BIOLOGY OF *GIARDIA LAMBLIA*

Detection of *N*-Acetyl-D-Glucosamine as the Only Surface Saccharide Moiety and Identification of Two Distinct Subsets of Trophozoites by Lectin Binding

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The process of differentiation of parasitic protozoa from one developmental stage to the next is complex and poorly understood. Knowledge of the mechanisms underlying this process is of basic biological importance and of potential value in designing strategies directed toward interruption of the life cycle of these parasites, in order to control spread of the disease they cause. One such parasite is *Giardia lamblia* which is a common cause of diarrheal disease around the world (1). This organism exists in two developmental forms: trophozoite and cyst. The trophozoite is the motile flagellated form that colonizes the proximal small intestine and causes disease; and the cyst is the infective form, excreted via the feces into the external environment where it survives for prolonged periods until ingested by a susceptible host. Infection is initiated by ingestion of cysts, which undergo excystation upon exposure to low gastric pH to yield the trophozoite form. Differentiation of trophozoite to cyst occurs by an unknown mechanism. However, a major structural difference between trophozoite and cyst is the presence of a thick wall surrounding the cyst (2).

Synthesis of the cyst wall therefore appears to be an essential step in the process of encystation. Information regarding the biochemical composition of both the cyst wall and the trophozoite surface membrane is a fundamental prerequisite to understanding the mechanisms underlying encystation. In a recent study, we analyzed surface carbohydrate moieties of *Giardia* cysts and identified the polysaccharide chitin as a major structural component of the cyst wall (3). This was based on the finding that affinity-purified chitinase specifically destroyed the cyst wall, as shown by EM, as well as by direct lectin-binding studies. Of 13 lectins with varying sugar specificities only the *N*-acetyl-D-glucosamine (D-GlcNAc)[1]–

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[1] *Abbreviations used in this paper:* D-GlcNAc, *N*-acetyl-D-glucosamine; LEA, *Lycopersicon esculentum* (tomato) agglutinin; S-WGA, succinylated wheat germ agglutinin; WGA, wheat germ agglutinin.

specific lectins wheat germ agglutinin (WGA), succinylated wheat germ agglutinin (S-WGA), and *Lycopersicon esculentum* (tomato) agglutinin (LEA) bound specifically to cyst walls. The binding of WGA was completely abolished by pretreating cysts with purified chitinase, confirming the presence of chitin in the cyst wall.

The present study was undertaken to analyze the carbohydrate residues of the trophozoite surface membrane, and in particular to determine whether chitin or oligomeric D-GlcNAc is also present on the trophozoite surface. Using the techniques of lectin binding and glycosidase digestion we find that, as was the case with the cyst wall, D-GlcNAc is the only detectable carbohydrate moiety on the trophozoite surface. In contrast to the cyst wall, the trophozoite D-GlcNAc residues are resistant to chitinase treatment, which has no effect on WGA binding. However, treatment of the trophozoites with *N*-acetyl-β-D-glucosaminidase results in abolition of WGA binding suggesting that the lectin is reacting with terminal β-linked D-GlcNAc residues. These residues are present in a number of trophozoite surface glycoproteins. A striking finding is the identification of two distinct subsets of trophozoites, one of which reacts with WGA and another which does not.

Materials and Methods

Materials. Affinity-purified chitinase (from *Serratia marcescens*) was a gift from Dr. E. Cabib, National Institutes of Health, Bethesda, MD. *N*-Acetyl-β-D-glucosaminidase (from jack beans), chitinase (from *Streptomyces griseus*), lysozyme (from chicken egg white), 3,3′-diaminobenzidine tetrahydrochloride, sugars, and FITC-conjugated lectins were obtained from Sigma Chemical Co., St. Louis, MO. LEA was purified (4) and conjugated to FITC (3) as described earlier. Neuraminidase (*Vibrio cholera*) was from Calbiochem-Behring Corp., La Jolla, CA. Biotinylated lectins and the Vectastain ABC kit were obtained from Vector Laboratories, Inc., Burlingame, CA. 4,000 Ci/mmol Bolton-Hunter reagent (^{125}I[*N*-hydroxysuccinimidyl *p*-hydroxyphenylproprionate] in benzene) was from New England Nuclear, Boston, MA.

Parasites. Trophozoites of the Portland-I strain of *Giardia lamblia* (originally provided by Dr. L. S. Diamond, National Institutes of Health, Bethesda, MD) were maintained in axenic culture in TYI-S-33 medium as described earlier (5). Parasites in late log phase were harvested by chilling on ice for 10 min, centrifuged at 800 *g* for 5 min, and washed three times in PBS (20 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride).

FITC Lectin Binding Studies of Live Trophozoites. A total of 10^6 washed trophozoites were incubated with 10 μg/ml of the following FITC-labeled lectins in PBS at 23°C for 30 min; WGA, Con A, peanut agglutinin, phytohemagglutinin, LEA, soybean agglutinin, *Lens culinaris* agglutinin, and *Helix pomatia* agglutinin. They were then washed three times in PBS, resuspended in 100 μl of PBS, and examined with an ICM microscope (Carl Zeiss, Inc., Thornwood, NY) with phase contrast and fluorescence optics. The inhibition of FITC-lectin binding to trophozoites was studied by incubating the FITC-lectin with the following specific sugar inhibitors for 30 min before incubation with the trophozoites: 200 mM D-GlcNAc and 1 mM *N,N′,N″*-triacetylchitotriose for WGA and LEA; 200 mM *N*-acetyl-D-galactosamine for phytohemagglutinin, soybean agglutinin, and *Helix pomatia* agglutinin; 200 mM D-mannose for Con A and *Lens culinaris* agglutinin; and 200 mM D-galactose for peanut agglutinin.

