Involvement of sulfated glycosaminoglycans on the development and attachment of *Trypanosoma cruzi* to the luminal midgut surface in the vector, *Rhodnius prolixus*

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(*Received 18 March 2011; revised 28 May, 30 June and 10 August 2011; accepted 12 August 2011; first published online 9 September 2011*)

SUMMARY

In the present study, we investigated the involvement of sulfated glycosaminoglycans in both the *in vivo* development and adhesion of *T. cruzi* epimastigotes to the luminal surface of the digestive tract of the insect vector, *Rhodnius prolixus*. Pre-incubation of *T. cruzi*, Dm 28c epimastigotes with heparin, chondroitin 4-sulfate, chondroitin 6-sulfate or protamine chloridrate inhibited *in vitro* attachment of parasites to the insect midgut. Enzymatic removal of heparan sulfate moieties by heparinase I or of chondroitin sulfate moieties by chondroitinase AC from the insect posterior midgut abolished epimastigote attachment *in vitro*. These treatments also reduced the labelling of anionic sites exposed at the luminal surface of the perimicrovillar membranes in the triatomine midgut epithelial cells. Inclusion of chondroitin 4-sulfate or chondroitin 6-sulfate and to a lesser extent, heparin, in the *T. cruzi*-infected bloodmeal inhibited the establishment of parasites in *R. prolixus*. These observations indicate that sulfated glycosaminoglycans are one of the determinants for both adhesion of the *T. cruzi* epimastigotes to the posterior midgut epithelial cells of the triatomine and the parasite infection in the insect vector, *R. prolixus*.

Key words: *Trypanosoma cruzi*, *Rhodnius prolixus*, triatomid-parasite interaction, posterior midgut, peritrophic and perimicrovillar membrane, glycosaminoglycans.

INTRODUCTION

Chagas disease continues to represent an endemic health threat affecting an estimated 28 million people, living mostly in the large area of South and Central America with approximately 21 000 deaths per year. Chagas disease is a complex zoonosis, with a large number of vertebrate reservoirs and triatomine insects participating in the transmission chain, making disease eradication practically impossible (WHO, 2002, 2007; Moncayo and Silveira, 2009). *Trypanosoma cruzi*, the aetiological agent of Chagas disease (Chagas, 1909, 1911), has, in its life cycle, different morphological and functional forms that occur in its vertebrate and invertebrate hosts. The parasite presents replicative amastigotes and non-dividing bloodstream trypomastigotes in mammals, whereas the insect midgut shelters the development of dividing epimastigotes and infective metacyclic trypomastigotes which are deposited together with feces and urine on the vertebrate host (Garcia *et al*. 2007).

Several macromolecules involved in parasite internalization, transformation into amastigotes, intracellular multiplication and release of new infective forms in mammalian cells in vertebrate hosts have been characterized (Burleigh and Woolsey, 2002; Tan and Andrews, 2002) but, there is only limited information concerning molecules modulating the life cycle of *T. cruzi* within the invertebrate hosts (Azambuja *et al*. 2005; Garcia *et al*. 2010).

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Parasitology (2011), **138**, 1870–1877.
doi:10.1017/S0031182011001521

Adhesion to perimicrovillar membranes (PMM) (Terra, 1990) in the insect midgut is an essential step for parasite division since the blockade of PMM development by either endocrine manipulation or oral administration of antiserum raised against *Rhodnius prolixus* PMM and midgut tissue reduced the development of the parasite inside the vector (Gonzalez and Garcia, 1992; Nogueira *et al*. 1997; Gonzalez *et al*. 1998, 1999, 2006). Glycoinositolphospholipids (Nogueira *et al*. 2007), hydrophobic proteins which bind to some glycopolypeptides with *M*r of 13 to 97 kDa from *R. prolixus* PMM (Alves *et al*. 2007) and calpain (Ennes-Vidal *et al*. 2011) are cell surface molecules involved in the adhesion of *T. cruzi* epimastigotes to the insect midgut epithelium.

Many pathogens use surface sulfated glycosaminoglycans (S-GAGs) as adhesion receptors (Tonnaer *et al*. 2006; Dinglasan *et al*. 2007; Sinnis *et al*. 2007; Akhtar and Shukla, 2009). S-GAGs are large and linear complex carbohydrate molecules negatively charged and composed of disaccharide repeating units and include dermatan sulfate, keratan sulfate, heparin (Hep), heparan sulfate (HS) and chondroitin sulfate (CS) (Nader *et al*. 2004; Volpi, 2006; Yamada and Sugahara, 2008; Nadanaka and Kitagawa, 2008; Dreyfuss *et al*. 2009). Those composed of HS, but not of CS, mediate both attachment and invasion of mouse cardiomyocytes by *T. cruzi* trypomastigotes (Calvet *et al*. 2003; Oliveira *et al*. 2008; Bambino-Medeiros *et al*. 2011).

The occurrence of S-GAGs in internal organs of triatomine vectors of Chagas disease has previously been reported by Dietrich *et al*. (1987). Their tissue distribution has also been determined by histochemical metachromatic staining (Costa-Filho *et al*. 2004). Regarding the digestive tract, CS and HS were the only S-GAGs detected in both *Triatoma brasiliensis* and *Rhodnius prolixus* (Souza *et al*. 2004; Costa-Filho *et al*. 2004).

The aim of the present study was to investigate the possible participation of S-GAG components of the midgut of *R. prolixus* in both the *in vivo* protozoan development and the *in vitro* attachment of *T. cruzi* to the luminal surface of triatomine posterior midgut epithelium.

MATERIALS AND METHODS

Insect rearing and feeding procedures

Rhodnius prolixus (Hemiptera: Reduviidae) were reared and maintained in the laboratory at 28 °C and relative humidity of 60–70%, as described by Azambuja and Garcia (1997). Randomly chosen fifth-instar male nymphs were identified, as previously described (Gillet, 1935; Lent and Juberg, 1969), starved for 30 days after the last ecdysis and then allowed to feed on citrated human blood using an artificial membrane apparatus (Garcia *et al*. 1984).

Parasites

The Dm 28c clone of *T. cruzi* was grown at 28 °C in a Brain Heart Infusion (DIFCO) culture medium, containing hemin and folic acid and supplemented with 20% heat-inactivated fetal calf serum (Garcia and Azambuja, 1997). For both *in vivo* and *in vitro* experiments, parasites were collected during the exponential growth phase, washed 3 times by centrifugation at 3000 ***g*** in 0·15 M NaCl, 0·01 M phosphate-buffer, pH 7·2 (PBS) and immediately used (Carvalho-Moreira *et al*. 2003).

