Anti-trypanosomal factor in the haemolymph of "Glossina"

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Anti-trypanosomal factor in the haemolymph of *Glossina*

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Summary

The motility of cultured procyclic forms of *Trypanosoma brucei brucei* ANTat serodeme derived from EATRO 1125 was greatly reduced when incubated in vitro with the haemolymph of *Glossina morsitans morsitans* at dilutions as low as 1:512 after incubation periods of 1–2 h at 27° C. This effect was demonstrated in the haemolymph of male and female, teneral and non-teneral *G.m. morsitans* but was abolished by heat inactivation. A significant reduction in the motility of cultured forms of *Trypanosoma (S.) dionisii* was also observed when incubated in *G.m. morsitans* haemolymph but little effect was seen on culture forms of *Crithidia fasciculata* and *Leishmania hertigi*. An anti-trypanosomal factor was also demonstrated in the haemolymph of *Glossina austeni*, *G. palpalis gambiensis* and *G. tachinoides*.

Key words: anti-trypanosomal factor; haemolymph; *Glossina; Trypanosoma brucei brucei.*

Introduction

Antimicrobial factors have been described in the haemolymph of many different species of insects (see Boman, 1981; Chadwick, 1975). Humoral responses of insects to foreign organisms have been shown in the form of lysozyme activity (Anderson and Cook, 1979; Powning and Davidson, 1973) a variety of bactericidins (Boman et al., 1978; Natori, 1977; Hultmark et al., 1980; Steiner et al., 1981) and agglutinating factors (Scott, 1971) in the haemolymph.

Infections of *Trypanosoma brucei* in the haemolymph of several species of

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Glossina (Mshelbwala, 1972; Otieno, 1973; Otieno and Darji, 1977) and the ability of trypanosomes to penetrate the midgut wall to the haemolymph (Evans and Ellis, 1975) have indicated the possibility of alternative sites of development of *Trypanozoon* species and alternative routes to the salivary glands. This possibility prompted our investigation of the potential immune and antimicrobial response of *Glossina* species to these invading and developing trypanosomes. Molyneux (1977, 1980) has reviewed the occurrence of trypanosome and other kinetoplastid parasites in the haemolymph of insects and arachnids. In previous studies we have examined the haemocytes of tsetse flies (East et al., 1980, and in preparation) and although we have observed evidence of the ability of tsetse haemocytes to react to implants our studies have found little evidence of interaction between haemocytes and *Trypanosoma brucei*.

The experiments described in this paper indicate the presence of an anti-trypanosomal humoral factor in the haemolymph of *Glossina*.

Materials and Methods

Tsetse flies. Puparia of *Glossina morsitans morsitans* and *Glossina austeni* were supplied by Dr. A. M. Jordan, Tsetse Research Laboratory, Langford, U.K.; *G. palpalis gambiensis* and *G. tachinoides* were supplied by Dr. J. Itard, Maison-Alforts, Paris. Puparia and emerged flies were maintained in an incubator at $27 \pm 1°$ C with $75 \pm 10\%$ relative humidity. A proportion of emerged flies were fed on "half-lop" rabbits four times a week. The tenerality, sex and age of all flies used in experiments was recorded. Flies were not fed in the 24 h before any experiment.

Parasites. Trypanosoma brucei brucei ANTat serodeme was obtained from Prof. K. Vickerman, University of Glasgow. This stock which had been cloned in Glasgow was initially derived from EATRO 1125 and is fly transmissible (Le Ray et al., 1977). It was originally isolated from bushbuck. Infections of this serodeme in Manchester white mice (Manchester University Medical School) were isolated in cultivation medium using the method described by Brun and Jenni (1977) but with SDM-79 medium (Brun and Schönenberger, 1979). The trypanosomes were cultivated in SDM-79 medium with 7.5% heat inactivated foetal calf serum (Sera-Lab, Crawley Down, U.K.) at 27° C with twice-weekly passages. Procyclic culture forms of *T. brucei* used in experiments were obtained between passages 25 and 40. *Leishmania hertigi hertigi* (Liverpool strain no. LV42; isolate no. C8), *Crithidia fasciculata* (LV116) and *Trypanosoma (Schizotrypanum) dionisii* (stock P2) were routinely maintained in 4N medium on human blood-agar at room temperature (20–24° C). For experimental purposes these parasites were grown in H0-MEM medium with 5% heat inactivated foetal calf serum (Berens et al., 1976).