FITC Lectin Binding Studies of Fixed Trophozoites. 25 μl of PBS containing 10^5 trophozoites was placed on a glass slide, air dried, and fixed in methanol for 10 min at 23°C. Slides were washed in PBS for 10 min and then incubated with 25 μl of FITC-conjugated lectin at a concentration of 10 μg/ml in PBS for 30 min at 23°C. The slides were then

washed three times in PBS for 5 min each time, mounted in PBS containing glycerol (10% vol/vol), and observed under the microscope. Inhibition studies were performed by preincubating the FITC-conjugated lectin with its specific sugar inhibitor as described above.

Cytochemical Visualization of Lectin Binding. A total of 10^5 washed trophozoites were placed on a glass slide, air dried, and fixed in methanol as described above. The slides were then processed for cytochemical visualization of lectin binding using the avidin biotin peroxidase technique as described earlier (6). The following biotinylated lectins were used at a concentration of 10 μg/ml; WGA, S-WGA, peanut agglutinin, Con A, *Ricinus communis* agglutinin I, *Dolichos biflorus* agglutinin, soybean agglutinin, *Ulex europaeus* agglutinin, and *Griffonia simplicifolia* agglutinin I.

Glycosidase Treatment of Fixed Trophozoites. 10^5 trophozoites were placed on a glass slide, air dried, fixed in methanol as described above, and incubated in a moist chamber with the following glycosidases: *N*-acetyl-β-D-glucosaminidase, 2.5 mU in 0.1 M sodium acetate, pH 5.0, containing 150 mM sodium chloride for 48 h at 23°C; muramidase (*N*-acetyl-muramyl hydrolase) 40 U in PBS for 30 min at 23°C; neuraminidase 25 mU in PBS for 30 min at 37°C; chitinase (*Streptomyces griseus*), 75 mU in PBS for 48 h at 23°C; affinity-purified chitinase (*Serratia marcescens*) 45 mU in PBS for 48 h at 23°C.

[^{125}I]WGA-binding Studies. WGA was labeled with ^{125}I Bolton-Hunter reagent (7), in the presence of 0.1 M D-GlcNAc to protect the sugar-binding sites. Free ^{125}I was removed by gel filtration on a Sephadex G 25 column. The minimum agglutinating dose of [^{125}I] WGA was found to be the same as that of the unlabeled lectin as determined by hemagglutination with rabbit erythrocytes. Specific activity ranged from 0.5 to 2×10^4 cpm per μg of protein; 71–76% of the radioactive material was precipitable by 10% TCA.

Washed trophozoites were fixed in 1% glutaraldehyde in PBS for 1 h at 23°C, incubated with 0.1 M glycine HCl, pH 5.0, for 30 min at 23°C, and washed three times in 0.1 M sodium acetate, pH 5.0, containing 150 mM sodium chloride. 5×10^8 glutaraldehyde-fixed trophozoites were treated with 2.5 U of *N*-acetyl-β-D-glucosaminidase in the same buffer for 48 h at 23°C. As a control the same number of trophozoites were treated with buffer alone under the same conditions.

[^{125}I]WGA-binding studies were performed on live or glutaraldehyde-fixed trophozoites as follows: 10^7 control or *N*-acetyl-β-D-glucosaminidase-treated trophozoites were incubated with increasing concentrations of [^{125}I]WGA in 100 μl of PBS containing 2 mg/ml of BSA for 1 h at 4°C and then washed three times in PBS containing 2 mg/ml of BSA. The amount of [^{125}I]WGA bound to the trophozoites was determined using a γ counter (Beckman Instruments, Inc., Palo Alto, CA). Appropriate corrections were made for nonspecific binding to the tubes, which accounted for 3–5% of the total counts bound. Specificity of binding was ascertained by performing the binding studies in parallel in the presence of 200 mM D-GlcNAc for each concentration of WGA used and subtracting the counts bound in the presence of the sugar from those bound in its absence.

Electron Microscopic Studies of WGA Binding to G. Lamblia Trophozoites. Aliquots of glutaraldehyde-fixed trophozoites (10^6 each) were treated with *N*-acetyl-β-D-glucosaminidase (25 mU for 48 h at 23°C), or with purified chitinase (90 mU for 48 h at 23°C), or with PBS alone as a control. After the indicated times the trophozoites were washed thrice with PBS and fixed in Trumps fixative (1% glutaraldehyde, 4% formalin). They were then incubated with biotinylated WGA and further processed for cytochemistry using the avidin–biotin–peroxidase complex technique as described earlier (6). Trophozoites were then postfixed in 2% aqueous osmium tetroxide, dehydrated in graded ethanol, stained en bloc with 5% uranyl acetate, and embedded in Epon 812. Sections were viewed with a Philips 300 electron microscope.