Chemicals and reagents

Hep, C 4-S, C 6-S, chondroitinase AC (Chase AC) (EC 4.2.2.5) from *Arthrobacter aurescens* and heparinase I (Hep I) (EC 4.2.2.7) from *Flavobacterium heparinum* were purchased from Sigma Chemical Co. (St Louis, MO, USA). Protamine chloridrate (Prot) (Roche) was kindly gifted by Dr Walmir Nelson Moreira (UFF).

Trypanosoma cruzi *infection and parasite development assay*

To conduct *in vivo* experiments, control insects were allowed to feed on a mixture of heat-inactivated citrated human blood and *T. cruzi* epimastigotes at a final concentration of 3×10^3 parasites/ml of blood (Garcia *et al*. 1995). For other groups, Hep, C 4-S or C 6-S GAGs were added immediately before feeding to the infected bloodmeal at a final concentration of 1·0 μg/ml. At different intervals post-feeding/infection, 8–10 insects were dissected, the whole digestive tract removed, homogenized in 1 ml of PBS, and the number of parasites quantified using a Neubauer haematocytometer under phase-contrast microscopy (Gonzalez *et al*. 1999). Each experiment was repeated at least 3 times with groups of 40 insects.

In vitro *interaction between* R. prolixus *PMM and epimastigotes*

After washing in PBS, epimastigotes were resuspended in fresh BHI to a density of $2{\cdot}5 \times 10^7$ cells/ml. Samples of an interaction medium composed of 200 μl of this parasite suspension together with posterior midguts, freshly dissected and washed from insects 10 days after non-infectious feeding, were placed into Eppendorf microtubes (Alves *et al*. 2007) and incubated for 30 min at 25 °C. Some experiments were performed with parasites previously incubated (30 min, 25 °C) in PBS with Hep, C 4-S, or C 6-S at different final concentrations

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(0·01, 0·1 or 1·0 μg/ml of interaction medium). Parasites were also incubated in the same conditions with protamine chloridrate (250–1000 UI/ml). For other experiments, posterior midguts were previously incubated with different enzymes to remove specific GAGs (Garcia-Abreu *et al*. 2000) or, alternatively, with only the reaction medium without the specific enzyme (non-treated control). For these enzyme experiments, incubation occurred for 2 h at 25 °C either in 100 mM sodium acetate and 10 mM calcium acetate, pH 7·0 containing Hep I (1·5 units/50 μl) for the removal of HS or in 50 mM Tris-HCl, pH 8·0, containing 5 mM EDTA and 15 mM sodium acetate with chondroitinase AC (0·25 units/50 μl) for the removal of CS, or in reaction medium alone. The treated-posterior midguts were then washed in fresh BHI and immediately added to the interaction medium. After incubation (30 min, 25 °C) with the parasites, all midgut preparations were spread onto glass slides to count the number of attached parasites. A Zeiss microscope with reticulated ocular, equipped with a video microscopy camera, was used for counting parasites attached to 100 randomly chosen epithelial cells in 10 different fields of each midgut preparation. For each experimental group, 10 insect midguts were used (Nogueira *et al*. 2007).

Localization of anionic groups

To localize exposed anionic sites, posterior midguts obtained from insects 10 days after non-infectious feeding were previously incubated for 2 h at 25 °C with either Hep I (1·5 units/50 μl) or Chase AC (0·25 units/50 μl). Following the work of Houk *et al*. (1986), fragments of these treated-posterior midguts and also from non-treated midguts (control) were collected, washed in PBS, incubated for 30 min in 50 mM ammonium chloride to block free aldehyde groups and then for 15 min in a solution containing colloidal iron hydroxide particles in PBS buffer, pH 1·8 (Gasic *et al*. 1968). Subsequently, the fragments were washed twice in PBS, fixed with 2·5% glutaraldehyde in 0·1 M sodium-cacodylate buffer, pH 7·2, for 2 h at room temperature, washed in 0·1 M cacodylate buffer, pH 7·2, and post-fixed with 1% osmium tetroxide in 0·1 M cacodylate buffer, pH 7·2 for 1 h at room temperature in the dark. The tissue fragments were then dehydrated in acetone, infiltrated and embedded in Epoxy resin and polymerized at 60 °C for 3 days. Approximately 50 thin sections of each group were analysed. These thin sections, each one containing 5 to 10 different cells depending on the magnification used, were examined in a Zeiss 900 transmission electron microscope. The results were only considered when almost 100% of the insects in each experimental group displayed the same posterior midgut ultrastructural arrangement. (Nogueira *et al*. 1997; Gonzalez *et al*. 1998; Albuquerque Cunha *et al*. 2009).

Data analysis

Significance of the results was analysed using ANOVA and Turkey's test according to Stats Direct Statistical Software, version 2.2.7 for Windows 98 (Armitage *et al*. 2002). The difference between treated and control insects was considered to be not statistically significant when $P > 0{\cdot}05$. Probability levels are specified in the text.

RESULTS

Influence of S-GAGs on epimastigote attachment to midgut epithelium

Previous incubation of the parasites with all the S-GAGs tested partially inhibited the attachment of *T. cruzi* to posterior epithelial midgut cells in the interaction medium (Fig. 1). In the preparations obtained from the control parasites, about 120·7 ± 13·3 epimastigotes attached per 100 midgut cells were observed. Similar adhesion rates were obtained when flagellates were previously incubated with 0·01 μg/ml of Hep, C 4-S or C 6-S ($P > 0{\cdot}05$). In contrast, attachment of only 15·3 ± 3·3 and 7·6 ± 2·7 parasites per 100 cells of the midgut epithelium were recorded when the flagellates were incubated with either 0·1 or 1·0 μg/ml of Hep ($P < 0{\cdot}0001$), respectively. In the same way, parasite incubation with either 0·1 or 1·0 μg/ml of C 4-S reduced the *T. cruzi* attachment to 32·6 ± 12·8 and 7·3 ± 2·6 epimastigotes per 100 midgut cells ($P < 0{\cdot}0001$), respectively. Similar results were observed after parasite incubation with 0·1 or 1·0 μg/ml of C 6-S.

