Multiple effects of the lectin-inhibitory sugars D-glucosamine and N-acetyl-glucosamine on tsetse-trypanosome interactions

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SUMMARY

We are studying early events in the establishment of *Trypanosoma brucei* in the tsetse midgut using fluorescent trypanosomes to increase visibility. Feeding flies with the lectin-inhibitory sugars D-glucosamine (GlcN) or N-acetyl-glucosamine (GlcNAc) has previously been shown to enhance fly susceptibility to infection with trypanosomes and, as expected, we found that both sugars increased midgut infection rates of *Glossina morsitans morsitans* with *T. brucei*. However, GlcNAc did not show the inhibitory effect on salivary gland infection rate reported previously for GlcN. Both sugars significantly slowed the movement of the bloodmeal along the midgut. GlcN also significantly increased the size of the bloodmeal taken and fly mortality. The most surprising finding was that GlcNAc stimulated trypanosome growth not only in the midgut, but also *in vitro* in the absence of any factor derived from the fly. Thus our direct comparison of the effects of GlcN and GlcNAc on the trypanosome-tsetse interaction has shown that these sugars impact on trypanosome growth and tsetse physiology in different ways and are not interchangeable as suggested in the literature. The sugars cause multiple effects, not restricted solely to the inhibition of midgut lectins. These findings have implications for current models of tsetse susceptibility to trypanosome infection.

Key words: tsetse, trypanosomes, *Trypanosoma brucei*, D-glucosamine, N-acetyl-glucosamine, parasite-vector interaction.

INTRODUCTION

Trypanosoma brucei undergoes complex cycles of differentiation and multiplication in its vector the tsetse fly, genus *Glossina*. Flies have low susceptibility to infection and resistance mechanisms operate at a number of levels and time-points. Trypanosomes must first establish infection in the tsetse midgut, which involves differentiation from bloodstream to procyclic forms followed by rapid multiplication initially in the gut lumen and then in the ectoperitrophic space. A process of trypanosome attrition is evident in some flies at around 3 days post-infected feed (Dipeolu and Adam, 1974; Welburn, Maudlin and Ellis, 1989; Gibson and Bailey, 2003). Tsetse midgut lectins have been implicated in trypanosome killing, because flies fed lectin-inhibitory sugars generally have higher midgut trypanosome infection rates than control flies (Maudlin and Welburn, 1987, 1988). A lectin specific for D-glucosamine (GlcN) and with lesser affinity for N-acetyl-D-glucosamine (GlcNAc) is present in the tsetse midgut (Ibrahim, Ingram and Molyneux, 1984; Ingram and Molyneux, 1988). Together with the observation that trypanosome infection is associated with the presence of endosymbiotic bacteria in the midgut, these results led to the current model of susceptibility involving the release of lectin-inhibitory sugars by bacterial digestion of chitin in the pupal stage, which neutralize the lectins in the early part of adult life (for reviews see Maudlin, 1991 and Welburn and Maudlin, 1999).

Chitin is primarily a polymer of GlcNAc with GlcN as a minor component (Merzendorfer and Zimoch, 2003). However, most studies on inhibition of tsetse midgut lectins have used GlcN (Maudlin and Welburn, 1987, 1988; Mihok *et al*. 1992, 1994) and only occasionally GlcNAc (Welburn *et al*. 1993; Welburn, Maudlin and Molyneux, 1994). Supporting data for the role played by lectins in the tsetse/trypanosome interaction is still limited since a trypanocidal lectin has not been purified from tsetse to date (Aksoy *et al*. 2003). Also, feeding sugars at high concentration may affect other aspects of fly midgut physiology such as efficiency of midgut digestion (Osir, Imbuga and Onyango, 1993; Mihok *et al*. 1994; Gibson and Bailey, 2003), with knock-on effects on the establishment of trypanosome infection.

