.

Interference of the breakdown of synthetic bradykinin by kininase

Erythrocyte and plasma kininases were prepared from human blood as described by Amundsen, Waaler, Dedichen, Laland, Laland & Thorsdalen (1964).

A volume of 0.5 ml. washed trypanosomes (10^{10} parasites/ml.) or an equivalent number of disintegrated trypanosomes was added to 500 ng of synthetic bradykinin and 0.5 ml. of either plasma or erythrocyte kininase preparation. The mixture was incubated at 37° C and samples tested at 5 min intervals on the isolated rat uterus preparation. The results were compared with the breakdown of bradykinin by plasma and erythrocyte kininases in the absence of trypanosomes.

Results

On addition of separated live trypanosomes or disintegrated trypanosomes to the isolated rat uterus preparation no contractions occurred ; thus, the organisms contained no detectable amounts of free kinin. Trypanosomes also did not contain any substrate for salivary kallikrein, nor could any kinin-forming activity be detected.

When separated live trypanosomes were incubated with synthetic bradykinin, no loss in activity was seen after 26 min of incubation but when similar experiments were performed with disintegrated parasites a 50% reduction in bradykinin content of the incubation mixture was seen in 21 min (Fig. 1). The trypanosomes thus con-

tained a low level of kininase activity, probably at intracellular sites, because it could only be detected after disintegration of the parasites.

The rate of breakdown of synthetic bradykinin by plasma and erythrocyte kininases was unaffected by the addition of either whole or disintegrated trypanosomes. Thus the trypanosomes contained no substances that interfered with the enzymic breakdown of kinin.

Studies of the kininase activity showed that plasma from infected and control rats had comparable activities because the time for a standard 1 in 40 dilution of plasma to break down 99% of 500 ng of synthetic bradykinin was 6 min for both normal and infected animals ($P>0.99$). The accumulation of kinins seen in trypanosomiasis cannot, therefore, be due to decreased activity of plasma kininase and as trypanosomes contain a low level of kininase of their own, a slight increase in the rate of breakdown of kinins might be expected.

In a series of ten experiments the kininogenase activity fell from 38.0 ± 5.4 ng/ml. bradykinin equivalents in the control rats to 9.5 ± 1.0 ng/ml. bradykinin equivalents in rats infected with *T. (T.) brucei* 427. The precursor level fell from 223.7 ± 24.9 ng/ml. bradykinin equivalents to 110.8 ± 10.2 ng/ml. bradykinin equivalents during the infection. These results show ($P<0.01$) that during the infection the kininogenase precursor in the blood is activated and the kinin-forming enzyme so formed will convert kininogen to free kinin.

Discussion

Kinins are found in the blood of animals with trypanosomiasis and these substances may be important in the pathogenesis of the disease (Boreham & Goodwin,