Influence of protamine chloridrate and S-GAG-specific degradative enzymes on epimastigote attachment to midgut epithelium

While the control group exhibited an adhesion rate of 109·7 ± 39·8 epimastigotes/100 midgut epithelium cells (Fig. 2), a partial reduction of the adhesion rates to 45·7 ± 39·5 ($P < 0{\cdot}01$), 30·9 ± 6·8 ($P < 0{\cdot}001$) and 11·7 ± 8·3 ($P < 0{\cdot}0001$) were observed when the posterior midguts were previously incubated with 250, 500 and 1000 UI Prot/ml of interaction medium, respectively.

After midgut incubation with either chondroitinase AC (Chase AC) or heparinase I (Hep I), the adhesion rates decreased to 21·1 ± 18·3 and 11·3 ± 8·5 epimastigotes/100 midgut epithelium cells ($P < 0{\cdot}001$), respectively (Fig. 3). No statistical difference was detected among the control group and the groups incubated with reaction medium without the respective specific enzymes ($P > 0{\cdot}05$).

Histochemical localization of anionic sites in the posterior midgut of R. prolixus

Posterior midgut preparations obtained from control group insects showed a typical posterior columnar

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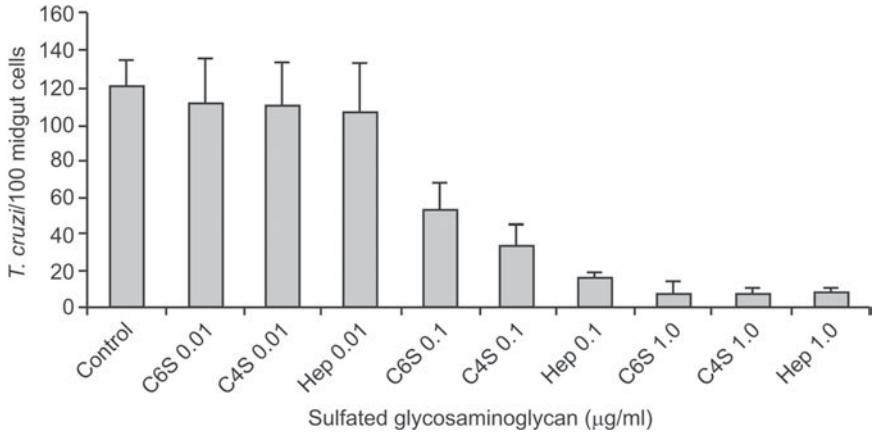

Fig. 1. Effect of sulfated glycosaminoglycans (S-GAGs) on *in vitro* attachment of *Trypanosoma cruzi* Dm 28c clone to posterior midgut epithelium obtained from male fifth instar larvae *Rhodnius prolixus* 10 days after the bloodmeal. Epimastigotes were previously incubated with different concentrations of S-GAGs (0·01, 0·1 and 1·0 μg/ml of BHI medium containing 250×10^5/ml), washed and added to the interaction medium. Adhered epimastigotes were counted per 100 epithelial cells in 10 different fields of each midgut preparation using a Zeiss microscope. Each group represents means ± S.E. of parasites attached in 10 midguts. Non-treated (Control), Chondroitin 6-S (C6S), Chondroitin 4-S (C4S) and Heparin (Hep). Non-treated (control) groups were performed without S-GAGs in the same pre-incubation conditions.

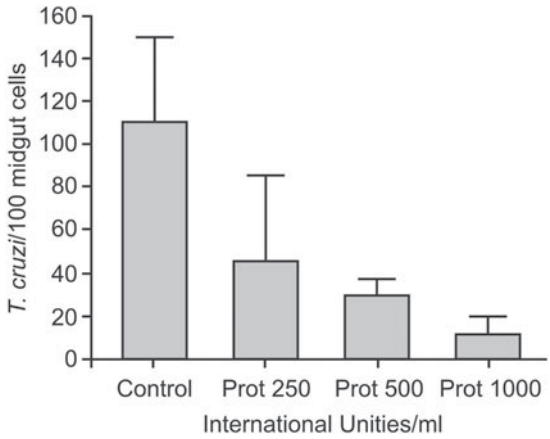

Fig. 2. Effect of protamine treatment on *in vitro* attachment of *Trypanosoma cruzi* Dm 28c clone to posterior midgut epithelium obtained from male fifth instar larvae *Rhodnius prolixus* 10 days after the bloodmeal. Midguts were previously incubated with different concentrations of protamine chloridrate, washed, and then added to the interaction medium containing the flagellates (250×10^5/ml). Adhered epimastigotes were counted per 100 epithelial cells in 10 different fields of each midgut preparation using a Zeiss microscope. Each group represents means ± S.E. of parasites attached in 10 midguts. Non-treated (Control) and protamine (Prot).

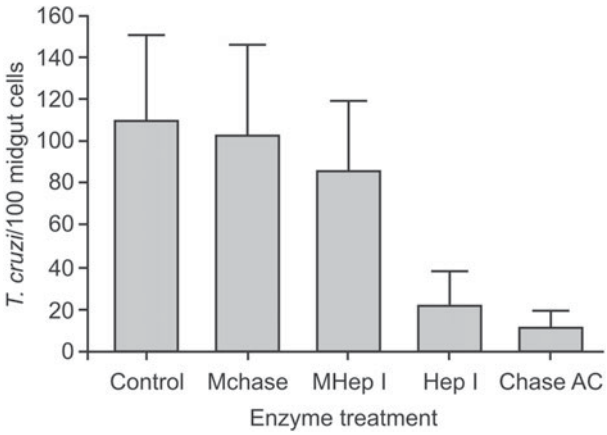

Fig. 3. Effect of sulfated glycosaminoglycans (S-GAGs) removal on *in vitro* attachment of *Trypanosoma cruzi* Dm 28c clone to posterior midgut epithelium obtained from male fifth instar larvae *Rhodnius prolixus* 10 days after the bloodmeal. Midguts were previously incubated with different enzymes – to selectively remove S-GAGs – at a final concentration of 1·5 units/50 μl (for heparinase I) and 0·25 units/50 μl (for chondroitinase AC), washed, and then added to the interaction medium containing the flagellates (250×10^5/ml). Adhered epimastigotes were counted per 100 epithelial cells in 10 different fields of each midgut preparation using a Zeiss microscope. Each group represents means ± S.E. of parasites attached in 10 midguts. Non-treated (Control), medium for Chase AC without the enzyme (Mchase AC), medium for Hep I without the enzyme (MHep I), chondroitinase AC (Chase AC) and heparinase I (Hep I).