In all experiments, parasites, before addition to the tsetse haemolymph, were pelleted from the cultivation medium by centrifugation (10 min at 1000 g) and washed twice by resuspension in Cunningham's medium (Cunningham, 1977) without serum supplement to give a final concentration of 4.0×10^6–5.0×10^6 parasites/ml. No antibiotic was used in the maintenance of experimental media.

Inoculations. Male and female non-teneral *G.m. morsitans* were hand held and inoculated in the thorax and abdomen with 2 μl of Cunningham's medium containing 1,000 to 20,000 cultivated procyclic *T. brucei* using a micro-injector (ARP, Wilmslow, U.K.). The presence of trypanosomes in the haemolymph was monitored up to 120 h after inoculation. Cultures of *C. fasciculata* were inoculated into a similar number of flies by the same procedure. Before inoculation one wing was removed from the flies which were maintained in the conditions described above.

Haemolymph collection and dilution. Haemolymph was collected by either cutting the arthrodial membrane beside the haustellum or removing the forelegs and then gently squeezing the

294

thorax. Haemolymph exuding from the cut was collected in an SMI micropipette (Alpha Laboratories, London) and injected directly into a 550 μl polypropylene microtube at 4° C. The haemolymph from 30–40 flies was collected for any one experiment, providing a total pooled volume of up to 30 μl of haemolymph, which was immediately centrifuged at 12,000 g for 2 min in a Hawksley microcentrifuge to remove haemocytes and other debris.

The haemolymph was serially diluted in Cunningham's medium (Cunningham, 1977), which has an amino acid composition based on that of adult *G.m. morsitans* haemolymph, in microtitre plates with 10 μl wells (Walter Sarstedt Labs., Leicester, U.K.). A dilution series, using two-fold dilutions in a series of 12 wells, was established in 4 μl volumes of haemolymph-medium. The addition of the flagellates in 4 μl of Cunningham's medium produced a final range of dilutions from 1:2 to 1:4096. The time taken from the start of haemolymph collection to the addition of the parasites was 1–1½ h.

Experimental conditions and evaluations. Four μl volumes of cultivated procyclic trypanosomes were added to the haemolymph dilutions producing a final trypanosome concentration of 2.0×10^6–2.5×10^6/ml. All experiments involved a duplicate or triplicate dilution series. Standard controls of 4 μl of trypanosomes added to 4 μl of Cunningham's medium were also set up in triplicate. Following the addition of trypanosomes to the diluted haemolymph the microtitre dishes were maintained in an incubator at 27° C. Observations were made on the motility of the procyclics for up to 3 h using a Leitz Diavert inverted microscope at 320× magnification. The motility of the trypanosomes was assessed on a subjective six point scale ranging from 5+ to 0 in which 5+ denotes highly motile cultures (similar to the standard controls); 1+ a condition in which few parasites per field show motility; and 0, complete immotility.

Evaporation from the 8 μl volumes in the wells was monitored during several experiments. To produce a high humidity in the sealed plates and reduce evaporation, the microtitre plates were lined with strips of filter paper soaked with distilled water. Under these conditions at 27° C a volume reduction of 1–2 μl was consistently observed during the first 2 h. Melanization of the haemolymph at various dilutions was observed and recorded.

Control experiments

a) In some experiments the haemolymph from non-teneral and teneral male and female *G.m. morsitans* was heat-inactivated at 56° C for 30 min after collection and centrifugation in microfuge tubes. Heat-inactivated haemolymph was diluted as described above.

b) It was considered that proteases released by damaged tissue during the collection of haemolymph could be responsible for any change observed in trypanosome motility. To examine this possibility freshly collected haemolymph from male and female non-teneral *G.m. morsitans* was pooled in 20 μl volumes in microfuge tubes and then incubated for 1 h at room temperature with a 1 mM concentration of the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF) (Fahrney and Gold, 1963). Haemolymph incubated with PMSF and haemolymph incubated separately with the absolute ethanol solvent used for this inhibitor were diluted in the manner previously described.

c) Final trypanosome concentrations of 2.5×10^6, 4.5×10^6 and 7.5×10^6/ml were used in one experiment to examine the influence of trypanosome concentration on the effect of haemolymph. In this and other experiments the reversibility of the haemolymph effect on trypanosome motility was examined by removing 2 μl volumes from experimental wells at various time intervals and adding these to 4 μl of fresh Cunningham's medium.