In recent work we explored the time-course of events in the early establishment of *T. brucei* in

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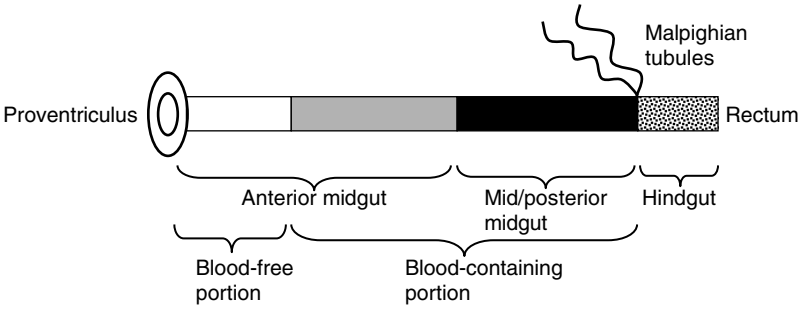

Fig. 1. Diagram of the tsetse mid- and hindgut. The part of the midgut occupied by the bloodmeal is shaded light grey to represent bright red undigested blood, and black to represent blackish-red digested blood.

the tsetse midgut using fluorescent trypanosomes to increase visibility and facilitate accurate counting (Gibson and Bailey, 2003). The aims of this study were to examine whether the addition of GlcN or GlcNAc to the infective bloodmeal perturbs the normal time-course and pattern of events.

MATERIALS AND METHODS

Tsetse flies

Experimental tsetse flies were from the Bristol laboratory colony of *Glossina morsitans morsitans* originally from Zimbabwe. Flies were kept at 25 °C and 70% relative humidity, and fed on sterile defibrinated horse blood via a silicone membrane. Bloodmeals were supplemented as required with GlcN, GlcNAc or glucose using filter-sterilized 1 M stock solutions in distilled water (60 mM final concentration); an equivalent volume of horse serum was added to control bloodmeals. Equal numbers of male and female flies were used for experiments, being given the infected bloodmeal for their first feed 24–48 h post-eclosion. For determination of bloodmeal size, flies were caged individually and weighed before and after the bloodmeal; for other experiments, flies were caged in groups of 5. Flies were kept for up to 5 days after the initial infective feed without further feeding, and thereafter offered maintenance feeds on horse blood as above.

Trypanosomes

Bloodstream forms of *Trypanosoma brucei gambiense* group 2 clone E21 (derived from MHOM/CI/78/TH2 [78E]; (Mehlitz *et al*. 1982)) were used to infect flies. E21 carries a gene for green fluorescent protein (GFP) under control of the procyclin promotor (Biebinger *et al*. 1997; Bingle *et al*. 2001); bloodstream forms become visibly fluorescent 2–4 h after commencement of differentiation to procyclics due to upregulation of the procyclin promotor (Gibson and Bailey, 2003). To standardize fly infection, cryopreserved aliquots of infected mouse blood were thawed and added to horse blood, giving a final concentration of approximately 4×10^6 trypanosomes per ml. In addition, bloodstream and procyclic form *T. b. brucei* J10 (MCRO/ZM/74/J10 [clone 1]) were used for *in vitro* growth experiments.

Dissection

Whole tsetse alimentary tracts, from the proventriculus to the rectum, were dissected in a drop of PBS at various time-points after feeding and arranged lengthways for measurements, as indicated in Fig. 1, using a calibrated graticule under a dissecting microscope. For trypanosome counts, defined sections of the gut (Fig. 1) were placed separately in 50 μl of PBS in a microcentrifuge tube and thoroughly disrupted using a Teflon pestle. The trypanosomes were fixed by adding paraformaldehyde to a final concentration of 0·1% (w/v) in PBS and counted under fluorescence using a haemocytometer. For determination of infection rates in experimentally infected flies, salivary glands and/or midguts were dissected in a drop of PBS, squashed under a coverslip on a slide and scored for presence or absence of trypanosomes.

In vitro *growth of procyclics*

Procyclic trypanosomes were grown at 27 °C in 1 ml wells containing Cunningham's Medium (CM; (Cunningham, 1977)) supplemented as required with GlcN, GlcNAc or glucose using filter-sterilized 1 M stock solutions in distilled water (60 mM final concentration). The contents of each well were thoroughly mixed and a sample counted daily using a haemocytometer. Experiments were carried out on either E21 or J10 bloodstream forms undergoing differentiation to procyclic forms, or established procyclics of *T. brucei* J10.