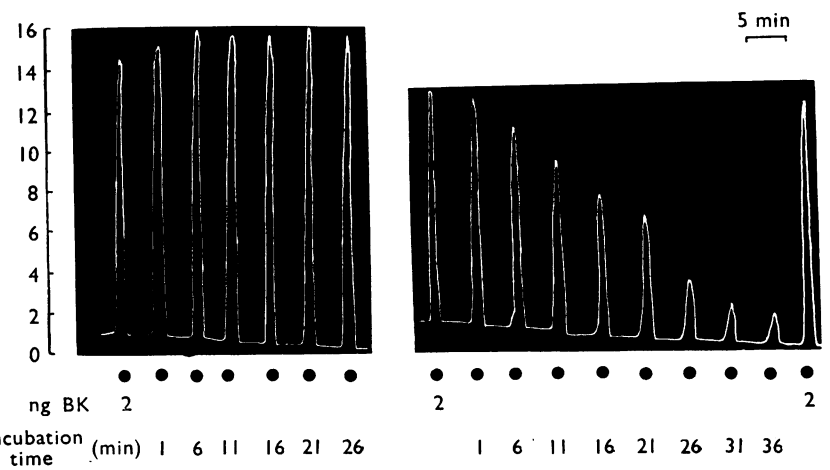

FIG. 1. Kininase activity of trypanosomes. The left hand trace shows the results of incubating 500 ng of synthetic bradykinin with 5×10^9 washed and separated *T. (T.) brucei* 427 parasites suspended in phosphate buffered saline, pH 7.8, at 37° C. A sample of 0.01 ml. of the incubation mixture was tested for activity on the isolated rat uterus preparation after various periods of incubation. The right hand trace shows a similar experiment using the same number of trypanosomes which had been ultrasonically disintegrated. Responses to 2 ng of synthetic bradykinin (BK) were obtained at the start and at the end of each experiment. 1 ng of synthetic bradykinin gave 38% of the response of 2 ng synthetic bradykinin on this preparation.

1967; Goodwin & Hook, 1968) so it is of interest to understand the mechanism of their release. Trypanosomes contain no detectable kinin-forming enzymes and no substrate on which salivary kallikrein can act but they do, however, contain a low level of kininase.

Kininase activity has been demonstrated in striated muscle cells, liver cells, cells of the mucosa of the alimentary canal (Amundsen & Nustad, 1965), squamous epithelial cells of the oral mucosa (Amundsen & Nustad, 1964) and leucocytes (Schwab, 1962; Greenbaum & Kim, 1967). In addition low levels of kininase activity have been found in cultures of *Escherichia coli* and *Pseudomonas æruginosa* but not in cultures of β-haemolytic streptococci (Amundsen & Rugstad, 1965; Rugstad, 1966, 1967). Because such large numbers of trypanosomes (5×10^9 parasites) are necessary to reduce the activity of solutions of synthetic bradykinin by 50% in 21 min, as tested on the isolated uterus, trypanosome kininase is unlikely to affect significantly the amount of kinin found in the plasma. Amundsen *et al.* (1964) have shown that urgocyton, a fluorescent substance obtained from liver extracts interferes with the breakdown of kinin. Trypanosomes have no such activity.

The point in the kinin system of the parasitized host at which trypanosomes seem to act is the activation of the precursor of the kininogenase. This cannot be a direct action because if washed separated trypanosomes are added to plasma in which kininase has been inhibited by 1,10-phenothroline hydrate no kinin is released. The mechanism must thus be an indirect one. It has been suggested that the antigen-antibody complex is implicated in kinin release (Boreham, 1968) and so it is possible that when trypanosome antigen combines with antibody to form complexes, activation of the kininogenase precursor and hence kinin release occurs. If this is so, these complexes may act like glass or kaolin and adsorb, and in some way alter, the structure of Hageman factor to cause its activation. Activated Hageman factor is known to activate prekallikrein (one of the kininogenase precursors) hence causing kinin release (Margolis, 1963). It is possible that Hageman factor first interacts with the enzyme permeability factor which then activates prekallikrein (Mason & Miles, 1962).

Several authors have described the activation of the kinin system by antigen-antibody complexes. In particular, Movat, DiLorenzo, Mustard & Helmel (1966). found that bovine serum albumin precipitated with antibovine serum albumin, caused activation of the kinin system *in vitro*. *In vivo* studies have shown that when an antigen-antibody reaction occurs, as in anaphylactic shock, kinins are liberated (Brocklehurst & Lahiri, 1963; Dawson & West, 1965).

The present findings are consistent with the hypothesis put forward earlier (Boreham, 1968) that the antigen-antibody complex causes the liberation of kinins in trypanosomiasis by activating the kininogenase precursor. The released kinins may be responsible, at least in part, for the vascular changes that occur in trypanosomiasis (Goodwin & Hook, 1968).

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REFERENCES

AMUNDSEN, E. & NUSTAD, K. (1964). Kinin-forming and destroying activities of saliva. *Br. J. Pharmac. Chemother.*, **23**, 440–444.