d) To assess the effects of diluted *G.m. morsitans* haemolymph on other trypanosomatids, cultures of *L.h. hertigi*, *C. fasciculata* and *T. dionisii* were centrifuged, washed and resuspended in Cunningham's medium and added to the haemolymph dilution series in 4 μl volumes, as described above, to give final concentrations of 2.0×10^6–2.5×10^6/ml.

e) The haemolymph of male and female non-teneral *G. austeni* and pooled male and female non-teneral *G.p. gambiensis* and *G. tachinoides* was collected and tested on cultured procyclic *T. brucei* using the methods described above.

Results

a) Inoculations

Haemolymph was examined from over 200 *G.m. morsitans* which had been inoculated with cultured procyclic *T. brucei*. In an experiment where 50 flies were inoculated with 1.3×10^4 procyclics small numbers of active trypanosomes were observed in haemolymph taken from each of 15 flies which were examined up to 4 h. After this time it became increasingly difficult to find trypanosomes in the haemolymph although parasites were found in all 15 flies examined up to 48 h. At 72 h trypanosomes were found in 1 out of 3 flies while 5 examined at 96 h and 6 at 120 h were negative. After haemolymph examination of these flies they were dissected and crop, gut, thoracic muscles and salivary glands were examined for parasites but none were found. Inoculated *C. fasciculata* were observed to survive and multiply in the haemolymph of 40 *G.m. morsitans;* by the sixth day after inoculation all flies were packed with flagellates.

b) Incubation of T.b. brucei in haemolymph in vitro

The clearance of trypanosomes from the haemolymph of *G.m. morsitans* following inoculation and the lack of evidence of any interaction between haemocytes and trypanosomes (East et al., in prep.) prompted the examination of the effects of haemolymph on cultured procyclic *T. brucei* in vitro.

Observations on the motility of cultured procyclic *T. brucei* in haemolymph showed effects dependent on both the dilution of the haemolymph and the time of exposure. The reduction in trypanosome motility due to haemolymph taken from male and female, teneral and non-teneral *G.m. morsitans* is shown in Tables 1 and 2. Although these tables and figures indicate slight differences in the effect on trypanosome motility of haemolymph collected from male and female, teneral and non-teneral flies, considering the subjectivity of the scale of motility used, the general effect must be considered to be similar. At the low dilutions of haemolymph, in the 1:4 to 1:128 range, the difference in the motility of the trypanosomes was marked after 1 h and only a few active trypanosomes were present in each well by 2 h. At a haemolymph dilution of 1:512 some reduction in motility was also observed (Tables 1 and 2). After 2 h of incubation the 1:1024 dilution was the highest showing trypanosome motility similar to the controls.

In the interpretation of the results two further points must be considered. Firstly, the greatest effects on trypanosome motility were observed in the 1:4 to 1:128 dilution range, whilst at the lowest dilution used (1:2) the trypanosomes present were always more active. At this 1:2 dilution there also appeared to be a reduction in the number of trypanosomes present; however, due to the small volume per well (8 μl), numbers present were not counted. Secondly, although the results were consistent over a number of experiments (Tables 1 and 2), a volume reduction in the wells of 1–2 μl liquid over 2 h was observed. This

Table 1. Motility of cultured procyclic *T.b. brucei* in the haemolymph of teneral *G.m. morsitans*

Haemolymph type and time		Dilutions of haemolymph												No. of expts
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	
Normal haemolymph														
Males	60 min	3+	3+	3+	2+	3+	4+	4+	5+	5+	5+	5+	5+	2
	120 min	2+	1+	1+	1+	1+	2+	3+	4+	5+	5+	5+	5+	
Females	60 min	3+	3+	2+	2+	2+	3+	3+	4+	4+	5+	5+	5+	3
	120 min	3+	2+	1+	1+	1+	1+	2+	3+	4+	5+	5+	5+	
Heat-inactivated haemolymph														
Males	120 min	4+	4+	4+	4+	5+	5+	5+	5+	5+	5+	5+	5+	1
Females	120 min	4+	4+	5+	5+	5+	5+	5+	5+	5+	5+	5+	5+	1

Table 2. Motility of cultured procyclic *T.b. brucei* in the haemolymph of non-teneral *G.m. morsitans*

