IN VITRO INHIBITION OF ATTACHMENT OF THE AMERICAN TRYPANOSOME, *TRYPANOSOMA RANGELI* ON THE SALIVARY GLAND OF ITS VECTOR, *RHODNIUS PROLIXUS*, USING THE PARASITE EXTRACT AND PARTIAL PURIFICATION OF AN ATTACHMENT INHIBITOR PROTEIN

H.R. BASSERI, Ph.D.,* AND N.A. RATCLIFFE **

From the *Department of Medical Entomology, School of Public Health, Tehran University of Medical Sciences, P.O. Box 14155-6446, Tehran, Iran, and the **Biomedical and Physiological Research Group, School of Biological Sciences, University of Wales Swansea, Singleton Park, Swansea SA2 8PP, United Kingdom.

ABSTRACT

The life cycle of the American trypanosome, *Trypanosoma rangeli*, in its invertebrate host, *Rhodnius prolixus*, is completed by invasion into the insect salivary glands. As a prerequisite, there is an initial recognition of the salivary gland receptors for the adhesion/invasion by *T. rangeli*. Therefore, the purpose of this study was to investigate the putative receptor molecules on either the salivary gland or parasites surfaces, which mediate the adhesion/invasion. Initially, the possible agglutination activity of *T. rangeli* epimastigotes against blood cell was examined. Then, in order to investigate the role of any receptor ligands on the surface of *T. rangeli* in adhesion/invasion of the parasite, GlcNAc was preferentially used to isolate a ligand protein. A 60kDa protein bound to the GlcNAc column and was isolated from *T. rangeli* (Tr-60) and the purity of this protein was confirmed by reverse phase HPLC. Tr-60 also exhibited a potent parasite inhibitory effect indicating that this protein (most probably a glycoprotein) may act as a ligand receptor. In vivo, the development and invasion of *T. rangeli* into the salivary gland was not interfered with by the presence of a sugar inhibitor of the *R. prolixus* hemolymph lectin.


**Keywords:** *Trypanosoma rangeli, Rhodnius prolixus, Salivary glands, Proteins, attachment.*

INTRODUCTION

*T. rangeli* needs an essential period of development before invasion into the salivary gland and the long epimastigote forms are responsible for invasion.1 The adhesion inhibition of *T. rangeli* onto *R. prolixus* salivary glands using sugars revealed that some carbohydrate-binding proteins on the surface of the trypanosome or gland acted as ligands mediating the parasite adhesion.2 Therefore, in this study, a sugar binding protein was used to purify some ligand proteins. Parasite adhesion/invasion is likely to be preceded by specific receptor-ligand interactions between moieties on the cell surface of the parasites and molecules of vector tissues.19 Such surface receptors are beginning to be characterized for *Try-
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**Parasites culture**

*Trypanosoma rangeli* Tejera 1920, strain Choachi was supplied by Dr. G. Schaub (University of Bochum, Germany). Trypanosomes were maintained at 25°C in 10 mL sterile centrifuge tubes containing LIT culture medium [0.068 M NaCl, 0.0054 M KC1, 0.056 M Na2HPO₄, 0.01 M glucose, 5 mg mL¹ trypose, 5 mg mL¹ LB (liver infusion broth, DIFCO), and 0.0025 mg mL¹ haemin (Sigma Co., Dorset, UK)], supplemented with 20% heat-inactivated fetal calf serum (FCS) (Takle and Young 1988).

**Insect and dissection of salivary glands**

*R. prolixus* was reared as described by Azambuja and Garcia (1997) and fed through a parafilm membrane on defibrinated horse blood (TCS Microbiologicals Ltd, Botolph, Claydon, Buckingham, UK) every 10–15 days and occasionally with rabbit blood (Harlan Sera Lab Ltd, Loughborough, UK) or fresh human blood. The salivary glands, the insects were anesthetized on ice for 10 min and then their heads were held firmly with No. 5 forceps (BIS Switzerland) and immersed in 150 mM NaCl and 10 mM sodium phosphate at pH 7.4 and 380 mM NaCl). The head and abdomen were then gently pulled apart. This allowed access to the salivary gland ducts, which remained attached to the head. The glands were then separated from the head.

**Preparation of T. rangeli extracts**

The epimastigotes long forms of *T. rangeli* from three week and short forms from one week old LIT medium were harvested and washed twice with PBS (containing 150 mM NaCl and 10 mM sodium phosphate at pH 7.4) by centrifugation at 10000 g IEC (Centra-M centrifuge, International Equipment Co. USA) for 10 minutes. The pellet was resuspended in 100 mL PBS to give a final concentration of 2.5 × 10⁶ cells/mL. Because of hard membrane and base on previous tests, the suspension of parasites was homogenised with an ultrasonic disintegration sonicator (Ultrasonic Disintegrator 150 W, MSE, UK) for 1 minute and then spun down at 10000 g for 10 minutes and the extracts were frozen at -20°C until use.

