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THE DISTRIBUTION OF AGGLUTININS AND LYTIC ACTIVITY AGAINST *TRYPANOSOMA RANGELI* AND ERYTHROCYTES IN *RHODNIUS PROLIXUS* AND *TRIATOMA INFESTANS* TISSUE EXTRACTS AND HAEMOLYMPH

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Haemolymph, heads, salivary glands, crops, midguts, hindguts, and Malpighian tubules from Rhodnius prolixus *and* Triatoma infestans *were extracted in phosphate or Tris buffer saline with calcium, and tested for agglutination and lytic activities by microtitration against both vertebrate erythrocytes and cultured epimastigote forms of* Trypanosoma rangeli. *Haemagglutination activity against rabbit erythrocytes was found in the crop, midgut and hindgut extracts of* T. infestans *but only in the haemolymph of* R. prolixus. *Higher titres of parasite agglutinins were found in* R. prolixus *haemolymph than* T. infestans, *whilst the converse occurred for the tissue extracts. In addition, the extracts of* T. infestans *salivary glands, but not those of* R. prolixus, *showed a trypanolytic activity that was heat-inactivated and was not abolished by pre-incubation with any of the sugars or glycoproteins tested.* T. infestans, *which is refractory to infection by* T. rangeli, *thus appears to contain a much wider distribution of agglutinating and trypanolytic factors in its tissues than the more susceptible species,* R. prolixus.

Key words: haemagglutinin – *Rhodnius prolixus* – *Triatoma infestans* – haemolymph – trypanolytic factor – *Trypanosoma rangeli*

Trypanosoma rangeli is a protozoan which is apparently non-pathogenic for vertebrates (Marinkelle, 1968; D'Alessandro & Mandel, 1969). It is of some medical interest as its geographical distribution overlaps that of *T. cruzi,* the etiological agent of Chagas' disease, and because both trypanosomes species can be transmitted by the same insect vectors. The triatomid, *Rhodnius prolixus,* is more susceptible than *Triatoma infestans* to infection with *T. rangeli,* and the development of the trypanosome is quite different in the two insects (Zeledon & Blanco, 1965). In *R. prolixus,* the trypanosomes multiply in the gut lumen and frequently pass from the gut to the haemocoel, where they multiply in the haemolymph without apparently any effective host reaction (Grewal, 1957; Tobie, 1968; Hecker et al., 1990). From the haemolymph, the parasites migrate to the salivary glands where they develop to the infective form. In *T. infestans,* however, the parasites develop in the gut of the insect at a lower rate than in *R. prolixus,* and it is unusual for the trypanosome to infect the haemocoel and the salivary glands, even if they are injected directly into the haemolymph (Zeledon & deMonge, 1966).

Insects are known to exhibit both cellular and humoral "immune" reactions against foreign particles (see Ratcliffe et al., 1985). A clear understanding of the molecular mechanisms of insect immunity, however, is wanting and essential in order to understand how some disease-causing organisms escape from the host defences. "Immune" factors, including agglutinins present in vector tissues, may be important determinants of the host-parasite relationship (Pereira et al., 1981; Ibrahim et al., 1984; Wallbanks et al., 1986; Maudlin & Welburn, 1987, 1988; Welburn et al., 1989, 1990; Ingram & Molyneux, 1988, 1990). For example, Welburn & Maudlin (1990) have recently reported that in *Glossina morsitans morsitans* a haemolymph lectin may be necessary for the normal maturation process of African trypanosomes.

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In spite of the medical and social importance of Chagas' disease in South America, very little is known about the "immune" system of their insect vectors (see, however, Pereira et al., 1981; Azambuja et al., 1986, 1989; Azambuja & Garcia, 1987; Takle & Lackie, 1987; Barraco & Loch, 1988; Gomes et al., 1988).

Using the triatomid vectors/*T. rangeli* model (see above), the present study thus aims to investigate the presence and distribution of aggutinins and trypanolytic factors in the tissues of *R. prolixus* and *T. infestans,* and correlate the results obtained with the mechanisms which may govern the insect-parasite interrelationship.

MATERIALS AND METHODS

Insect – Adult male and female *R. prolixus* and *T. infestans* were maintained on sterile defibrinated pig blood using an *in vitro* membrane feeding system (Bauer & Wetzel, 1976). They were starved for 4 weeks, fed once on heat-inactivated foetal calf serum (FCS) and then used 3 to 4 days after feeding.

Erythrocytes – Calf, rabbit, chicken, horse, goat, sheep and human (A, B, AB and O) red blood cells (RBC) were washed twice in buffered saline, then twice more in the same buffer containing 0.5% bovine serum albumin (BSA) before resuspension at a concentration of 2% (v/v). Two saline solutions were used: phosphate buffered saline (PBS, pH 7.4) or Tris buffered saline (TBS, 50 mM Tris, 80 mM NaCl, 10 mM $CaCl_2$, pH 7.4).