Haemolymph type and time		Dilutions of haemolymph												No. of expts
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	
Normal haemolymph														
Males	60 min	3+	2+	2+	2+	3+	3+	3+	4+	5+	5+	5+	5+	2
	120 min	2+	2+	1+	2+	2+	2+	2+	3+	4+	5+	5+	5+	
Females	60 min	3+	3+	3+	3+	3+	3+	4+	4+	5+	5+	5+	5+	4
	120 min	2+	1+	1+	1+	2+	2+	3+	3+	4+	5+	5+	5+	
Heat-inactivated haemolymph														
Females	120 min	4+	4+	4+	5+	5+	5+	5+	5+	5+	5+	5+	5+	1

12.5–25% volume reduction would have concentrated the anti-trypanosomal factor and so, for example, after 2 h of incubation the titre for the haemolymph could be considered closer to 1:512 than 1:1024.

The onset of changes in trypanosome motility appeared first as more sluggish or non-rhythmic movement. *T. brucei* procyclics did not show morphological changes in phase contrast or in stained smears and did not form agglutinated clumps. In some experiments a web-like agglutination pattern was observed. The reversibility of this phenomenon was examined in four experiments and, although not quantified, some trypanosomes placed in fresh Cunningham's medium did recover their motility; the extent of reversibility appeared to be dependent on the concentration of the haemolymph and time of exposure.

The melanization pattern of diluted haemolymph in the wells was also recorded in four experiments. The most intense melanization (haemolymph and Cunningham's medium turning dark brown-black) was observed after 1–2 h at the dilutions of 1:32 to 1:128. Although this was not always seen to have a direct effect on trypanosome motility, in one experiment, using haemolymph pooled from 30 non-teneral male flies, at the 1:64 to 1:256 dilutions a dark precipitate appeared to be enmeshing the trypanosomes.

These effects on trypanosome motility were not limited to the haemolymph of *G.m. morsitans*, as similar effects were observed using haemolymph collected from *G. austeni*, *G. tachinoides* and *G.p. gambiensis*. For these tsetse species, large reductions were observed in trypanosome motility (down to 2 + or 1 +) at dilutions as low as 1:32 after 1 h and 1:128 after 2 h.

c) Incubation of other trypanosomatids in haemolymph in vitro

The haemolymph of *G.m. morsitans* had differing effects on the motility of other trypanosomatid species (Table 3). Reduction in the motility of cultured *T. (S.) dionisii* were produced by haemolymph from male non-teneral and female teneral and non-teneral flies. The effect of the haemolymph on *T. dionisii* differed from that on *T.b. brucei* in that (a) the reduction in motility was not as marked (Table 3) and (b) the cells were observed to agglutinate in clumps and round-up before becoming immotile. In 5 other experiments using *C. fasciculata* or *L. hertigi* little change in motility was observed except at the lowest dilutions (Table 3). *C. fasciculata* incubated with haemolymph from male and female teneral and non-teneral flies showed sluggish behaviour at the lowest dilutions, no morphological change and no reduction in motility between 1–2 h in the range of dilutions which proved to be critical for *T. brucei*.

d) Incubation controls

The effects of haemolymph from male and female teneral and female non-teneral *G.m. morsitans* on the motility of *T.b. brucei* were abolished when the haemolymph was previously heated at 56°C for 20–30 min (Tables 1 and 2). Haemolymph incubated for 1 h with the protease inhibitor, PMFS, caused

298

Table 3. Motility of *T. dionisii*, *C. fasciculata* and *L. hertigi* in the haemolymph of *G.m. morsitans*

Haemolymph type* and time		Dilutions of haemolymph												No. of expts
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	
T. dionisii														
Tenerals	60 min	3+	3+	3+	2+	2+	3+	4+	5+	5+	5+	5+	5+	1
	120 min	3+	3+	3+	2+	2+	3+	3+	4+	4+	5+	5+	5+	
Non-tenerals	60 min	4+	3+	3+	4+	4+	4+	5+	5+	5+	5+	5+	5+	2
	120 min	3+	2+	2+	2+	3+	3+	4+	4+	5+	5+	5+	5+	
C. fasciculata														
Tenerals	120 min	4+	4+	4+	4+	5+	5+	5+	5+	5+	5+	5+	5+	1
Non-tenerals	120 min	4+	4+	4+	4+	4+	5+	5+	5+	5+	5+	5+	5+	3
L. hertigi														
Non-tenerals	120 min	4+	4+	4+	4+	5+	5+	5+	5+	5+	5+	5+	5+	1

* results for haemolymph from male and female flies taken together

similar reductions in trypanosome motility as did normal untreated haemolymph. This suggests that proteases released by damaged tissue during the collection of the haemolymph were not responsible for any of the effects on *T. brucei* motility.