Flow Microfluorometric Analysis of WGA Binding. For analysis of WGA binding by flow microfluorometry, 10^6 live trophozoites were incubated with 1 μg/ml of FITC-WGA in PBS with 0.02% sodium azide for 1 h at 4°C. The cells were then washed thrice in PBS with 0.02% sodium azide and analyzed in an EPICS 541, five-parameter cell sorter equipped with a 2-W 90-2 coherent laser (Coulter Electronics, Inc., Hialeah, FL). To

determine specificity of binding the FITC-WGA was preincubated with 1 mg/ml of *N,N',N"*-triacetylchitotriose before incubation with the trophozoites.

Surface Labeling of G. lamblia Trophozoites. *G. lamblia* trophozoites were labeled with ^{125}I Bolton-Hunter reagent as described earlier (8). Briefly, 100 μCi of ^{125}I-labeled Bolton-Hunter reagent in benzene were placed in a glass tube and evaporated with a stream of nitrogen. 10^8 washed trophozoites were then added to the tube and incubated for 10 min at 4°C. The labeled trophozoites were then washed thrice in PBS to remove the free ^{125}I and adjusted to a concentration of 10^8/ml in PBS. More than 95% of the trophozoites were viable as assessed by motility at the end of the labeling period.

Analysis of Carbohydrate Residues on G. lamblia Surface Glycoproteins by Western Blotting Using Lectins as Probes. Labeled trophozoites were lysed with 0.5% Triton X-100 in PBS containing 2 mM PMSF and centrifuged at 600 *g* to pellet nuclei. The supernatant was boiled with sample buffer and subjected to electrophoresis on a 10% SDS–polyacrylamide gel (9). Separated proteins were electrotransferred to nitrocellulose as described earlier (10). The nitrocellulose filter was blocked with 0.05% Tween 20 in PBS for 1 h at 37°C and then probed with the following biotinylated lectins at a concentration of 1 μg/ml in PBS containing 0.05% Tween-20 for 1 h at 23°C: WGA, S-WGA, Con A, phytohemagglutinin, and peanut agglutinin. The filter was then washed three times in PBS containing 0.05% Tween-20 and incubated with an avidin–biotin–peroxidase complex for 1 h at 23°C. After washing the filter three times with PBS, lectin-binding proteins were visualized by incubating the filter in 0.05 M Tris HCl, pH 7.5, containing 0.5 mg/ml of the substrate 3,3'diamino benzidine tetrahydrochloride and 0.001% hydrogen peroxide. The filter was then exposed to Kodak XRP film for 18 h at −70°C and the film developed using a Kodak X-omat processor. To ascertain specificity of binding, the biotinylated lectins were incubated with their respective specific sugar haptens at a concentration of 200 mM for 30 min before incubation with the nitrocellulose filter and the incubation was then continued in the presence of the sugar.

Results

D-GlcNAc Is the Only Detectable Saccharide Moiety on the Trophozoite Surface. Using a panel of 13 lectins (Table I) with varying sugar specificities to study the carbohydrate residues of *Giardia lamblia* trophozoites, the only detectable saccharide moiety on the trophozoite plasma membrane we found was D-GlcNAc. This conclusion is based on the exclusive binding of the D-GlcNAc-specific lectins WGA, S-WGA, and LEA to the trophozoite surface, demonstrated by fluorescence microscopy (Fig. 1*a*) or by cytochemistry using the avidin–biotin–peroxidase method of staining (Fig. 2). The binding of these lectins was mediated by their sugar-binding sites since it could be completely inhibited by preincubating the lectins with the specific hapten *N,N',N"*-triacetylchitotriose (Fig. 1*b*). Lectins with specificity for α- or β-linked D-galactose, α- or β-linked *N*-acetyl-D-galactosamine, α-linked D-mannose, α-linked D-glucose, and α-linked *L*-fucose did not react with the trophozoites. The sugar specificity of WGA includes terminal sialic acid as well as D-GlcNAc and its β 1 → 4–linked oligomers (11). However, the carbohydrate residues on the trophozoite surface that were recognized by WGA appeared to contain β 1 → 4–linked D-GlcNAc rather than sialic acid, since S-WGA which binds only to D-GlcNAc and not to sialic acid (11), reacted with the trophozoite in a manner indistinguishable from that of the unsuccinylated lectin (Table I). Further evidence that WGA receptors on the trophozoite contained D-GlcNAc, and not sialic acid, was provided by the finding that extensive neuraminidase digestion did not effect WGA binding (Table II) and also by the results obtained with LEA (Table I), since oligomers of β 1 → 4–

TABLE I
Lectin Binding to G. lamblia Trophozoites

Lectin	Major sugar specificity	Binding assessed by:	
		Fluorescence	Cytochemistry
WGA	D-GlcNAc β1→4; NeuNAc α1→	+	+
S-WGA	D-GlcNAc β1→4	+	+
LEA	(D-GlcNAc)$_3$	+	ND
PNA	D-Gal β1→3 D-GalNAc	−	−
PHA	D-GalNAc	−	−
DBA	D-GalNAc α1→	ND	−
HPA	D-GalNAc α1→ D-GlcNAc α1→	−	−
SBA	D-GalNAc α or β1→ D-Gal α or β1→	−	−
RCA I	D-Gal α or β1→	ND	−
GS I	D-Gal α1→	ND	−
Con A	D-Man α1→; D-Glc α1→	−	−
LCA	D-Man α1→; D-Glc α1→	−	ND
UEA I	L-Fuc α1→	ND	−