Statistical analyses

The chi-squared test was used for comparison of infection rates, mortality and percentage flies with trypanosomes in the blood-free anterior midgut, with *post-hoc* chi-squared tests performed on

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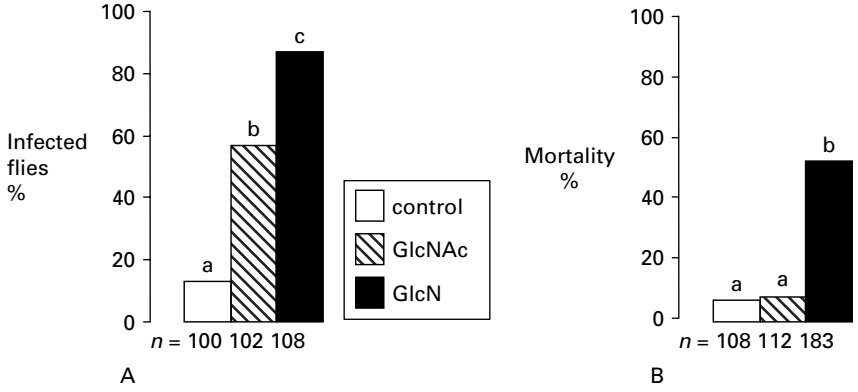

Fig. 2. Effect of N-acetyl-glucosamine (GlcNAc) and D-glucosamine (GlcN) on (A) the frequency of midgut *Trypanosoma brucei* infection, and (B) mortality in *Glossina morsitans morsitans*. Means with different letters are significantly different ($P<0{\cdot}05$; Chi-squared). Number of flies (n) is indicated at the base of each bar.

significant effects (no Bonferonni correction). ANOVA was used for comparison of bloodmeal consumption, counts of trypanosomes *in vivo* and *in vitro*, the percentage midgut full of bloodmeal and the percentage trypanosomes in the anterior midgut. Trypanosome counts were square-root transformed prior to analysis to normalise variances. *Post-hoc* tests (1-way ANOVA or Tukey) were performed on the significant effects. Comparison between bloodmeal weight and infection used a *t*-test and that between bloodmeal weight and percentage mid-gut full of bloodmeal used linear regression. The frequency distribution of trypanosome numbers in flies was analysed for conformity to a normal distribution using a single-sample Kolmogorov-Smirnov test, and a 2-sample test for comparison of distribution between treatments. All data were processed using the statistical package SPSS version 12.0.

RESULTS

Infection rates

We first carried out experiments to confirm that both GlcN and GlcNAc enhance midgut infection rates for *T. brucei* in *G. m. morsitans*, as reported previously (Maudlin and Welburn, 1987; Mihok *et al.* 1992; Welburn *et al.* 1993). Addition of GlcN or GlcNAc to the bloodmeal significantly increased the midgut infection rate compared to control flies dissected 10 days after the infected bloodmeal (GlcN: $X^2=49{\cdot}70$, $P<0{\cdot}001$; GlcNAc: $X^2=27{\cdot}43$, $P<0{\cdot}001$), with the effect of GlcN significantly greater than that of GlcNAc ($X^2=4{\cdot}28$, $P=0{\cdot}04$) (Fig. 2A). A similar effect was observed in flies dissected 5 weeks after the infected bloodmeal (not shown). Addition of glucose to the bloodmeal also significantly increased midgut infection rate compared to the control (glucose, 41·5%, $n=65$; control, 26·1%, $n=69$; $X^2=3{\cdot}85$; D.F.$=1$; $P=0{\cdot}05$), although the effect was significantly lower than for GlcNAc-fed flies used for comparison in the same experiment (GlcNAc, 61·9%, $n=63$; $X^2=3{\cdot}77$, $P=0{\cdot}05$).

In some studies GlcN has been reported to decrease the proportion of midgut infections that mature to salivary gland infections, i.e. transmission index (TI), and this is thought to be a consequence of inhibition of a lectin required for maturation (Maudlin and Welburn, 1988; Welburn *et al.* 1994). We tested whether GlcNAc had a similar effect. In flies dissected 5 weeks after the infective feed, there was no significant difference in TI between GlcNAc-fed flies and controls (TI: GlcNAc 31%, $n=45$; control 29%, $n=21$; $X^2=0{\cdot}07$, $P=0{\cdot}80$). The relatively high number of salivary gland infections in the GlcNAc-fed flies compared to controls reflected the fact that the midgut infection rate in the GlcNAc-fed flies was over twice that of controls (infection rates: GlcNAc, 82%, $n=55$; control, 40%, $n=53$; $X^2=14{\cdot}46$, $P<0{\cdot}001$).

No significant differences were observed between male and female flies in midgut infection rates. Only male flies were used for the salivary gland experiment, since the TI for male flies is significantly higher than that for females (Maudlin, Welburn and Milligan, 1991; Mihok *et al.* 1992).