Trypanosomes – *T. rangeli* (R 1306 strain) stock solutions were maintained in RPMI 1640 medium supplemented with 20% heat-inactivated FCS and 20 mM L-glutamine (Takle & Young, 1988), in 50 ml tissue culture plastic flasks (Nunclon). The parasites were harvested by centrifugation from 5 to 10 day old cultures (mainly epimastigote forms), washed and resuspended in PBS, TBS or RPMI 1640 medium to give a final concentration of $2,0 - 3,0 \times 10^6$ parasites ml^{-1}.

Haemolymph collection – Haemolymph (*ca* 10 μl) was collected by leg amputation and gently squeezing the thorax. Material from 10 insects, in each experiment, was pooled into polypropylene microtubes kept at 4 °C, and then centrifuged at 2800 g for 5 min to remove haemocytes and cell debris. The supernatant was assayed immediatly or stored at –20 °C with no detectable change in the original activity.

Preparation of tissue extracts – A method similar to that described by Pereira et al. (1981) was employed. The head (10 ml^{-1}), salivary glands (50 pairs ml^{-1}), crop (10 ml^{-1}), midgut (10 ml^{-1}), hindgut (20 ml^{-1}) and Malpighian tubules (10 ml^{-1}) were isolated, homogenized in cold PBS or TBS, using a 1 ml glass tissue grinder, and left at 4 °C for several hours with intermittent shaking. The chilled extracts were centrifuged at 2800 g for 10 min. The supernatants obtained were passed through 0.22 μm Millipore filters and assayed immediatly or stored at –20 °C with no detectable change in their activities.

Agglutination and trypanolytic tests – Two-fold serial dilutions of haemolymph or tissue extracts, each of 5 μl volume, were prepared in PBS or TBS in microtitre plates to give final dilution ranges of 1:2 to 1:4096. Five μl of either RBC or parasites were then added to the wells. The mixtures were incubated at 25 °C for up to 4 h and examined for agglutination and lytic activities using an inverted phase contrast microscope. The agglutinating or lytic end point titres were recorded as the reciprocal of those dilutions which just failed to give visible effects. All experiments were performed in duplicate, at least 3 times. Controls consisted of buffered salines alone and heat-inactivated test solutions (100 °C, 10 min). FCS and pig blood serum were also tested for agglutinating and lytic activities.

Sugar inhibition – A wide range of sugars and glycoproteins were tested for their abilities to inhibit the agglutination or the lytic activities of the samples (details in Millar & Ratcliffe, 1987). Monosaccharides (D[+]glucose, D[+]mannose, D[+]fucose, L[–]fucose, D[+]galactose, D[–]arabinose, L[+]arabinose, D[+]xylose, L[–]xylose, D[–]fructose, L[+]rhamnose), oligosaccharides (D[+]lactose, maltose, D[+]sucrose, D[+]turanose, stachyose, trehalose, D[+]cellobiose, D[+]raffinose), N-acetylated sugars (N-acetyl D-mannosamine, N-acetyl D-glucosamine, N-acetyl D-galactosamine, N-acetyl neuraminic acid), methyl glycosides (methyl D-galactoside, methyl D-mannopyranoside), amino sugars (D[+]glucosamine, D[+]galactosamine, D[+]mannosamine), glycoproteins

(fetuin, desialylated fetuin, bovine submaxillary mucin), were all tested at 0.2M (for carbohydrates) or 1% (for glycoprotein) concentrations.

RESULTS

The distribution of naturally-occurring haemagglutinins in the haemolymph and tissue extracts of both insects was quite different, and was detected only against rabbit RBC (Table I). *R. prolixus* haemolymph, in contrast to that of *T. infestans*, agglutinated rabbit erythrocytes. None of the *R. prolixus* tissue extracts, however, were active, in either PBS or TBS. This contrasts the *T. infestans* crop and midgut extracts which showed high levels of haemagglutination. Some activity was also found in the *Triatoma* hindgut, Malpighian tubules and salivary glands (Table I). The activity of all samples was inactivated by heat-treatment, but was not affected by any sugar tested in the inhibition assays. Tissues prepared after different extraction times were tested for their haemagglutination activities (Table II). The results showed that extraction time did not adversely affect the haemagglutinating titre and, in some cases, enhanced the activity obtained. For example, the *T. infestans* crop activity increased from 512 at 1 h to 2048 at 18 h incubation.

The agglutinating activity of the samples against *T. rangeli* (Table III) followed the same pattern observed against the RBC. There was a higher parasite agglutinin level in *R. prolixus* haemolymph than in that of *T. infestans*. The opposite was recorded in the tissue extracts, with *T. infestans* containing parasite agglutinins throughout the digestive tract, the titres of which were usually higher than those in *R. prolixus*. Although the parasites were agglutinated, there was no killing or lytic activity in gut extracts of both insect species against *T. rangeli*. Pig blood serum also agglutinated rabbit RBC and *T. rangeli* with a titre of 16 (Tables I and III).