Binding of lectins to trophozoites was assessed by fluorescence using FITC-conjugated lectins or cytochemistry using the avidin–biotin–peroxidase technique as described in Materials and Methods. +, specific binding of a particular lectin; −, no binding; ND, not done; PNA, peanut agglutinin; PHA, phytohemagglutinin; DBA, *Dolichos biflorus* agglutinin; HPA, *Helix pomatia* agglutinin; SBA, soybean agglutinin; RCA I, *Ricinus communis* agglutinin I; GSI, *Griffonia simplicifolia* agglutinin I; Con A, concanavalin A; LCA, *Lens culinaris* agglutinin; UEA I, *Ulex Europeaus* agglutinin I; NeuNAc, N-acetyl-neuraminic (sialic) acid; D-Gal, D-galactose; D-GalNAc, N-acetyl-D-galactosamine; D-Man, D-mannose; D-Glc, D-glucose; L-Fuc, L-fucose.

linked D-GlcNAc are the only saccharides known to react with this lectin (4). There was no difference in lectin binding between live or fixed trophozoites.

Identification of Distinct Subsets of G. lamblia Trophozoites by WGA Binding. Analysis of WGA binding to trophozoites by fluorescence microscopy, cytochemistry (Fig. 2), and EM revealed distinct subsets of WGA-positive and WGA-negative cells. The number of trophozoites that were WGA positive increased with increasing concentrations of WGA (Fig. 3). However at WGA concentrations between 1 and 10 μg/ml, only 80–85% of cells were consistently labeled with this lectin. There were no differences in viability or morphology between WGA-positive and WGA-negative cells. This finding of differences in WGA reactivity of trophozoites was confirmed by flow microfluorometry. As shown in Fig. 4*a*, two distinct subsets of WGA-positive and WGA-negative cells could be identified. Fluorescence-activated cell sorting of a heterogeneous population of trophozoites resulted in separation of the two subsets, one of which represented 80% of the total number of cells and was WGA positive, and another which was WGA negative as observed by subsequent fluorescence microscopy (data not shown). The specificity of FITC-WGA binding was confirmed by inhibition studies using the specific sugar hapten *N,N′,N″*-triacetylchitotriose (Fig. 4*b*).

Chitin Is Not Present on the Surface of Trophozoites. To identify the nature of the WGA receptors on the trophozoite surface, the cells were treated with glycosidases of known sugar specificity and then assessed for their ability to react

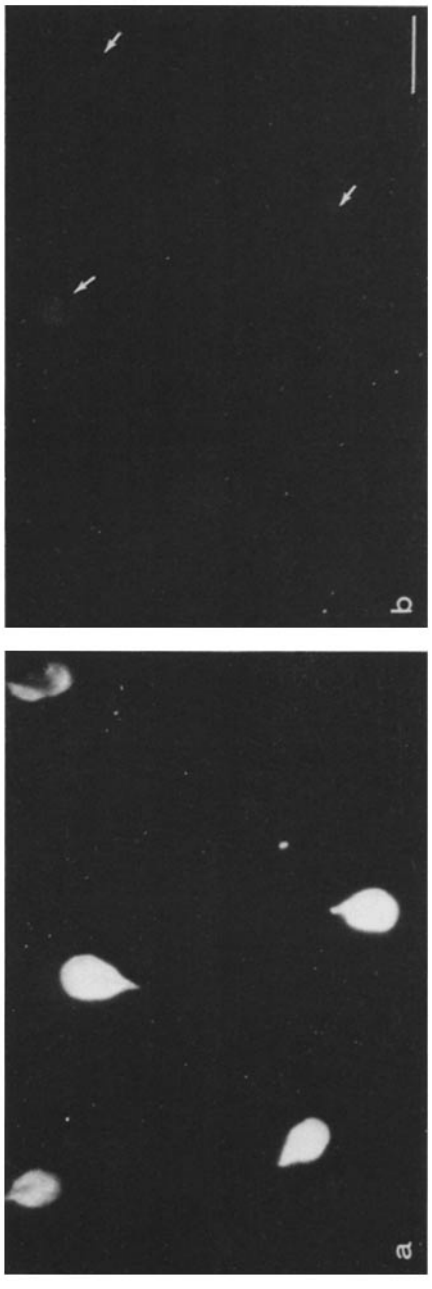

FIGURE 1. Specific binding of FITC-WGA to *G. lamblia* trophozoites. (*a*) Trophozoites were fixed in methanol and stained with FITC-WGA as described in Materials and Methods. (*b*) FITC-WGA binding to trophozoites was inhibited by preincubation with the specific-sugar hapten *N,N′,N″*-triacetylchitotriose. Bar, 6.6 μm.

TABLE II

Effect of Glycosidase Treatment on WGA Binding to G. lamblia Trophozoites

Glycosidase	Specificity	WGA binding
Neuraminidase (*V. cholera*)	terminal NeuNAc $\alpha\rightarrow$	+
Muramidase (chicken egg white)	D-GlcNAc $\beta 1\rightarrow 4$ MurNAc	+
Chitinase (*S. griseus*)	D-GlcNAc $\beta 1\rightarrow 4$ D-GlcNAc	+
Chitinase (*S. marcescens*)	D-GlcNAc $\beta 1\rightarrow 4$ D-GlcNAc	+
N-Acetyl-β-Glucosaminidase (from Jack beans)	terminal D-GlcNAc $\beta\rightarrow$	−

Fixed trophozoites were treated with various glycosidases as described in Materials and Methods and then assessed for WGA binding by fluorescence microscopy and cytochemistry using the avidin–biotin–peroxidase technique. +, specific binding; −, no binding; NeuNAC, *N*-acetyl-neuraminic acid; MurNAc, *N*-acetyl-muramic acid.