AMUNDSEN, E. & NUSTAD, K. (1965). Kinin-forming and destroying activities of cell homogenates. *J. Physiol., Lond.*, **179**, 479–488.

AMUNDSEN, E., NUSTAD, K. & WAALER, B. (1963). A stable substrate for the assay of plasma kinin-forming enzymes. *Br. J. Pharmac. Chemother.*, **21**, 500–508.

AMUNDSEN, E. & RUGSTAD, H. E. (1965). Influence of some pathogenic bacteria on kinin formation and destruction. *Br. J. Pharmac. Chemother.*, **25**, 67–73.

AMUNDSEN, E., WAALER, B. A., DEDICHEN, J., LALAND, P., LALAND, S. & THORSDALEN, N. (1964). Kininase inhibition by a fluorescent substance prepared from liver. *Nature, Lond.*, **203**, 1245–1248.

BOREHAM, P. F. L. (1966). Pharmacologically active peptides produced in the tissues of the host during chronic trypanosome infections. *Nature, Lond.*, **212**, 190–191.

BOREHAM, P. F. L. (1968). Immune reactions and kinin formation in chronic trypanosomiasis. *Br. J. Pharmac. Chemother.*, **32**, 493–504.

BOREHAM, P. F. L. & GOODWIN, L. G. (1967). A pharmacological and pathological study of the vascular changes which occur in chronic trypanosomiasis of the rabbit. *Int. Scient. Com. Trypanosom.*, **xi** (100), 83–84.

BROCKLEHURST, W. E. & LAHIRI, S. C. (1963). Formation and destruction of bradykinin during anaphylaxis. *J. Physiol., Lond.*, **165**, 39–40*P*.

DAVIES, G. E., HOLMAN, G. & LOWE, J. S. (1967). Role of Hageman factor in the activation of guinea-pig pre-kallikrein. *Br. J. Pharmac. Chemother.*, **29**, 55–62.

DAWSON, W. & WEST, G. B. (1965). The importance of bradykinin in anaphylactic shock. *J. Pharm. Pharmac.*, **17**, 246–247.

GOODWIN, L. G. & HOOK, S. V. M. (1968). Vascular lesions in rabbits infected with *Trypanosoma (Trypanozoon) brucei*. *Br. J. Pharmac. Chemother.*, **32**, 505–513.

GOODWIN, L. G. & RICHARDS, W. H. G. (1960). Pharmacologically active peptides in the blood and urine of animals infected with *Babesia rodhaini* and other pathogenic organisms. *Br. J. Pharmac. Chemother.*, **15**, 152–159.

GREENBAUM, L. M. & KIM, K. S. (1967). The kinin-forming and kininase activities of rabbit polymorphonuclear leucocytes. *Br. J. Pharmac. Chemother.*, **29**, 238–247.

HORTON, E. W. (1959). The estimation of urinary kallikrein. *J. Physiol., Lond.*, **148**, 267–282.

MARGOLIS, J. (1963). The interrelationship of coagulation of plasma and release of peptides. *Ann. N.Y. Acad. Sci.*, **104**, 133–145.

MASON, B. & MILES, A. A. (1962). Globulin permeability factors without kininogenase activity. *Nature, Lond.*, **196**, 587–588.

MOVAT, H. Z., DILORENZO, N. L., MUSTARD, J. F. & HELMEL, G. (1966). Activation of Hageman and a vascular permeability factor in serum by AG–AB precipitates. *Fedn Proc.*, **25**, 682.

RUGSTAD, H. E. (1966). Kininase production by some microbes. *Br. J. Pharmac. Chemother.*, **28**, 315–323.

RUGSTAD, H. E. (1967). Purification of a kinin inactivating enzyme from cultures of *Pseudomonas aeruginosa*. *Br. J. Pharmac. Chemother.*, **30**, 134–142.

SCHWAB, J. (1962). Kininases in leucocytes and other tissues. *Nature, Lond.*, **195**, 345–357.

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