midgut epithelium with homogeneously distributed microvilli covered by plexiform PMM connecting the microvilli to each other and projecting into the midgut lumen (Fig. 4A). Numerous anionic sites were detected in the luminal surface of PMM when the midgut tissue was previously incubated with colloidal iron. Some labelling was observed at both the bases of microvilli and microvillar membranes and between microvilli, but no electron-dense reaction was detected in the cytoplasm of this experimental group (Fig. 4B). Similar results were obtained in the posterior midguts pre-treated only with the reaction medium (without the enzymes) for either Chase AC or Hep I enzymes (not shown). However, no labelling at PMM or microvilli was

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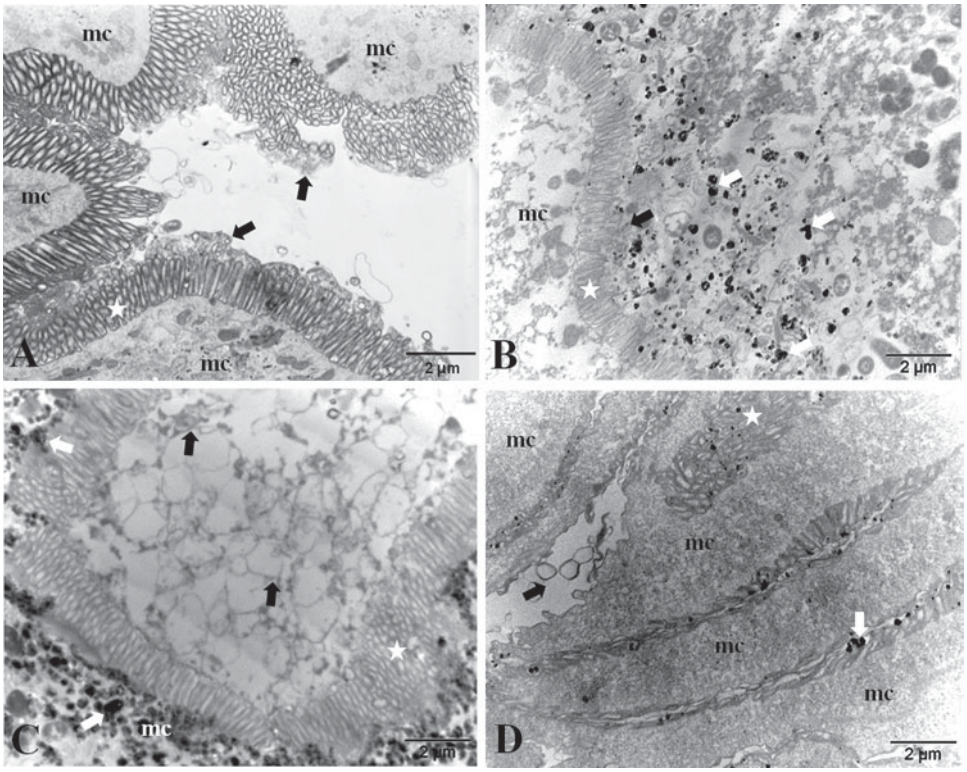

Fig. 4. Transmission electron microscopy of posterior midgut epithelium of male fifth instar larvae *Rhodnius prolixus* 10 days after feeding. (A) Control. The apical regions of these posterior midgut epithelial cells (mc) show numerous and homogeneous distributed microvilli (star) surrounded by luminal perimicrovillar membranes (black arrow). (B) After a single incubation with colloidal iron, the apical regions of these cells show perimicrovillar membranes (black arrow) with intense labelling of anionic sites detected in its luminal surface (white arrow). Microvilli (star). (C) After incubation with chondroitinase AC following incubation with colloidal iron, no labelling was observed either in perimicrovillar membranes (black arrow) or in microvilli (star) but, several anionic sites were detected in line at the apical cytoplasm near to the basis of microvilli (white arrow). (D) After incubation with heparinase I following incubation with colloidal iron, only few anionic sites were exposed at perimicrovillar membranes (white arrow) while no labelling was observed at apical cytoplasm or lumen (black arrow). Microvilli (star).

observed after previous treatment with chondroitinase AC, but in this group intense deposition of reaction products was noted in the cytoplasm at the basis of microvilli (Fig. 4C). Only sparse regions of labelling were present at PMM and microvilli of posterior midgut cells previously treated with Hep I enzyme. No cytoplasmic reaction was detected in this experimental group (Fig. 4D).

In vivo *experiments*

Control insects displayed high infection levels from $66{\cdot}6 \pm 6{\cdot}4 \times 10^2$ flagellates per insect midgut at 10 days after infection to $8{\cdot}3 \pm 5{\cdot}0 \times 10^3$ flagellates per insect midgut at 30 days after infection (Fig. 5). In contrast, all other experimental groups (treated with different GAGs) exhibited significant lower levels of *T. cruzi* infection. Hep-treated insects showed significantly reduced infection levels of $50 \pm 23{\cdot}4$ ($P < 0{\cdot}001$), $10 \pm 10{\cdot}0$ ($P < 0{\cdot}0001$) and $58{\cdot}3 \pm 20{\cdot}0$ ($P > 0{\cdot}01$) protozoans per insect midgut at days 10, 15 and 25 after infection, respectively. In the same period, C 4-S-treated insects also displayed significantly reduced infection levels of 10 ± 10 ($P < 0{\cdot}0001$), $33{\cdot}3 \pm 51{\cdot}6$ ($P < 0{\cdot}01$) and $41{\cdot}6 \pm 20$ ($P < 0{\cdot}001$) flagellates per midgut. No parasites were observed at 30 days of infection in both Hep and C 4-S-treated insects. Flagellates were never detected in C 6-S-treated insects throughout the whole experimental period.