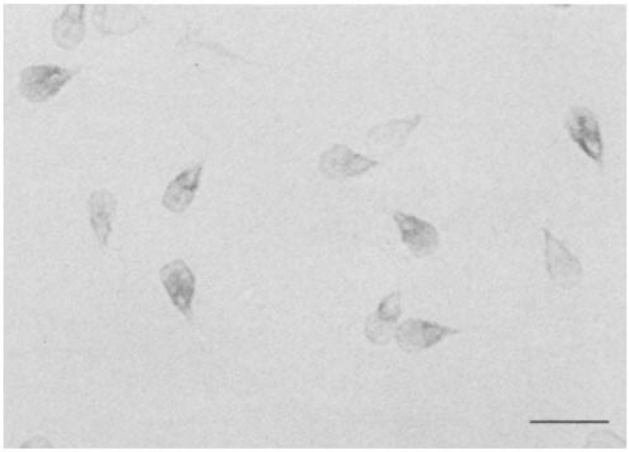

FIGURE 2. Identification of two subsets of trophozoites by WGA binding. Fixed trophozoites were stained with WGA by the avidin–biotin–peroxidase technique as described in Materials and Methods. Most of the trophozoites are stained with WGA, but a proportion are unstained. Bar, 10 μm.

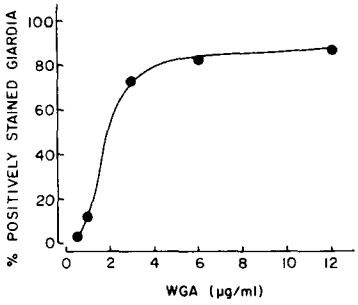

FIGURE 3. Proportion of trophozoites labeled with increasing concentrations of WGA. Fixed trophozoites were labeled with increasing concentrations of WGA using the avidin–biotin–peroxidase technique as described in Materials and Methods and the number of positively stained trophozoites were counted. The results are expressed as a percentage of the total number of cells.

with WGA. In contrast to the results obtained with cysts, chitin does not appear to be the receptor for WGA on the trophozoite surface, since neither commercially available chitinase from *S. griseus* nor affinity-purified chitinase from *S. marcescens* had any effect on WGA binding to the trophozoite (Table II; Fig. 6, *e* and *f*). However *N*-acetyl-β-D-glucosaminidase, which cleaves terminal β-linked D-GlcNAc, completely abolished WGA binding to the trophozoite surface mem-

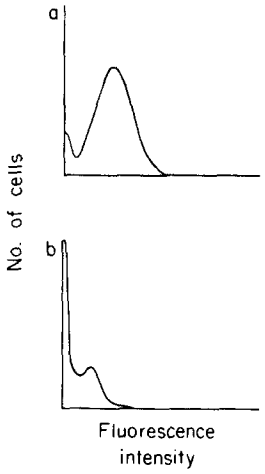

FIGURE 4. Identification of two subsets of trophozoites by flow cytometry. (*a*) Live trophozoites were stained with FITC-WGA and analyzed by flow microfluorometry as described in Materials and Methods. Two distinct peaks of fluorescence intensity can be seen which correspond to the two subsets of trophozoites, one of which binds WGA and the other which does not. (*b*) Binding of FITC-WGA to trophozoites was inhibited by the specific sugar *N,N',N"*-triacetylchitotriose, resulting in an increase in the number of trophozoites which were not bound by the lectin.

brane as shown by fluorescence microscopy, cytochemistry (Fig. 5*b*), and EM (Fig. 6, *c* and *d*). This finding was confirmed by [^{125}I]WGA-binding studies which showed that *N*-acetyl-β-D-glucosaminidase treatment of trophozoites markedly reduced binding of the iodinated lectin (Fig. 7). Other glycosidases such as neuraminidase and muramidase had no effect on WGA binding to the trophozoites, confirming that the WGA was not binding to sialic acid or murein, respectively (Table II). *N*-Acetyl-β-D-glucosaminidase treatment did not result in any structural alterations to the trophozoite surface as shown by EM (Fig. 6, *c* and *d*), in contrast to chitinase treatment of the cyst which resulted in destruction of the cyst wall (3). These results indicate that the WGA receptors on the trophozoite surface contain terminal β-linked D-GlcNAc residues and that chitin is not present in the trophozoite form of the parasite.

[^{125}I]WGA-binding Studies. To quantitate the interaction of WGA with the trophozoite surface membranes, we performed binding studies using ^{125}I-labeled WGA. As shown in Fig. 6*a*, increasing concentrations of [^{125}I]WGA resulted in binding of the labeled lectin in a saturable fashion. There was no difference in binding of [^{125}I]WGA to live or fixed trophozoites. Treatment of fixed trophozoites with *N*-acetyl-β-D-glucosaminidase resulted in a marked reduction in [^{125}I] WGA binding, confirming that WGA was reacting with terminal β-linked D-GlcNAc residues on the surface membrane. When the binding data were plotted according to the method of Steck and Wallach (12), as shown in Fig. 8, the number of lectin-binding sites was determined to be 3×10^5/cell with an apparent K_a of 4×10^6 M^{-1}.