**Hemagglutination assay**

Two-fold serial dilutions of long and short epimastigote form extract were prepared in sterile 60 well microplates (5 µL) (Nunc, Gibco, U.K.) to give a final dilution range of 1:2 to 1:1024. Five microliters of 2% rabbit erythrocytes in PBS were added to each well and the mixtures were agitated for 1 minute and then incubated for 2 hours at room temperature. The end points of agglutinin were examined under a stereomicroscope.
and by the flow characteristics of the erythrocyte pellets when the plate was held at an angle. As controls, 100 μL of the extract was heat-inactivated for 20 minutes at 100°C. All samples were prepared 3 times and the assay repeated each time.

**Attachment inhibition assay using T. rangeli extract**

The salivary glands of 4th and 5th instar nymphs of *R. prolixus* (4 glands each time) were dissected as above and then preincubated with the extract of *T. rangeli* for 1 hour at room temperature. The salivary glands were then quickly washed and incubated with the living trypanosomes (2 × 10^6 cells/mL) in PBS for 6 hours at 25°C. Each salivary gland was washed in PBS and the attached trypanosomes were counted by counting from the upper surface area of the posterior and anterior lobes of the salivary glands perpendicular to the viewing direction, using 100 squares in an eyepiece grid graticule measuring 0.09 mm^2. Controls consisted of the same number of the salivary glands that had been preincubated with heat-inactivated trypanosome extract and untreated salivary glands in PBS. The experiment was repeated five times with 2 replications each.

**Partial purification of a T. rangeli attachment protein**

*T. rangeli* epimastigote long forms were harvested from a large volume (5000 mL) of 3 week old LIT culture medium by centrifugation at 14000g for 30 minutes at 4°C. The pellets were resuspended in 10 mM PBS (pH 7.4) containing 380 mM sucrose, and washed three times by centrifugation at 10000g for 15 minutes each and then resuspended in 5 mL PBS containing 1% sodium dodecylsulfate (SDS). Trypanosomes were homogenized with an ultrasonicator (Ultrasonic Disintegrator 150 watt; MSE, UK) and centrifuged at 10000g. The supernatant was then applied to a GlcNAC-agarose column for protein purification. Five milliliters of the supernatant were loaded onto a 1 mL GlcNAC-agarose column (Sigma Co., Dorset, UK) and centrifuged at 10000g. The supernatant was then applied to a GlcNAC-agarose column for protein purification. Five milliliters of the supernatant were loaded onto a 1 mL GlcNAC-agarose column (Sigma Co., Dorset, UK) and centrifuged at 10000g. The supernatant was then applied to a GlcNAC-agarose column. The mobile phase was 50% CH_3CN: 50% H_2O containing 1% trifluoroacetic acid (TFA) (Sigma Co., Dorset, UK). Ten microliters of the eluted protein was injected and the purity of the protein solution was detected at a wavelength of 220 nm.

**RESULTS**

**Hemagglutination assay**

The trypanosome extract showed a weak hemagglutinin activity (titre < 16) against rabbit red blood cells. In contrast, no hemagglutinin activity was observed in the extract of short epimastigotes and of the eluted protein. The heat treatment of the long epimastigote extract at 100°C did not completely destroy the agglutinin activity (Table I).

**Attachment inhibition assay using T. rangeli extract**

The extract of *T. rangeli* epimastigote long forms showed a high inhibitory effect (85.7% reduction in comparison with the PBS control) on the attachment of live parasites to the surface of *R. prolixus* salivary glands.
Inhibition of *T. rangeli* Attachment on *R. prolixus*

### Table I. Hemagglutination activity of *T. rangeli* extract (long and short epimastigotes forms) and the eluted protein (Tr-60) of long form extract from a GlcNAc-agarose column against rabbit erythrocytes.

<table>
<thead>
<tr>
<th>Extract source</th>
<th>Exp. 1 Concentration mg/mL</th>
<th>End point</th>
<th>Exp. 2 Concentration mg/mL</th>
<th>End point</th>
<th>Exp. 3 Concentration mg/mL</th>
<th>End point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long epimastigotes</td>
<td>1.65</td>
<td>3-4</td>
<td>1.95</td>
<td>3-4</td>
<td>2.48</td>
<td>3-4</td>
</tr>
<tr>
<td>Control (heat-inactivated)</td>
<td>1.65</td>
<td>2-3*</td>
<td>1.95</td>
<td>1-2*</td>
<td>2.48</td>
<td>1-2*</td>
</tr>
<tr>
<td>Tr-60</td>
<td>ND</td>
<td>0</td>
<td>0.041</td>
<td>0</td>
<td>0.083</td>
<td>0</td>
</tr>
<tr>
<td>Short epimastigotes</td>
<td>2.50</td>
<td>0</td>
<td>2.28</td>
<td>0</td>
<td>2.07</td>
<td>0</td>
</tr>
<tr>
<td>BSA</td>
<td>2%</td>
<td>0</td>
<td>2%</td>
<td>0</td>
<td>2%</td>
<td>0</td>
</tr>
</tbody>
</table>

*The control was inactivated by heating at 100°C for 20-30 minutes. ND, not done.