DISCUSSION

Throughout its life cycle in both vertebrate and invertebrate hosts, *T. cruzi* undergoes adhesion to specific host tissues which is crucial for development in different host tissues since it triggers a variety of interaction events such as cell internalization, replication and transformation to infective stages (Burleigh and Woolsey, 2002; Tan and Andrews, 2002). Previous observations concerning the dynamics of parasite interaction with its triatomine vector showed that the insect midgut (particularly the PMM in the posterior midgut and the hydrophobic rectal cuticle) contains components exposed on the surface that mediate epimastigote attachment followed by parasite multiplication in the posterior

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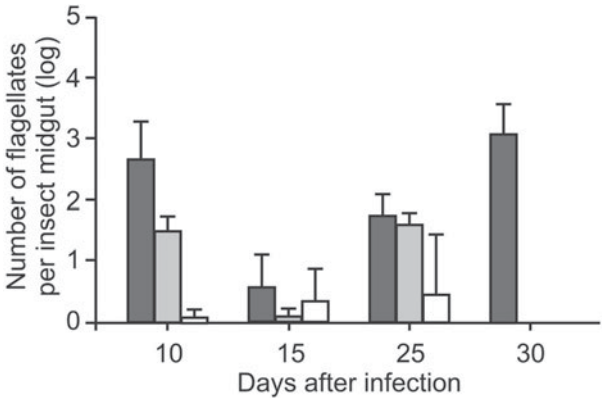

Fig. 5. Effect of sulfated glycosaminoglycans (S-GAGs) on *in vivo* development of *Trypanosoma cruzi* Dm 28c clone in the midgut of male fifth instar larvae *Rhodnius prolixus* after infection. Insects were fed on citrated and complement-inactivated human blood containing 3×10^3 flagellates/ml. Each S-GAG was added to the bloodmeal at a dose of 1·0 μg/ml. Non-treated (black), heparin (grey), chondroitin 4-S (white). Parasites were not detected in the chondroitin 6-S group. Each group represents means ± S.D. of flagellates in 10 insect midguts.

midgut and metacyclogenesis in the rectum (Garcia *et al*. 2007; Schaub, 2009). Furthermore, research analysing the parasite tropism and chemotaxis to insect midgut tissues established that molecules inhibiting the parasite attachment to insect midgut surfaces *in vitro* also often efficiently block the *in vivo* development of *T. cruzi* in its invertebrate host (Alves *et al*. 2007; Nogueira *et al*. 2007), and may constitute key factors in avoiding parasite transmission by its vectors. Since S-GAGs are synthesized by triatomines (Garcia *et al*. 1986; Dietrich *et al*. 1987) and are present in the digestive tract of *T. brasiliensis* and *R. prolixus* (Costa-Filho *et al*. 2004; Souza *et al*. 2004), it is possible that these molecules may be important for the attachment of *T. cruzi* to the midgut epithelium of *R. prolixus* nymphs and posterior development of the protozoan in the vector. Our data support this idea since previous incubation of *T. cruzi* with S-GAGs before contact with *R. prolixus* posterior midguts in the interaction medium inhibited the attachment of epimastigotes to the triatomine luminal surface of midgut epithelium *in vitro* in a dose-dependent manner. In addition, there was a severe reduction of the flagellate population in the digestive tract of *R. prolixus* when nymphs were infected with epimastigotes of *T. cruzi* and simultaneously orally treated with very low doses of 1·0 μg of Hep, C 4-S or C 6-S GAGs per ml of the infective bloodmeal.

Polypeptides of 13 to 97 kDa from *R. prolixus* PMM, some of them related to N-acetylglucosamine and N-acetylgalactosamine, have been shown to bind to either hydrophobic proteins or glycoinositolphospholipids (GIPLs) on the surface of epimastigotes (Gonzalez *et al*. 2006; Alves *et al*. 2007; Nogueira *et al*. 2007; Albuquerque-Cunha *et al*. 2009). Similarly, S-GAGs have disaccharide repeating units composed of hexosamine (*D*-glucosamine or *D*-galactosamine) and either hexuronic acid (*D*-glucuronic or *L*-iduronic acid) or galactose and are covalently bound to a protein core forming a structure known as a proteoglycan (Didraga *et al*. 2006; Taylor and Gallo, 2006; Gandhi and Mancera, 2008).

Calvet and co-workers (2003) have examined the role of S-GAGs in the attachment of *T. cruzi* trypomastigotes to mouse cardiomyocytes and demonstrated that HS, but not CS mediate not only the attachment but also the invasion process. Subsequently, the same group reported that *T. cruzi* trypomastigotes have heparin-binding proteins, which specifically bind to HS (Oliveira *et al*. 2008). Bacteria, viruses and protozoa also often utilize HS and/or CS as receptors for adherence as with pneumococcal binding to mucosal epithelial cells (Tonnaer *et al*. 2006) and human papillomavirus and herpex simplex virus for attachment and invasion to host cell surfaces (reviewed by Akhtar and Shukla, 2009; Sapp and Bienkowska-Haba, 2009).

In mosquitoes, HS binds to the circumsporozoite protein of *Plasmodium,* and S-GAG is presumably engaged in the infection and transmission of the *Plasmodium* parasite (Sinnis *et al*. 2007) and a mosquito CS has been shown to interact *in vivo* with *Plasmodium falciparum* during invasion of the midgut (Dinglasan *et al*. 2007).

Previously it has also been shown that incubation of *T. cruzi* epimastigotes with sialic acid and mannose inhibited the attachment of flagellates to the midgut epithelium of *R. prolixus*. This suggests the involvement of both negatively charged specific carbohydrates in the midgut and carbohydrate binding proteins on the *T. cruzi* surface in the attachment process (Nogueira *et al*. 2007; Alves *et al*. 2007). Moreover, carbohydrate residues have been suggested as important molecules in the development of *T. cruzi in vitro* and in the insect vector (Pereira *et al*. 1981; Tyler and Engman, 2000, 2001; Bonay *et al*. 2001; Bourguignon *et al*. 2006). In the present investigation, the previous incubation of *R. prolixus* posterior midguts with Prot, Hep I or Chase AC enzymes before contact with *T. cruzi* in the interaction medium also inhibited the attachment of epimastigotes to the triatomine luminal surface of the midgut epithelium. The ultrastructural analysis of the posterior midgut demonstrated that, in contrast with control insects, anionic sites on the luminal surface of PMM were rarely detected following treatment with Hep I or Chase AC enzymes, which efficiently remove negatively charged HS or CS, respectively. It is important to mention that *T. cruzi* morphology and motility were not affected after treatment with either GAGs or enzymes

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(Chase AC and Hep I) during both *in vivo* and *in vitro* experiments (data not shown). Similar results on *T. cruzi* viability and longevity were obtained after exposure to HS by Calvet *et al*. (2003) and Bambino-Medeiros *et al*. (2011).