Fly mortality rates

While fly mortality was similar for control and GlcNAc-fed flies, GlcN resulted in a significant increase in fly deaths compared to controls ($X^2=34{\cdot}89$, $P<0{\cdot}001$) (Fig. 2B). Half of these deaths occurred within the first 2 days of feeding GlcN. There were no significant differences between males and females in mortality rate. Decreased fly survival after feeding GlcN was also observed by Mihok *et al.* (1992) and Osir *et al.* (1993); in the latter study, only GlcN showed this lethal effect among a range of sugars tested, including GlcNac.

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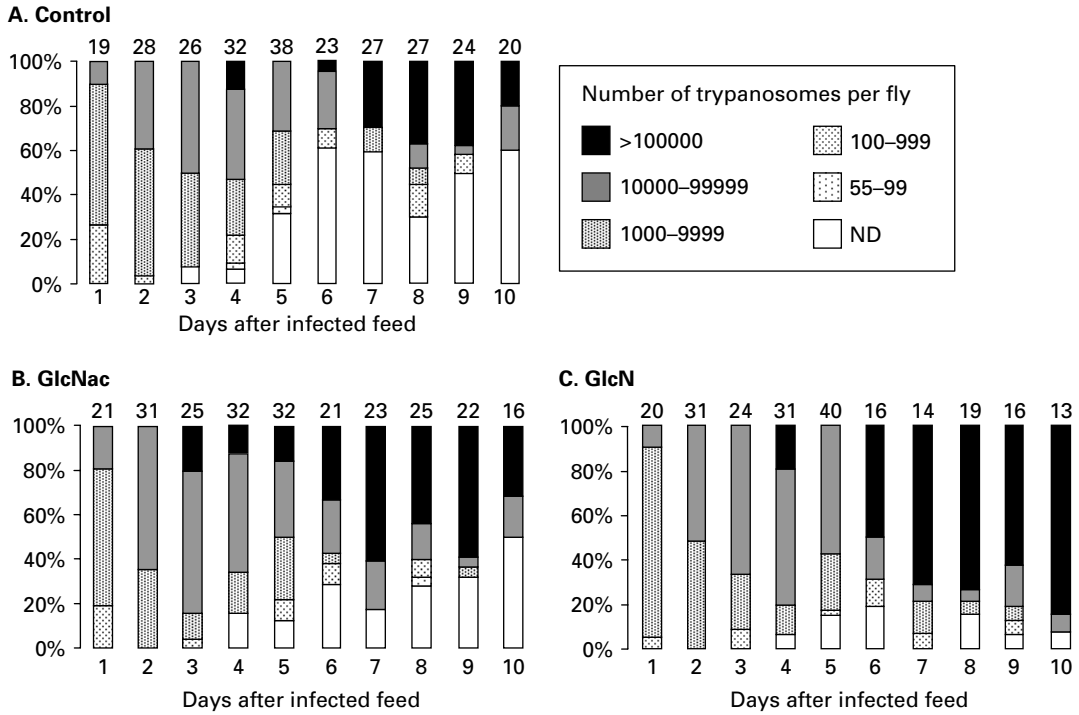

Fig. 3. Effect of N-acetyl-glucosamine (GlcNAc) and D-glucosamine (GlcN) on the frequency distribution of number of trypanosomes in individual flies on days 1–10 following the infected feed. Figures above bars indicate number of flies. N.D. Indicates no detectable trypanosomes i.e. <55.

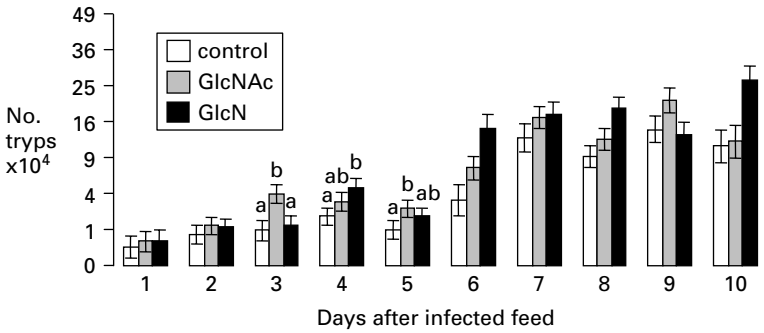

Fig. 4. Effect of N-acetyl-glucosamine (GlcNAc) and D-glucosamine (GlcN) on the total number (back-transformed square root $\pm$ S.E.) of trypanosomes in infected flies on days 1–10 following the infected feed. For each time-point, means with different letters are significantly different ($P<0{\cdot}05$; Tukey *post-hoc*).