Identification of D-GlcNAc–containing Glycoproteins on Trophozoite Surface Membranes. To ascertain whether the D-GlcNAc residues recognized by WGA were present on trophozoite surface membrane glycoproteins, proteins from surface-labeled trophozoites were separated by SDS PAGE, transferred to nitrocellulose, probed with biotinylated lectins, and identified using an avidin–biotin–peroxidase complex as described in Materials and Methods. Surface-labeled proteins were then identified by autoradiography of the nitrocellulose filter and matched

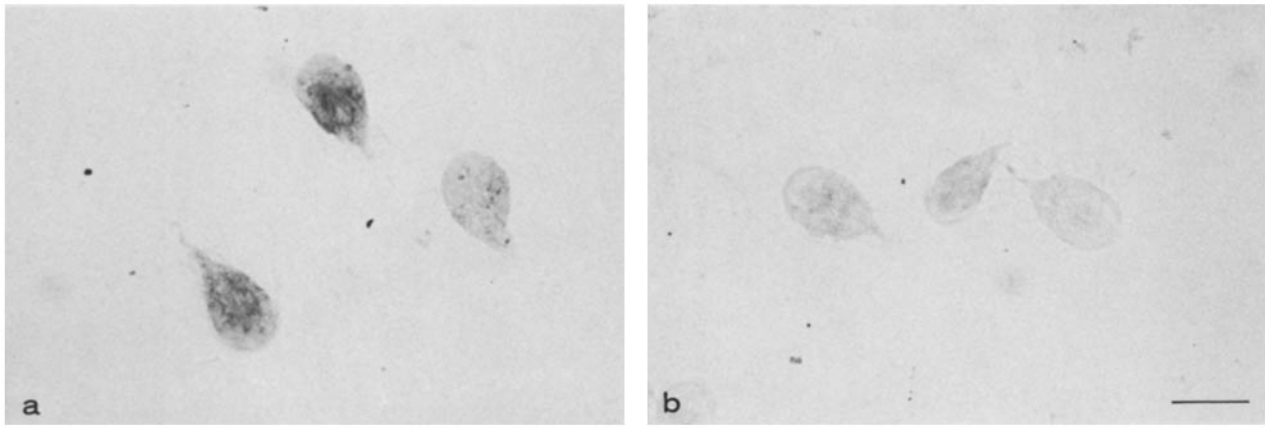

FIGURE 5. Abolition of WGA binding to trophozoites by treatment with *N*-acetyl-β-D-glucosaminidase. Fixed trophozoites were treated with PBS alone as a control (*a*) or with *N*-acetyl-β-D-glucosaminidase (*b*) and then assessed for their ability to bind WGA by the avidin–biotin–peroxidase technique as described in Materials and Methods. Bar, 5 μm.