A very interesting result was the detection of trypanolytic and haemolytic activities in the salivary glands of *T. infestans* against *T. rangeli* and sheep, human AB and horse RBC (Table IV). All other RBC, including rabbit, gave negative results. The trypanolytic activity was destroyed by heat treatment of the samples, but was not abolished by any sugar tested in the sugar inhibition assays. No such lytic activity was found in the salivary glands of *R. prolixus*.

TABLE I

Haemagglutination activity of haemolymph and tissue extracts of *Rhodnius prolixus* and *Triatoma infestans* against rabbit erythrocytes

Insect	Tissue[b,c] tested	Buffer solution[a]	
		PBS	TBS + Ca^{++}
R. prolixus	Haemolymph[d]	128	64
	Head	0	0
	Salivary gland	0	0
	Crop	0	0
	Midgut	0	0
	Hindgut	0	0
	Malpighian tubules	0	0
T. infestans	Haemolymph	0	0
	Head	0	0
	Salivary gland	8	8
	Crop	256	2048
	Midgut	16	256
	Hindgut	4	8
	Malpighian tubules	0	8
	Buffer solution	0	0
	Pig blood serum	8	16
	Foetal calf serum	0	0

a: used for both tissue extraction and the haemagglutination assay.
b: the haemagglutination activity of the samples was inactivated by heat treatment.
c: not affected by any sugar tested in sugar inhibition assays.
d: not tested for sugar inhibition.

TABLE II

Haemagglutination activity of *Rhodnius prolixus* and *Triatoma infestans* tissue extracts against rabbit erythrocytes, after different times of extraction

Insect	Source of agglutinin activity	Time of extraction [hours]				
		1	2	4	8	18
R. prolixus	Head	0	0	0	0	0
	Salivary gland	0	0	0	0	0
	Crop	0	0	0	0	0
	Midgut	0	0	0	0	0
	Hindgut	0	0	0	0	0
	Malpighian tubules	0	0	0	0	0
T. infestans	Head	0	0	0	0	0
	Salivary gland	0	2	2	4	8
	Crop	512	512	512	1024	2048
	Midgut	128	128	256	256	256
	Hindgut	8	8	8	8	8
	Malpighian tubules	8	8	8	8	8

TABLE III

Agglutination activity of haemolymph and tissue extracts of *Rhodnius prolixus* and *Triatoma infestans* against *Trypanosoma rangeli*[a]

Insect	Tissue tested	Buffer used to prepare the extracts		
		None[b]	PBS	TBS + Ca^{++}
R. prolixus	Haemolymph	16		
	Head		0	0
	Salivary gland		0	0
	Crop		0	8
	Midgut		0	2
	Hindgut		0	0
	Malpighian tubules		0	0
T. infestans	Haemolymph	4		
	Head		0	0
	Salivary gland		0	0
	Crop		4	8
	Midgut		0	16
	Hindgut		0	2
	Malpighian tubules		0	2
	Buffer solution	0		
	Pig blood serum	16		
	Foetal calf serum	0		

a: parasites prepared in RPMI 1640 medium.
b: material prepared without dilution.

TABLE IV

Haemolytic and trypanolytic activities of salivary gland extracts of *Triatoma infestans* and *Rhodnius prolixus*

Insect	*T. rangeli*[a]	Erythrocytes		
		Sheep	Human AB	Horse
R. prolixus	0	0	0	0
T. infestans	16-64[b,c]	32[b]	32[b]	32[b]

a: T. rangeli resuspended in RPMI 1640 medium, PBS or TBS + Ca^{++}.
b: activity lost on heat treatment of the tissue extract.
c: not affected by any sugar tested in sugar inhibition assays.

DISCUSSION

In the present study, we showed that gut tissues of *T. infestans* have a higher concentration and wider distribution of haemagglutinins and parasite agglutinins than the gut tissues of *R. prolixus*. In contrast, however, the haemolymph of *R. prolixus* contains higher levels of agglutinins than *T. infestans* against rabbit erythrocyte and cultured epimastigote forms of *T. rangeli*.