Trypanosome numbers in the fly gut

If sugars inhibit trypanocidal lectins, we would expect to observe not only an increase in midgut infection rates in sugar-fed flies compared to controls, but also an increase in the relative numbers of trypanosomes present. We therefore counted numbers of trypanosomes per midgut in groups of flies dissected at 24-h intervals over 10 days. A quantitative experiment of this nature has not been reported before; Mihok *et al*. (1992) counted trypanosome numbers in the midgut at a single 5-week time-point only.

The basic pattern of infection for all treatments was as observed previously (Gibson and Bailey, 2003): trypanosomes multiplied exponentially during the first 24–72 h, but thereafter, trypanosome numbers started to fall in some flies, leading to the complete elimination of infection, while in other flies, trypanosome numbers continued to increase or remained at high levels (Fig. 3). Separation into these 2 categories should produce a bimodal frequency distribution, and indeed divergence from a normal distribution could be clearly demonstrated by day 4 for the control group, using a single sample Kolmogorov-Smirnov test (Table 1A); however, this was not evident for the GlcNac- and GlcN-fed flies. In these groups, particularly GlcN, there were many flies in the highest infection categories and relatively few in the low and non-detectable infection categories (Fig. 3). Comparison of frequency distributions between treatments using a 2-sample

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Table 1. Statistical analysis of frequency distribution of trypanosome numbers per fly

(*P*-values from Kolmogorov-Smirnov test. Asterisks indicate values that show a significant difference from normal distribution.)

(A) Single sample comparison

Day	Control	N-acetyl-glucosamine	D-glucosamine
1	0·648	0·807	0·536
2	0·576	0·913	0·150
3	0·196	0·524	0·784
4	0·028*	0·097	0·101
5	0·005*	0·053	0·168
6	0·004*	0·190	0·422
7	0·000*	0·763	0·946
8	0·007*	0·196	0·989
9	0·007*	0·274	0·785
10	0·035*	0·158	0·194

(B) Two-sample comparison

Day	Control *vs.* N-acetyl-glucosamine	Control vs. D-glucosamine	N-acetyl-glucosamine *vs.* D-glucosamine
1	0·171	0·122	0·620
2	0·070	0·223	0·607
3	0·000*	0·179	0·001*
4	0·270	0·056	0·355
5	0·095	0·051	0·778
6	0·050	0·012*	0·349
7	0·002*	0·003*	0·371
8	0·721	0·040*	0·143
9	0·297	0·011*	0·580
10	0·913	0·002*	0·020*

Kolmogorov-Smirnov test showed significant differences between control and GlcN fed flies on days 6–10 (Table 1B).

Fig. 4 shows the mean number of trypanosomes per fly at each time-point for all treatments; flies without detectable infection were excluded from the analysis. It can be seen that overall the GlcN- and GlcNAc-fed flies had consistently higher numbers of trypanosomes present in the gut than controls. Pooling all time-points (=independent samples), statistical analysis showed significant differences between each sugar and the control treatment, but there was no significant difference between GlcN and GlcNac treatments (control: $4{\cdot}50\times10^4$, CI $4{\cdot}04$–$4{\cdot}99\times10^4$; GlcN: $8{\cdot}28\times10^4$, CI $7{\cdot}70$–$8{\cdot}88\times10^4$; GlcNAc: $7{\cdot}19\times10^4$, CI $6{\cdot}65$–$7{\cdot}75\times10^4$; $F=12{\cdot}98$, $P<0{\cdot}001$, D.F. = 2, 541). However, there was a significant interaction between treatment and day ($F=1{\cdot}78$, $P=0{\cdot}02$, D.F. = 18, 541). In particular, GlcNAc-fed flies had more trypanosomes than controls or GlcN flies on day 3 ($P<0{\cdot}05$) (Fig. 4). Interestingly, a difference between GlcNac and the other treatments on day 3 was also evident from analysis of the frequency distribution in individual flies (Table 1B). GlcNac appeared to be causing a sudden growth spurt in trypanosomes in the midgut and we investigated this further in trypanosomes grown *in vitro* (see below).