So, our present results demonstrate that HS and CS present on the luminal surface of *R. prolixus* midgut are involved in *T. cruzi* epimastigote binding. The high negative charge of both S-GAGs might act as a non-specific step for *T. cruzi* adhesion to the luminal midgut and reduce the level of these negative binding sites on the luminal midgut since enzymatic removal of either HS or CS inhibits the attachment of the parasites. Similarly, the pre-incubation of midgut preparations with protamine – a cationic polypeptide that can bind to negatively charged Hep and neutralize its antithrombin-mediated anticoagulant properties (Ni Ainle *et al*. 2009). This reduces the level of negative binding sites available on the midgut, probably by binding to S-GAGs, resulting in the inhibition of *T. cruzi* attachment. Curiously, *T. cruzi* trypomastigotes selectively bind to HS GAGs, as demonstrated in *in vitro* binding assays with mouse cardiomyocytes (Calvet *et al*. 2003), whereas *T. cruzi* epimastigotes show a less selective profile by binding to both HS and CS GAG. This may suggest the presence of different GAG-binding sites between the two forms of the parasite. These observations also indicate that S-GAGs are one of the determinants of parasite infection in the insect vector and that the recognition mechanisms involved might depend upon the physical-chemical nature of both GAGs in the insect midgut and carbohydrate binding proteins on the surface of *T. cruzi*.

ACKNOWLEDGEMENTS

We thank Dr Walmir Nelson Moreira from the pharmacy of Hospital Universitário Antonio Pedro (HUAP) of the Universidade Federal Fluminense (UFF) who kindly gave us the protamine chloridrate. We also thank Rodrigo Mexas, Heloisa Maria Nogueira Diniz and Genilton José Vieira (Laboratório de Análise e Reprodução de Imagens/ Fiocruz) for the photography treatment, Professor Norman Ratcliffe (University of Swansea, UK) for the English revision and the anonymous referee for scientific suggestions.

FINANCIAL SUPPORT

This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Fundação Oswaldo Cruz (FIOCRUZ, PAPES). L. C. F. Silva, P. Azambuja and E. S. Garcia are CNPq senior research fellows. D. P. Castro is a researcher with a FAPERJ Post-doc fellowship. D. P. Mattos is a student with FAPERJ scientific initiation fellowship.

REFERENCES

Akhtar, J. and Shukla, D. (2009). Viral entry mechanisms: cellular and viral mediators of *Herpex simplex* virus entry. *The FEBS Journal* **276**, 7228–7236.

Albuquerque-Cunha, J. M., Gonzalez, M. S., Garcia, E. S., Melllo, C. B., Azambuja, P., Almeida, J. C. A., de Souza, W. and Nogueira, N. F. S. (2009). Cytochemical characterization of microvillar and perimicrovillar membranes in the posterior midgut epithelium of *Rhodnius prolixus*. *Arthropod Structure and Development* **38**, 31–44.

Alves, C. R., Albuquerque-Cunha, J. M., Melllo, C. B., Garcia, E. S., Nogueira, N. F. S., Bourguingnon, S. C., de Souza, W., Azambuja, P. and Gonzalez, M. S. (2007). *Trypanosoma cruzi*: Attachment to perimicrovillar membrane glycoproteins of *Rhodnius prolixus*. *Experimental Parasitology* **116**, 44–52.

Armitage, P., Berry, G. and Matthews, J. N. S. (2002). Comparison of several groups and experimental design. In *Statistical Methods in Medical Research* 4th Edn. (ed. Armitage, P.), pp. 208–256. Blackwell Science Publishing, Oxford, UK.

Azambuja, P. and Garcia, E. S. (1997). Care and maintenance of triatomine colonies. In *Molecular Biology of Insect Disease Vectors: a Methods Manual* (ed. Crampton, J. M., Beard, C. B. and Louid, C.), pp. 56–64. Chapman and Hall, London, UK.

Azambuja, P., Ratcliffe, N. A. and Garcia, E. S. (2005). Towards an understanding of the interactions of *Trypanosoma cruzi* and *Trypanosoma rangeli* within the reduviid insect host, *Rhodnius prolixus*. *Academia Brasileira de Ciências* **77**, 397–404.

Bambino-Medeiros, R., Oliveira, F. O. R., Calvet, C. M., Vicente, D., Toma, L., Krieger, M. N., Meirelles, M. R. S. L. and Pereira, M. C. S. (2011). Involvement of host cell heparan sulfate proteoglycan in *Trypanosoma cruzi* amastigote attachment and invasion. *Parasitology*. doi:10.1017/S0031182010001678.

Bonay, P., Molina, R. and Fresno, M. (2001). Binding of mannose-specific carbohydrate-binding protein from the cell surface of *Trypanosoma cruzi*. *Glycobiology* **11**, 719–729.

Bourguignon, S. C., Mello, C. B., Santos, D. O., Gonzalez, M. S. and Souto-Pádron, T. (2006). Biological aspects of the *Trypanosoma cruzi* (Dm 28c clone) intermediate form, between epimastigote and trypomastigote obtained in modified liver infusion tryptose (LIT) medium. *Acta Tropica* **98**, 103–109.

Burleigh, B. A. and Woolsey, A. M. (2002). Cell signaling and *Trypanosoma cruzi* invasion. *Cellular Microbiology* **4**, 701–711.

Calvet, C. M., Toma, L., Souza, F. R., Meirelles, M. R. S. L. and Pereira, M. C. (2003). Heparan sulfate proteoglycans mediate the invasion of cardiomyocytes by *Trypanosoma cruzi*. *Journal of Eukaryotic Microbiology* **50**, 97–103.

Carvalho-Moreira, C. J., Spata, M. C. D., Coura, J. R., Garcia, E. S., Azambuja, P., Gonzalez, M. S. and Mello, C. B. (2003). In vivo and in vitro metacyclogenesis tests of two strains of *Trypanosoma cruzi* in the triatomine vectors *Triatoma pseudomaculata* and *Rhodnius neglectus*: short, long-term and comparative study. *Experimental Parasitology* **103**, 102–111.

Chagas, C. (1909). Nova tripanosomíase humana. Estudos sobre a morfologia e o ciclo evolutivo do *Schizotrypanum cruzi* n. gen.,n. sp., agente etiológico de nova entidade mórbida do homem. *Memórias do Instituto Oswaldo Cruz* **1**, 159–218.