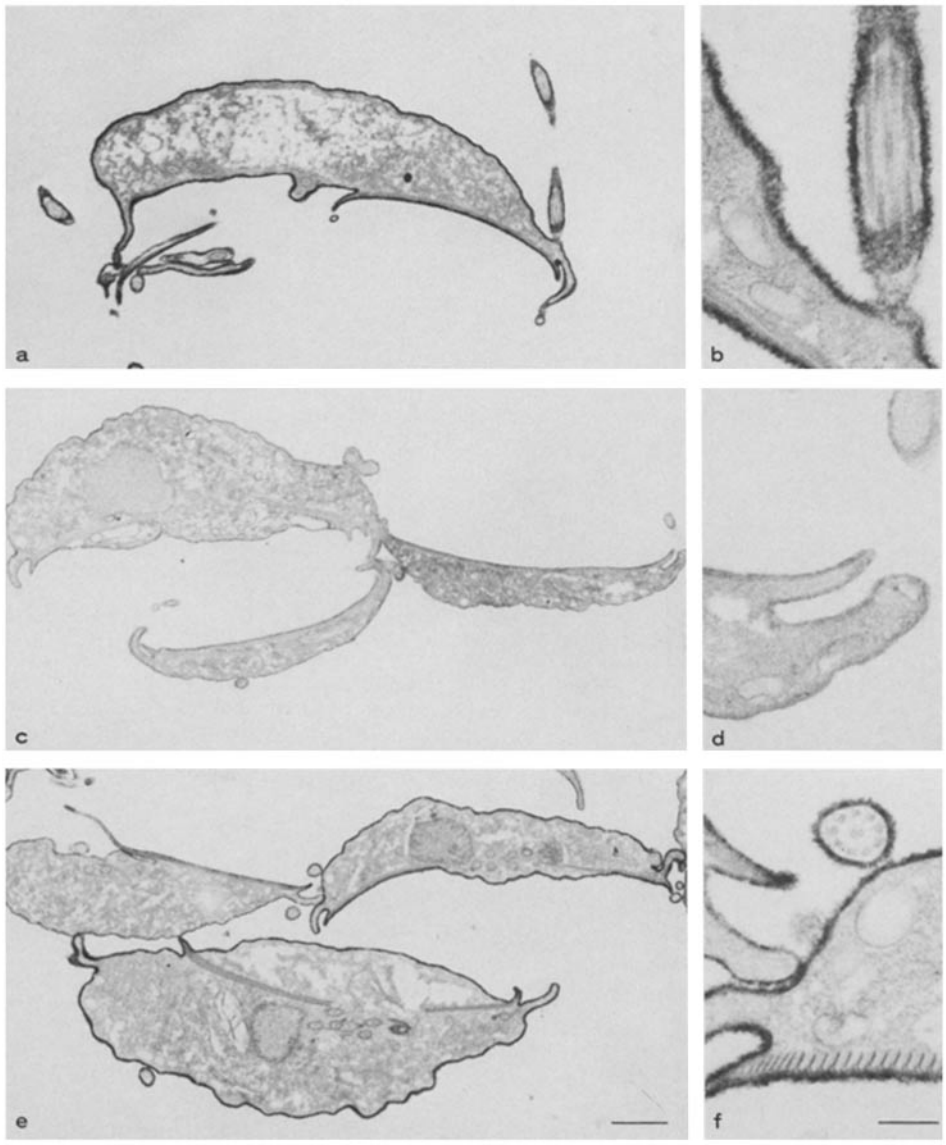

FIGURE 6. Electron micrographs of trophozoites stained by WGA after treatment with *N*-acetyl-β-D-glucosaminidase or chitinase. Trophozoites were treated with PBS alone as a control (*a* and *b*), or with *N*-acetyl-β-D-glucosaminidase (*c* and *d*), or with chitinase (*e* and *f*), stained with WGA using the avidin–biotin–peroxidase technique and processed for EM as described in Materials and Methods. *N*-Acetyl-β-D-glucosaminidase completely abolished WGA binding to the trophozoite (*c* and *d*) whereas chitinase (*e* and *f*) had no effect on WGA binding. Bar, *a*, *c*, and *e*, 1.11 μm; *b*, *d*, and *f*, 0.22 μm.

with the lectin-binding proteins. Once again the D-GlcNAc–specific lectins WGA and S-WGA were the only ones that reacted specifically with surface glycoproteins. Lectins with specificity for D-Glc or D-Man, D-Gal-β1→3-D-GalNAc, and D-GalNAc did not react with surface glycoproteins. As shown in Fig. 9, lane *b*, a number of proteins were recognized by WGA, many of which corresponded in

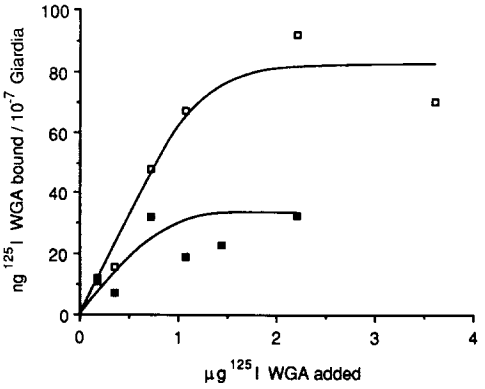

FIGURE 7. Effect of *N*-acetyl-β-D-glucosaminidase treatment on binding of [^{125}I]WGA to trophozoites. Trophozoites were treated with PBS alone as a control or with *N*-acetyl-β-D-glucosaminidase and then assessed for binding to increasing concentrations of [^{125}I]WGA as described in Materials and Methods.

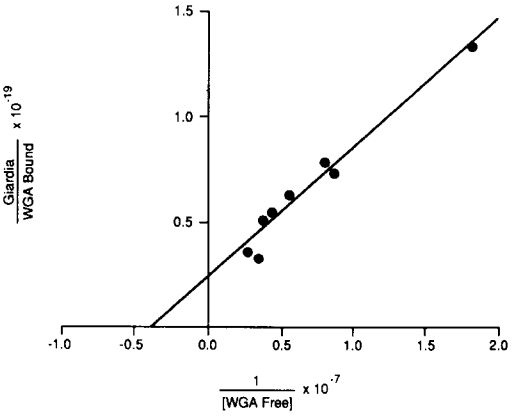

FIGURE 8. Binding of [^{125}I]WGA to *G. lamblia* trophozoites. [^{125}I]WGA binding studies were performed as described in Materials and Methods. The data have been plotted by the method of Steck and Wallach (12) according to the equation: c/WGA bound = $1/(K \times n) \times 1$/WGA free + $1/n$, where c is the concentration of cells, WGA is the molar concentration of WGA, n is the number of lectin molecules bound/cell, and K is the association constant of WGA.

molecular weight to ^{125}I surface-labeled proteins (Fig. 9, lane *a*), suggesting that they were present on the surface membrane. The binding of WGA to these glycoproteins was specific, since it could be completely inhibited by the specific sugar hapten *N,N′,N″*-triacetylchitotriose (Fig. 9, lane *c*). Similar results were obtained with S-WGA, confirming that the WGA was reacting with D-GlcNAc and not sialic acid residues (data not shown).

Discussion

A systematic analysis of *G. lamblia* trophozoite surface carbohydrate residues using lectins and glycosidases of known sugar specificity revealed that β-linked D-GlcNAc is the only detectable saccharide moiety on the plasma membrane of this parasite. This finding is highly unusual since eukaryotic cells, including a number of other protozoan parasites, are known to express a variety of other sugar residues such as sialic acid, D-mannose, D-galactose, *N*-acetyl-D-galactosamine, and L-fucose in addition to D-GlcNAc on cell surface glycoconjugates (13–16). In fact, variation in lectin reactivity has been exploited to study differences in the expression of carbohydrate residues on the cell surface at various stages of differentiation (15–18). The present study confirms the earlier findings of Hill et al., who also found that WGA was the only one of six lectins studied that reacted with *G. lamblia* trophozoites (19).