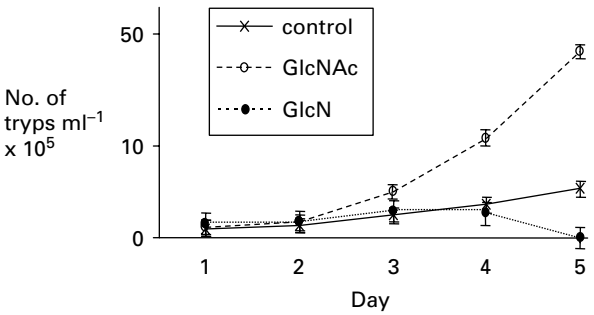

Fig. 5. The effect of sugars on the growth of newly differentiated procyclics of *Trypanosoma brucei* E21 *in vitro* (back-transformed square root ± S.E.). Cultures maintained in Cunningham's medium (control) or with 60 mM (final concentration) N-acetyl-glucosamine (GlcNAc) or D-glucosamine (GlcN) ($n=8$).

Overall, females had significantly more trypanosomes ($7{\cdot}28\times10^4$, CI $6{\cdot}83$–$7{\cdot}75\times10^4$) than males ($5{\cdot}86\times10^4$, 95%, CI $5{\cdot}45$–$6{\cdot}29\times10^4$) ($F=5{\cdot}16$, $P=0{\cdot}02$, D.F. = 1, 541). However, this did not translate into infection rate (see above), corroborating the results of Mihok *et al.* (1992). Greater numbers of trypanosomes may simply be a consequence of the larger bloodmeal taken by females, as parasite numbers were already significantly higher in females 24 h after the infected feed.

Trypanosome distribution in the fly gut

The number of trypanosomes in different sections of the gut (Fig. 1) was counted to determine the relative proportions present. The percentage of trypanosomes in the anterior midgut section differed between treatments and day. During the first 5 days, GlcNAc-fed flies had a significantly higher percentage of trypanosomes in the anterior section than did control or GlcN flies (control: 41·8% ± 2·6%; GlcN: 41·2% ± 2·5%; GlcNAc: 49·0% ± 2·5%) ($F=3{\cdot}042$; D.F. = 2, 367; $P=0{\cdot}049$ ANOVA; $P<0{\cdot}05$ *post-hoc* Tukey). Trypanosomes were often present in the blood-free part of the anterior section (control 46·2%, GlcNAc 67·9%, GlcN 77·8%), with a significantly lower number in controls than GlcNAc ($\chi^2=4{\cdot}24$; D.F. = 1; $P=0{\cdot}039$) or GlcN-fed flies ($\chi^2=7{\cdot}813$; D.F. = 1; $P<0{\cdot}005$). However, between days 6 and 10, this picture reversed and GlcN-fed flies had a significantly lower percentage of trypanosomes in the anterior (54·0% ± 3·8%) than did control or GlcNAc-fed flies (control: 75·1% ± 4·3%; GlcN: 70·2% ± 3·9%) ($F=7{\cdot}709$, D.F. = 2, 189, $P=0{\cdot}001$; $P<0{\cdot}005$ *post-hoc* Tukey). We assume that from 6 days onwards the trypanosomes are mostly, if not all, within the ectoperitrophic space.

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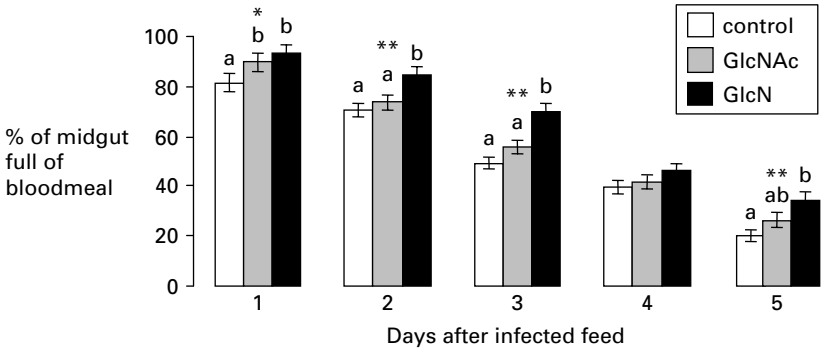

Fig. 6. Effect of N-acetyl-glucosamine (GlcNAc) and D-glucosamine (GlcN) on the percentage (±S.E.) of tsetse midgut full of bloodmeal on days 1–5 following the sugar feed. Means with different letters are significant at * $P<0{\cdot}05$ or ** $P<0{\cdot}005$ (1-way ANOVA, *post-hoc*).

Numbers of trypanosomes in the midgut and hindgut were compared in flies dissected from days 1 to 5. While the average number of trypanosomes in the midgut was $8{\cdot}6\times10^4\pm5{\cdot}8\times10^3$, the average for the hindgut was only $31\pm8{\cdot}3$ with most flies having no detectable trypanosomes in this section. This is consistent with the observation that trypanosomes penetrate the peritrophic matrix (PM) directly (Ellis and Evans, 1977), rather than passing around the open posterior end.