Finally comparisons were made using *T. brucei* concentrations of 2.0×10^6 ml, 4.0×10^6 ml and 6.0×10^6 ml. Little difference was observed in the effect of the haemolymph at the various dilutions.

Discussion

The results show the presence of an anti-trypanosomal factor in the haemolymph of *G.m. morsitans* which within 2 h severely reduces the motility of cultured procyclic *T. brucei* at dilutions as low as 1:128 to 1:256, and has some effect on the trypanosome motility down to 1:512 dilution. The presence of the factor in teneral and non-teneral male and female *G.m. morsitans* suggests that it is present in the normal haemolymph of adult laboratory bred flies which has not been induced by infection and is not derived from proteins absorbed across the midgut from blood meals (Nogge and Gianetti, 1979). The factor is not restricted to *G.m. morsitans*; similar effects were observed in experiments on *G. austeni*, *G. palpalis gambiensis* and *G. tachinoides*.

Infections of *T. brucei* have been observed in the haemolymph of *G. morsitans* and other tsetse species following experimental infections (Mshelbwala, 1972; Otieno, 1973) and in wild caught *G. pallidipes* where 3 out of 955 dissected had infections of the haemocoele (Otieno and Darji, 1977). Otieno et al. (1976) also inoculated blood stream forms of *T. brucei* into the haemocoele of *G. morsitans* and detected trypanosomes for up to 6 days with various morphological changes. The survival of *T. brucei* in the haemolymph compared with our observations could be due to different serodemes or zymodemes of *T. brucei* behaving differently on exposure to haemolymph. Similarly the natural occurrence of haemocoelic infections in nature seems uncommon from observations of Otieno and Darji (1977) and Croft and East (unpublished observations). Although Evans and Ellis (1975) showed the ability of midgut forms of *T.b. rhodesiense* to penetrate midgut cells to the haemocoele and developing forms of *T. brucei* were observed in the haemocoele of *G. morsitans*, *G. tachinoides* and *G. palpalis* (Mshelbwala, 1972) the role of haemocoelic infections and a possible "alternative route" for these trypanosomes to the salivary glands has yet to be clearly defined. The presence of an anti-trypanosomal factor in the haemolymph of *Glossina* species could reduce the significance of the role of haemocoelic infections.

The studies described in this paper have also suggested that the factor is specifically anti-trypanosomal, as it has been shown to reduce the motility of cultured *T. brucei* and the bat trypanosome *T. dionisii* but has little effect on less closely related trypanosomatids *C. fasciculata* and *L. hertigi*. The survival and

300

multiplication of *C. fasciculata* in the haemocoele in vivo following inoculation compared with the rapid disappearance of *T. brucei* from the haemolymph following inoculation supports this suggestion. In this respect *Glossina* differed from other insect species; *T. brucei* was able to develop and grow in the haemolymph of the moth larvae *Galleria mellonella* (Hoare, 1938), and the cricket *Acheta domesticus* had a strong cellular response to inoculations of *Crithidia* (Schmittner and McGhee, 1970).

Although the amino-acid content of *Glossina* haemolymph has been analysed (Cunningham and Slater, 1974; Tobe et al., 1975) and absorption of proteins across the midgut recognised (Nogge and Giannetti, 1979), there are still many haemolymph factors to be examined especially with regard to the nature of the anti-microbial response. Although examination of the haemolymph of other parasitized insects has shown changes in amino acid and carbohydrate content (Mack et al., 1979a, b; Schmidt and Platzer, 1980) and inducible anti-microbial activity (Boman et al., 1978; Anderson and Cook, 1979), recent studies have also shown the potential of relatively specific bactericidal components (Hultmark et al., 1980; Steiner et al., 1981). Seaman and Robert (1968) also showed that cockroaches can produce a factor which immobilizes injected ciliates. Further studies will be made of the nature of the factor in *Glossina* haemolymph which appears to be specifically anti-trypanosomal, not induced, heat-inactivated, and the effects of which are partially reversible.

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