### Table II. In vitro attachment inhibition assay. An extract of *T. rangeli* was loaded onto a lmL GlcNAc-Agarose column. GlcNAc-binding protein (Tr-60) was eluted using 1mg/mL GlcNAc in 10mM PBS. The eluted protein (Tr-60) as well as original extract were used to treat the Rhodnius Salivary glands and washed. The salivary glands were then treated with the living parasites for one hour and the number of attached trypanosomes on each salivary gland was counted under a light microscope.

<table>
<thead>
<tr>
<th>Blocking agent</th>
<th>Protein concentration* mg/mL</th>
<th>Mean of attached parasites/0.09mm²</th>
<th>+SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original extract</td>
<td>1.65-2.48</td>
<td>25.65</td>
<td>3.01</td>
</tr>
<tr>
<td>Boiled original extract</td>
<td>1.65-2.48</td>
<td>92.4</td>
<td>9.16</td>
</tr>
<tr>
<td>Tr-60</td>
<td>0.041-0.083</td>
<td>65.66</td>
<td>6.18</td>
</tr>
<tr>
<td>Boiled Tr-60</td>
<td>0.041-0.083</td>
<td>119.95</td>
<td>12.34</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>180</td>
<td>14.42</td>
</tr>
</tbody>
</table>

*The number of attached parasites is significantly less (p<0.05) than controls.

SDS-Polyacrylamide Gel Electrophoresis and HPLC

The molecular mass of the eluted protein was estimated according to the wide range marker ca. 60kDa (Tr-60) (Fig 2) while the whole protein extract loaded on the gel, showed numerous bands. The purity of the eluted protein (Tr-60) was also tested by reverse phase HPLC (Fig. 3). When this protein solution was injected into the HPLC analytical system, a peak appeared at the retention time of 13-14 minutes (rT=13.85) after loading (Fig. 3) which was 51.6% of the area of the graph. The remaining peaks belonged to PBS (rT= 2.60 and 2.98) and GlcNAc (rT=3.59 and 4.45) which have low molecu-
Fig. 1. In vitro attachment inhibition assay. The extract of *T. rangeli* and the protein eluted (Tr-60) from a GlcNAc-agarose column were used to incubate *R. prolulas* salivary glands. The salivary glands were then treated with the living parasites for one hour and the number of attached trypanosomes on the salivary glands were counted under a light microscope. Controls consisted of boiled original protein, boiled eluted protein and PBS protein-free.

* The number of attached parasites is significantly less (p<0.05) than controls.

**DISCUSSION**

The in vitro adhesion inhibition assay using extracts of long epimastigotes of *T. rangeli* revealed significant lar weights and were observed at the beginning of the graph.

Fig. 2. 10% SDS-PAGE of *T. rangeli* (epimastigotes long form) extract and eluted proteins (Tr-60). The parasite extract was loaded onto 1ml GlcNAc-Agarose column and after wash, the proteins were eluted using 1 mg/mL GlcNAc. The yielded protein and the whole parasite extract were applied to SDS-PAGE. The molecular mass of the eluted protein was estimated according to s wind range marker.

Fig. 3. HPLC analysis of Tr-60. The *T. rangeli* extract was prepared and loaded onto a GlcNAc-agarose column. The GlcNAc-binding protein (Tr-60) was eluted and applied for High Performance Liquid Chromatography. A protein peak appeared at a retention time 13.85 minutes conforming the single band in SDS-PAGE gel. The rest of peaks belonged to buffer (rT=2.60 and 2.98)and GlcNAc (3.59 and 4.45), which have low molecular weights and were observed at the beginning of the graph.
Inhibition of T. rangeli Attachment on R. prolixus adhesion inhibition by blocking the receptors on the salivary gland surface. This process was specific to the extract from the epimastigote long form as no such inhibition occurred with the short form of the parasites. These results indicate that T. rangeli long epimastigotes possess the appropriate ligands for binding to R. prolixus salivary glands, which mediate the initial attachment of the parasites. In fact, in each parasite stage, specific molecules associated with the surface membrane are most probably involved in interactions with the hosts. As previously has been described, in the insect-parasite phase of T. rangeli, only long epimastigotes can attach to the surface of R. prolixus salivary gland and, this process can be blocked by pre-incubation of parasites or salivary glands with specific sugars.