Chagas, C. (1911). Nova entidade mórbida do homem; resumo geral de estudos etiológicos e clínicos. *Memórias do Instituto Oswaldo Cruz* **3**, 219–275.

Costa-Filho, A., Souza, M. L. S., Martins, R. C. L., dos Santos, A. V. F., Silva, G. V., Comaru, M. W., Moreira, M. F., Atella, G. C., Allodi, S., Nasciutti, L. E., Masuda, H. and Silva, L. C. F. (2004). Identification and tissue-specific distribution of sulfated glycosaminoglycans in the blood-sucking bug *Rhodnius prolixus* (Linnaeus). *Insect Biochemistry and Molecular Biology* **34**, 251–260.

Costa-Filho, A., Werneck, C. C., Nasciutti, L. E., Masuda, H., Atella, G. C. and Silva, L. C. F. (2001). Sulfated glycosaminoglycans from ovary of *Rhodnius prolixus*. *Insect Biochemistry and Molecular Biology* **31**, 31–40.

Dietrich, C. P., Nader, H. B., Toma, L., Azambuja, P. and Garcia, E. S. (1987). A relationship between the inhibition of heparan sulfate and chondroitin sulfate synthesis and the inhibition of molting by selenate in the hemipteran *Rhodnius prolixus*. *Biochemical and Biophysical Research Communications* **146**, 652–658.

Didraga, M., Barroso, B. and Bischoff, R. (2006). Recent developments in proteoglycan purification and analysis. *Current Pharmaceutical Analysis* **2**, 323–27.

Dinglasan, R. R., Alaganan, A., Ghosh, A. K., Saito, A., van Kuppevelt, T. H. and Jacobs-Lorena, N. (2007). *Plasmodium falciparum* ookinetes require mosquito midgut chondroitin proteoglycans

for cell invasion. *Proceedings of the National Academy of Sciences, USA* **104**, 15882–15887.

Dreyfuss, J. L., Regatieri, C. V., Jarrouge, T. R., Cavalheiro, R. P., Sampaio, L. O. and Nader, H. B. (2009). Heparan sulfate proteoglycans: structure, protein interactions and cell signaling. *Anais da Academia Brasileira de Ciências* **81**, 409–429.

Ennes-Vidal, V., Menna-Barreto, R. F. S., Santos, A. L. S., Branquinha, M. H. and d'Avila-Levy, C. M. (2011). MDL28170, a calpain inhibitor, affects *Trypanosoma cruzi* metacyclogenesis, ultrastructure and attachment to *Rhodnius prolixus* midgut. *PLoS ONE* **6**, e18371. doi:10.1371/journal.pone.0018371.

Gandhi, N. S. and Mancera, R. L. (2008). The structure of glycosaminoglycans and their interactions with proteins. *Chemical Biological and Drug Design* **72**, 455–482.

Garcia, E. S. and Azambuja, P. (1997). Infection of triatomines with *Trypanosoma cruzi*. In *Molecular Biology of Insect Disease Vectors: A Methods Manual*. (ed. Crampton, J.M., Beard, C.B. and Louid, C.), pp. 146–155. Chapman and Hall, London, UK.

Garcia, E. S., Azambuja, P., Forster, H. and Rembold, H. (1984). Feeding and molt inhibition by azadirachtins A, B and 7-acetylazadirachtin A in *Rhodnius prolixus* nymphs. *Zeitschrift für Naturforschung* **29**, 1155–1158.

Garcia, E. S., Azambuja, P., Nader, H. B. and Dietrich, C. P. (1986). Biosynthesis of sulfated glycosaminoglycans in the hemipteran *Rhodnius prolixus*. Enhancement of chondroitin sulfate synthesis by phenylxyloside. *Insect Biochemistry* **16**, 347–352.

Garcia, E. S., Genta, F. A., Azambuja, P. and Schaub, G. A. (2010). Interactions between intestinal compounds of triatomines and *Trypanosoma cruzi*. *Trends in Parasitology* **26**, 499–505.

Garcia, E. S., Gonzalez, M. S., Azambuja, P., Baralle, F. E., Frainderaich, D., Torres, H. N. and Flawia, M. M. (1995). Induction of *Trypanosoma cruz* metacyclogenesis in the gut of hematophagous insect vector, *Rhodnius prolixus*, by hemoglobin and peptides carrying α^{D}-globin sequences. *Experimental Parasitology* **81**, 255–261.

Garcia, E. S., Ratcliffe, N. A., Whitten, M. M., Gonzalez, M. S. and Azambuja, P. (2007). Exploring the role of insect host factors in the dynamics of *Trypanosoma cruzi-Rhodnius prolixus* interactions. *Journal of Insect Physiology* **53**, 11–21.

Garcia-Abreu, J., Mendes, F. A., Onofre, G. R., Freitas, M. S., Silva, L. C. F., Moura Neto, V. and Cavalcante, L. A. (2000). Contribution of heparan sulfate to the non-permissive role of the midline glia to the growth of midbrain neuritis. *Glia* **29**, 260–272.

Gasic, G. J., Berwick, L. and Sorrentino, M. (1968). Positive and negative colloidal iron as cell surface electron stains. *Laboratory Investigations* **18**, 63–71.

Gillet, J. D. (1935). The genital sterna of the immature stages of *Rhodnius prolixus* (Hemiptera). *Transactions of the Royal Entomological Society of London* **83**, 1–5.

Gonzalez, M. S., Hamedi, A., Albuquerque-Cunha, J. M., Nogueira, N. F. S., de Souza, W., Ratcliffe, N. A., Azambuja, P., Garcia, E. S. and Mello, C. B. (2006). Antiserum against perimicrovillar membranes and midgut tissue reduces the development of *Trypanosoma cruzi* in the insect vector, *Rhodnius prolixus*. *Experimental Parasitology* **114**, 297–304.

Gonzalez, M. S. and Garcia, E. S. (1992). Effect of azadirachtin on the development of *Trypanosoma cruzi* in different species of triatomine insect vectors: Long-term and comparative studies. *Journal of Invertebrate Pathology* **60**, 201–205.

Gonzalez, M. S., Nogueira, N. F. S., Feder, D., de Souza, W., Azambuja, P. and Garcia, E. S. (1998). Role of the head in the ultrastructural midgut organization in *Rhodnius prolixus*: evidence from head transplantation experiments and ecdysone therapy. *Journal of Insect Physiology* **44**, 553–560.