The lack of haemagglutinating activity in the *Rhodnius* tissue extracts in our experiments was quite surprising, since Pereira et al. (1981) reported agglutinins in the crop and midgut of *R. prolixus* against rabbit RBC. To investigate a possible loss of activity in our samples during the prolonged extraction procedure, material from both insect species was tested after different extraction times, with no adverse effect on the haemagglutinating titres. The difference in our results and those of Pereira et al. (1981) may be due to variation in the feeding or haemagglutination protocols adopted for the insects in the two studies. We observed that the pig blood serum, on which our insects were normally fed, agglutinates rabbit RBC at low titres. To avoid the detection of haemagglutinin not naturally present in *Rhodnius* or *Triatoma* tissues, but derived from the pig blood, we used tissue extracts from insects that were firstly starved and then allowed to feed on heat-inactivated FCS, which does not agglutinate rabbit RBC. We have also found that enzyme-treatment of test erythrocytes introduces artificially high readings in haemagglutination assays with *Rhodnius* and *Triatoma* tissues and should be avoided (unpublished observations).

The possible role played by agglutinins or lectin-like molecule detected in host insect tissues on the vector competence is still unclear. Previously, Maudlin & Welburn (1987) showed by *in vivo* carbohydrate inhibition experiments that lectin secreted in the midgut of tsetse played an important role in the establisment of gut trypanosomatid flagellate infection. Midguts from a refractory line of *Glossina morsitans morsitans* to trypanosome infection exhibited higher levels of RBC agglutination than those from a the susceptible strain. Subsequent *in vitro* incubation of midgut extracts from both susceptible and refractory flies with the flagellates induced significant killing of *Trypanosoma brucei rhodesiense*, although the tissues of the refractory flies were more effective at killing over the 24 h incubation period. *In vivo* studies also showed that the refractory line of *G. m. morsitans* with high agglutinin levels took 170 h to kill 50% of the infection while the susceptible line with low agglutinin titres only killed 5% after 168 h (Welburn et al., 1989).

The presence of agglutinin in gut tissue of *T. infestans* may thus be responsible for the resistance of the insect to develop *T. rangeli*

gut infection at the same extent as it occurs in *R. prolixus.* There was, however, no evidence of lytic activity against the parasite by the gut extracts during the 4 h incubation period of the experiments. The lytic activity against *T. rangeli* detected in the salivary gland extract of *T. infestans,* but not in *R. prolixus,* may be an important factor contributing to the low rate of *T. rangeli* intestinal infection in *T. infestans.* This is the first time such an effect has been reported from the salivary glands and the characterization of such trypanolytic factor is now required. Welburn et al. (1989) showed morphological evidence that the midgut homogenates of refractory *G. g. mositans,* which agglutinated rabbit RBC also had a lytic effect on trypanosomes. We could not, however, correlate the haemagglutinating and the lytic activities present in the *T. infestans* salivary glands, as they exhibited different titres, and both activities were unaffected by any of the sugars used in the inhibition assays.

All of the 31 sugars tested in the inhibition assays were also found to be non-inhibitory for the agglutinins detected in *T. infestans* gut extracts. This finding is not unusual, as there are many reports of invertebrate agglutinins which are not affected by the simple sugars often used in inhibition tests (Bretting et al., 1976; Lackie, 1981; Stein et al., 1982; Hapner, 1983; Castro et al., 1987). This result may reflect either the presence of a group of heterogeneous agglutinin molecules or of one agglutinin with a complex binding site, as has been described by Möck & Renwrantz (1986).

Also of significance is the recent observation by Welburn & Maudlin (1990) that the agglutinin present in the haemolymph of *G. g. morsitans* may be important in the development of the parasite and the maturation infection *in vivo.* They observed that *Glossina palpalis palpalis,* which has a low haemolymph lectin titre, is a poor vector for *Trypanosoma congolensi* since they mature few infections. In contrast, *G. m. morsitans* which has high transmission indices for *T. congolensi* also has a high haemolymph lectin titre. The presence of haemagglutinin in *R. prolixus* haemolymph, the susceptible triatomine species to *T. rangeli* haemolymph and salivary gland infections, may also be essential for the trypanosomes to develop to the correct stage for infection. During natural and experimental *T. rangeli* infections of *Rhodnius* haemocoele, the parasite reproduces intensely free in the haemolymph and within the haemocytes (Tobie, 1970; Watkins, 1971). The agglutinin present in the *Rhodnius* haemolymph may thus be essential for trypanosome development. Notori (1991) has recently shown a dual function for insect haemolymph lectins in both immune recognition and development in *Sarcophaga* since the agglutinin not only removes foreign antigens from the blood but also produces a signal essential for the development of the imaginal discs.

Finally, we have recently purified the *Rhodnius* haemolymph lectin, which is galactose-specific, and intend to test its role in *T. rangeli* development. This lectin is not derived from the pig blood serum, since the agglutinating activity of the latter is not affected by incubation with galactose. Isola et al. (1983) failed to detect any effect of *T. infestans* whole blood or cell-free haemolymph on the growth and development of cultured epimastigote forms of *T. cruzi.* The incubation of the *T. cruzi* with *T. infestans* midgut homogenate, however, led to differentiation of the parasite to metacyclic epimastigotes (Isola et al., 1986). The role of these insect lectin has thus yet to be clearly defined.

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