Trypanosome growth in vitro

We tested whether the significant increase in numbers of trypanosomes observed in GlcNAc-fed flies on day 3 compared to control and GlcN-fed flies (Fig. 4) could be reproduced *in vitro*. Samples from the infective bloodmeals fed to flies at time zero were cultured *in vitro*, allowing the bloodstream form trypanosomes (E21) to differentiate into procyclics and multiply. The starting concentrations of GlcN and GlcNAc were 60 mM and these levels were maintained by subculturing in CM supplemented with 60 mM sugar (Fig. 5). No difference between treatments was detected in ability to differentiate to procyclics and growth on days 1 and 2, but from day 3 trypanosomes grown in 60 mM GlcNAc multiplied faster than in control or 60 mM GlcN wells, and by 5 days had grown to a significantly higher density than controls ($F=33{\cdot}441$, D.F. = 2, 17, $P<0{\cdot}001$ ANOVA; $P<0{\cdot}001$ *post-hoc* Tukey). In contrast, 60 mM GlcN appeared to have an inhibitory or toxic effect on trypanosome growth *in vitro* and cultures died out by day 5. Similar results were produced for differentiating bloodstream forms and established procyclics of *T. b. brucei* J10 (not shown).

The toxic effect of GlcN may have been due to its acidity: the pH of CM fell from 7·6 to 6·9 on addition of 60 mM GlcN, and similarly the pH of horse blood fell from 7·6 to 7·0; addition of 60 mM GlcNAc resulted in no change to the pH of CM and a drop from pH 7·6 to 7·4 for horse blood. As no attempt has been made to neutralize the acidity of GlcN in previous studies, we did not adjust the pH in our protocols.

Effect of sugars on bloodmeal size and transit of bloodmeal along midgut

Increased infection rates could result from sugar-fed flies taking in more trypanosomes by imbibing a larger infected bloodmeal and indeed we found that the addition of GlcN to the bloodmeal significantly increased the amount of blood consumed by flies ($F=6{\cdot}10$, $P=0{\cdot}003$, D.F. = 2, 315). Flies took an average bloodmeal of $12{\cdot}9\pm0{\cdot}6$ mg ($n=108$), which increased to $15{\cdot}6\pm0{\cdot}6$ ($n=109$) when GlcN was added to the feed. The addition of GlcNAc also resulted in an increase in size of the bloodmeal, but this was not significant ($14{\cdot}6\pm0{\cdot}6$ mg, $n=104$; $P=0{\cdot}08$). The average bloodmeal taken by females, $15{\cdot}1\pm0{\cdot}5$ mg ($n=134$), was significantly larger than that by males, $13{\cdot}7\pm0{\cdot}4$ mg ($n=187$) ($F=4{\cdot}80$, $P=0{\cdot}029$, D.F. = 1, 315), with no significant interaction between sex and sugar addition.

In preliminary studies it was noticed that the bloodmeal appeared to move more slowly along the midgut when GlcN was added. To investigate this further, we compared the rates of midgut emptying in sugar-fed and control flies at 24-h intervals after feeding. The length of midgut filled with blood relative to the total length (Fig. 1) significantly decreased each day ($F=189{\cdot}4$, $P<0{\cdot}001$, D.F. = 1, 274), with females having a significantly fuller gut on average ($60{\cdot}8\pm1{\cdot}1\%$, $n=154$) than males ($56{\cdot}0\pm1{\cdot}1\%$, $n=150$) ($F=9{\cdot}78$, $P=0{\cdot}002$, D.F. = 1, 274). The addition of either sugar caused an overall significant ($P<0{\cdot}005$, Tukey *post-hoc*) slowing of transit of the bloodmeal along the midgut: averaging over all days examined, percentage midgut full of blood was $65{\cdot}7\%\pm1{\cdot}4\%$ for GlcN and $57{\cdot}4\%\pm1{\cdot}3\%$ for GlcNAc compared to $52{\cdot}2\%\pm1{\cdot}3\%$ for control flies. On a daily basis, GlcN-fed flies had significantly the most full midguts, and control the least, on all but day 4 (Fig. 6).