Several functions have been ascribed to sugar-binding proteins on the surface of protozoans, one of the main roles being the interaction between these molecules as receptors with host cell surface carbohydrate, during parasite attachment/invasion. One of the candidates for the attachment molecules may be lectins which are known to mediate cell-to-cell interaction. These molecules have been reported from several parasitic protozoans such as Leishmania species promastigotes and amastigotes, Plasmodium falciparum, Tritrichomonas foetus and Entamoeba histolytica.

In the present study, the extract of long epimastigotes of T. rangeli showed low agglutinin activity against rabbit blood cells so that the attachment molecules may well be lectin-like in this parasite. The effect of carbohydrate inhibitors on attachment of some protozoans to their host cells suggests the presence of lectin or lectin-like molecular activity in the Protozoa. Several authors have studied surface protein receptor in protozoan adhesion. For example, the 82kDa glycoprotein on the surface trypomastigotes of T. cruzi which is involved in mammalian cell invasion. Jungrey (1985) also reported that P. falciparum merozoites had lectin-like proteins on their surface that bound to red blood cell carbohydrate receptors and the merozoite invasion in vitro could be specifically blocked by GlcNAc, GalNAc and NeuNAc. Recently, three merozoite surface proteins have been identified that are specific for GlcNAc binding and could be involved in the binding of merozoites to the glycoconjugates on the surface of the red blood cells.

It has been shown the presence of six sugars on the surface of R. prolixus salivary glands and also those sugars were employed for the attachment inhibition assays in vitro by authors. The results showed T. rangeli adhesion was blocked strongly by GlcNAc indicating the presence of specific ligand on the parasites and salivary gland. Therefore, on the hypothetical possibility that the attachment protein may bind to a sugar affinity column, in the present study, GlcNAc was preferentially chosen to purify a ligand protein from T. rangeli using a GlcNAc-agarose affinity column. The isolated protein has apparent molecular mass at 60 kDa by SDS/PAGE and its purity was confirmed with HPLC (Fig. 3). This protein even at low concentration (0.083 mg/mL), showed attachment inhibitory effect (Fig. 1) suggesting that T. rangeli receptors specifically interact with glycosylated salivary gland surface molecules such as GlcNAc moieties which are present on the salivary gland. In addition, the results showed that the molecule causing the inhibitory effect from both the trypanosomes extract and the Tr-60 were not completely heat-sensitive.

WGA-labelled lectin has a high affinity for GlcNAc, exhibited strong and specific labelling of the salivary gland basal membrane and cell layer. This interaction may confirm the previous finding, indicating the high inhibitory effect of pre-incubating parasites in GlcNAc and subsequent experiments, described here, show the interaction of this sugar with protein receptors on the surface of T. rangeli. This interaction was not affected by extraction of parasites using solvent, indicating that the binding to Tr-60 was mediated by glycoproteins rather than glycolipids. Generally, these results strongly show the presence of carbohydrates on the cell-surface of R. prolixus salivary gland which are linked to glycoproteins by N-glycosylation.

Several authors have reported the role of glycosylated proteins in invasion of parasitic protozoans into the host cells. For example, Barreau et al., (1995) reported the presence of carbohydrate-binding proteins on the surface of the salivary gland of Aedes aegypti serving as receptors for invasion of the sporozoite of Plasmodium gallinaceum. The role of carbohydrate-mediated adherence has also been shown in a mannose-binding protein of Acanthamoeba and a galactose/GalNAc-binding protein of Entamoeba. Finally, in the present study, the 60 KDa protein isolated from T. rangeli epimastigote long form can recognize GlcNAc specific receptor present on R. prolixus salivary glands and are involved in the parasites adhesion. In addition, an attempt to find the sequence of Tr-60 using Edman chemistry failed probably due to modification of the N-terminus of the protein.

CONCLUSION

Experiments described in this study, show the blocking of the attachment of T. rangeli long epimastigotes in vitro to the surface of R. prolixus salivary glands using the extract of trypanosomes. Also, the presence of low agglutinin activity in the parasites extract may indicate the epimastigote long form possesses lectin-like molecules. GlcNAc, which showed the highest inhibitory potency with respect to blocking the trypanosome ad-
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hesion on the salivary gland surface, was preferentially used to purify a ligand protein of *T. rangeli*. A 60 kDa protein-bind GlcNAc isolated from *T. rangeli* (Tr-60) showed a potent parasite adhesion inhibitory effect indicating that this protein may act as a ligand receptor in the interaction between the trypomastigote and the gland cell surface. The presence of SDS (sodium dodecyl sulphate) in the buffer did not affect the interaction of this glycosylated protein with the salivary gland cells indicating that the Tr-60 could be a glycoprotein. The purity was confirmed by reverse phase HPLC.

REFERENCES


Inhibition of *T. rangeli* Attachment on *R. prolixus*