Gonzalez, M. S., Nogueira, N. F. S., Mello, C. B., de Souza, W., Schaub, G. A., Azambuja, P. and Garcia, E. S. (1999). Influence of brain on the midgut arrangement and *Trypanosoma cruzi* development in the vector, *Rhodnius prolixus*. *Experimental Parasitology* **92**, 100–108.

Houk, E. J., Hardy, J. L. and Chiles, R. E. (1986). Mesenteronal epithelial cell surface charge of the mosquito, *Culex tarsalis* Coquillett, binding of colloidal iron hydroxide, native ferritin and cationized ferritin. *Journal of Submicroscope Cytology and Pathology* **18**, 385–396.

Lent, H. and Juberg, J. (1969). O Genero *Rhodnius* Stal, 1859, com um estudo sobre a genitalia das especies (Hemiptera, Reduviidae, Triatominae). *Revista Brasileira de Biologia* **29**, 487–560.

Moncayo, A. and Silveira, A. C. (2009). Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. *Memórias do Instituto Oswaldo Cruz* **104**, 17–30.

Nadanaka, S. and Kitagawa, H. (2008). Heparan sulphate biosynthesis and disease. *Journal of Biochemistry* **144**, 7–14.

Nader, H. B., Lopes, C. C., Rocha, H. A. O., Santos, E. A. and Dietrich, C. (2004). Heparins and heparinoids: occurrence, structure and mechanism of antithrombotic and hemorrhagic activities. *Current Pharmacological Design* **10**, 951–966.

Ni Ainle, F., Preston, R. J. S., Jenkis, P. V., Nel, H. J., Johnson, J. A., Smith, O. P., White, B., Fallon, P. G. and O'Donnell, J. S. (2009). Protamine sulfate down-regulates thrombin generation by inhibiting factor V activation. *Blood* **114**, 1658–1665.

Nogueira, N. F. S., Gonzalez, M. S., Garcia, E. S., Mello, C. B. and de Souza, W. (1997). Effects of azadirachtin on the fine structure of the midgut of *Rhodnius prolixus*. *Journal of Invertebrate Pathology* **69**, 58–63.

Nogueira, N. F., Gonzalez, M. S., Gomes, J. E., De Souza, W., Garcia, E. S., Azambuja, P., Nohara, L. L., Almeida, I. C., Zingales, B. and Colli, W. (2007). *Trypanosoma cruzi*: Involvement of glycoinositolphospholipids in the attachment to the luminal midgut surface of *Rhodnius prolixus*. *Experimental Parasitology* **116**, 120–128.

Oliveira, F. O. R., Alves, C. R., Calvet, C. M., Toma, L., Bouças, R. I., Nader, H. B., Côrtes, L. M. C., Krieger, M. A., Meirelles, M. N. S. L. and Pereira, M. C. S. (2008). *Trypanosoma cruzi* heparin-binding proteins and the nature of the host cell heparan sulfate-binding domain. *Microbial Pathogenesis* **44**, 329–338.

Pereira, M. E. A., Andrade, A. F. B. and Ribeiro, J. M. C. (1981). Lectins of distinct specificity in *Rhodnius prolixus* interact selectively with *Trypanosoma cruzi*. *Science* **211**, 597–600.

Sapp, M. and Bienkowska-Haba, M. (2009). Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus. *The FEBS Journal* **276**, 7206–7216.

Schaub, G. A. (2009). Interactions of trypanosomatids and triatomines. In *Advances in Insect Physiology* **37**, 177–242.

Sinnis, P., Coppi, A., Toida, T., Toyoda, H., Hinoshita-Toyoda, A., Xie, J., Kemp, M. M. and Linhardt, R. J. (2007). Mosquito heparan sulfate and its potential role in malaria infection and transmission. *The Journal of Biological Chemistry* **282**, 25376–25384.

Souza, M. L. S., Sarquis, O., Gomes, T. F., Moreira, M. F., Lima, M. M. and Silva, L. C. F. (2004). Sulfated glycosaminoglycans in two hematophagous arthropod vectors of Chagas disease, *Triatoma brasiliensis* and *Rhodnius prolixus* (Hemiptera: Reduviidae). *Comparative Biochemistry and Physiology* B **139**, 631–634.

Tan, H. and Andrews, N. W. (2002). Don't bother to knock – the cell invasion strategy of *Trypanosoma cruzi*. *Trends in Parasitology* **18**, 427–428.

Taylor, K. R. and Gallo, R. L. (2006). Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. *FASEB Journal* **20**, 9–22.

Terra, W. R. (1990). Evolution of digestive system of insects – review. *Annual Review of Entomology* **35**, 181–200.

Tonnaer, E. L. G. M., Hafmans, T. G., van Kuppevelt, T. H., Sanders, E. A. M., Verwejj, P. E. and Curfs, J. H. A. J. (2006). Involvement of glycosaminoglycans in the attachment of pneumococci to nasopharyngeal epithelial cells. *Microbes and Infection* **8**, 316–322.

Tyler, K. M. and Engman, D. M. (2000). Flagellar elongation induced by glucose limitation is preadaptive for *Trypanosoma cruzi* differentiation. *Cell Motility and the Cytoskeleton* **46**, 269–278.

Tyler, K. M. and Engman, D. M. (2001). The life cycle of *Trypanosoma cruzi* revisited. *International Journal for Parasitology* **31**, 472–481.

Volpi, N. (2006). Advances in chondroitin sulfate analysis: application in physiological and pathological states of connective tissue and during pharmacological treatment of osteoarthritis. *Current Pharmaceutical Design* **12**, 639–58.

Yamada, S. and Sugahara, K. (2008). Potential therapeutic application of chondroitin sulfate/dermatan sulfate. *Current Drug Discovery Technologies* **5**, 289–301.

World Health Organization (2002). *Chagas' disease*. UNPD World Bank WHO, Special Program for Research and Training in Tropical Disease (TDR), Strategic Direction for Research. World Health Organization, Geneva, Switzerland.

World Health Organization (2007). *Reporte sobre la enfermedad de Chagas*. UNICEF UNPD World Bank WHO, Special Program for Research and Training in Tropical Disease (TDR), Strategic Direction for Research. World Health Organization, Geneva, Switzerland.