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Since addition of GlcN significantly increased bloodmeal size (see above), this might lead to slower bloodmeal transit along the midgut. However, there was no correlation between the amount of blood consumed by flies (with/without GlcN added) and the percentage of their midgut full (GlcN $R^2 = 0{\cdot}065$, $P = 0{\cdot}19$; control $R^2 = 0{\cdot}007$, $P = 0{\cdot}57$). This also held for female flies, which consumed significantly more blood than males and had a greater percentage of their midgut full of bloodmeal yet showed no correlation between these two amounts. Therefore, it appears that females have slower digestion than males, which agrees with the findings of Langley (1967).

DISCUSSION

We have found that GlcN and GlcNAc impact on trypanosome growth and tsetse physiology in multiple and different ways. Thus the effect of these sugars is more complex than simple inhibition of midgut lectins (Maudlin and Welburn, 1987; Welburn *et al*. 1993) and the two sugars are not interchangeable as frequently suggested in the literature (Maudlin and Welburn, 1994; Welburn and Maudlin, 1999; Dale and Welburn, 2001). While both sugars enhanced midgut infection rates as shown previously (Maudlin and Welburn, 1987, 1988; Mihok *et al*. 1992; Welburn *et al*. 1993, 1994), direct comparison here showed that GlcNAc had significantly less effect than GlcN. Both sugars significantly slowed the transit of the bloodmeal along the midgut and increased food consumption, although this latter effect was only significant for GlcN. In addition, GlcN caused high mortality in experimental flies and its acidity lowered the pH of the bloodmeal.

These effects, including increased trypanosome infection rates, can be interpreted as direct effects of the sugars on fly physiology, for example by inhibition of digestive enzymes or trypanocidal lectins. However, we also made the surprising discovery that GlcNAc is a direct growth stimulant for procyclic trypanosomes *in vitro* in the absence of any factor from the fly, suggesting that GlcNAc enhances midgut infection rates by a direct effect on trypanosome growth as well as, or possibly instead of, any inhibitory effect on fly defence. Presumably GlcNAc, as the major building block of chitin, is readily available within the fly, and the use of GlcNAc by trypanosomes could be an adaptation for growth in the fly midgut. We are currently investigating this phenomenon in more detail.

In the lectin hypothesis of tsetse susceptibility to trypanosome infection, GlcN and GlcNAc inhibit a trypanocidal lectin(s), thus promoting trypanosome establishment, but also inhibit maturation, because the same lectin(s) is purported to be a trigger for maturation of *T. brucei* (Welburn and Maudlin, 1999; Maudlin and Welburn, 1988, 1994). However, other studies have failed to demonstrate any effect of GlcN or GlcNac on maturation (Mihok *et al*. 1992; Welburn *et al*. 1993). Likewise, here we found no difference in the transmission index between control and GlcNAc-fed flies; the higher salivary gland infection rate with GlcNAc is due to enhanced midgut infection rates rather than more efficient maturation. GlcNAc, with its lower toxicity to flies than GlcN, is therefore preferable to GlcN for increasing infection rates in experimental flies.

The slowing of transit of the bloodmeal along the midgut, significantly greater with GlcN, may be a result of inhibition of the protease trypsin. Osir *et al*. (1993) showed that trypsin in midgut homogenates of *G. m. morsitans* is specifically and competitively inhibited by GlcN, but not by GlcNAc. However, the relationship between protease levels and trypanosome infection in tsetse is not clear (Mihok *et al*. 1994). The position of the infected bloodmeal within the midgut may also have a bearing on the success of infection. The anterior section is considered to be devoid of digestive enzymes and trypanocidal lectins (Aksoy *et al*. 2003), thus potentially providing a more favourable environment for trypanosomes. Slowed transit of the bloodmeal along the gut would prolong the sojourn of trypanosomes in the anterior section, which would be particularly advantageous if parasites enter the ectoperitrophic space by direct penetration of the peritrophic matrix (PM) rather than passing around the open posterior end. The latter route is discredited by the extremely low numbers of trypanosomes found in the hindgut section for the first 5 days after the infected feed in this study and that of Gibson and Bailey (2003), and by the experimental evidence that trypanosomes penetrate the PM directly (Ellis and Evans, 1977).

Addition of sugars to the bloodmeal did not change the normal time-course of infection: trypanosome attrition began at day 3 and by day 6 most flies appear to either have lost or established a midgut infection. However, we noticed a few flies in all treatments that had unusually low numbers of parasites on days 6–10, and this may indicate continued action of the tsetse immune response in limiting infection (Aksoy *et al*. 2003).

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