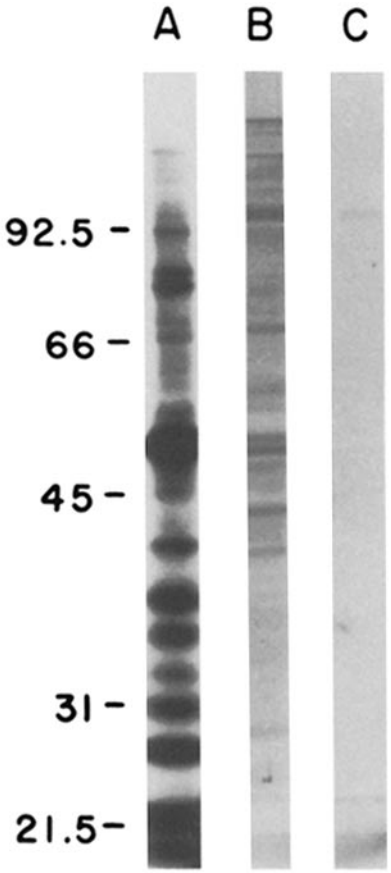

FIGURE 9. Identification of D-GlcNAc-containing glycoproteins on *G. lamblia* surface glycoproteins. ^{125}I surface-labeled proteins were subjected to electrophoresis on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with WGA as described in Materials and Methods. Lane *A*, autoradiograph of ^{125}I surface-labeled proteins. Lane *B*, WGA-binding glycoproteins. Lane *C*, inhibition of WGA binding to glycoproteins by the specific sugar hapten *N,N',N"*-triacetylchitotriose. Molecular mass markers (in kD) are as follows: phosphorylase B, 92.5; BSA, 66; ovalbumin 45, carbonic anhydrase, 31; soybean trypsin inhibitor 21.5.

Whereas trophozoites of *G. lamblia* exhibited exclusive reactivity with D-GlcNAc–specific lectins, not all the trophozoites reacted with these lectins. Using one of these lectins, WGA, to study the variation in binding, two distinct subsets of WGA-positive and WGA-negative cells were identified, both by direct observation of fluoresceinated or biotinylated lectin binding to the cell surface as well as by microfluorometric analysis of cells labeled with FITC-labeled WGA. These studies indicated that only about 80–85% of trophozoites were consistently labeled at concentrations of the lectin ranging from 1 to 10 μg/ml, and two subsets of cells could be separated by fluorescence-activated cell sorting. Since D-GlcNAc–specific lectins were the only ones that reacted with the trophozoite surface, it would appear that the WGA-negative subset of cells do not express major cell surface carbohydrate moieties.

Lectins have been widely used to identify and separate subpopulations of mammalian cells such as lymphocytes, based on differences in cell surface carbohydrates (17, 18). Stage specific variations (15, 16) as well as variations in different phases of growth (20) in the expression of surface saccharide moieties in parasitic protozoa have also been identified by lectin-binding studies. The biological significance of the finding of two subsets of trophozoites remains to be determined. However, it is possible that one or the other of these subsets may

represent a stage of differentiation of the trophozoite form of the parasite to the cyst. Recently Gillin et al. were able to induce a subset of *G. lamblia* trophozoites to encyst in culture by exposing them to various bile salts (21). It is therefore possible that the encysting trophozoites may be represented by one of these subsets of cells which is destined to differentiate into cysts. This possibility could be further investigated by separating the two subsets by flow cytometry and determining whether either one or both can be induced to encyst by exposing them to the appropriate bile salts. Since our studies were performed with asynchronous cultures of trophozoites it is also possible that the subpopulations of cells may represent cell cycle variations in the expression of surface carbohydrate moieties. These possibilities are being investigated in ongoing studies.

In an earlier study of the surface carbohydrate residues of *Giardia* cysts, we identified the polysaccharide chitin as a major structural component of the cyst wall (3). There is no structural analogue to the cyst wall on the trophozoite surface. However one of the aims of this study was to determine whether chitin was also present on the trophozoite surface. The findings of the present study indicate that although there is an abundance of β-linked D-GlcNAc residues on the surface of trophozoites, these residues are not present in the form of chitin. This is based on the finding that affinity-purified chitinase had no effect on the binding of WGA to the trophozoite surface, in contrast to chitinase treatment of the cyst which resulted in destruction of the cyst wall as well as in abolition of WGA binding. We have made identical observations with trophozoites of the murine parasite *G. muris* (Ward, H. D., G. T. Keusch, and M. E. A. Pereira, unpublished observations). The presence of chitin thus appears to be limited to the cyst form of the parasite and suggests that chitin synthesis is developmentally regulated. In yeast, chitin is synthesized from the substrate UDP *N*-acetyl-D-glucosamine by the action of the enzyme chitin synthetase (22). The finding that the presence of chitin is developmentally regulated is in agreement with the study of Gillin et al. who showed that encysting cultures of *G. lamblia* trophozoites exhibited increased levels of chitin synthetase activity as compared to nonencysting cultures (21).

Since the D-GlcNAc residues on the trophozoite surface did not appear to be in the form of polysaccharides such as chitin, the next possibility to investigate was whether these moieties were present on surface membrane–bound glycoproteins. The results of this study indicate that a number of surface proteins were specifically and exclusively bound by WGA and S-WGA, indicating the presence of β-linked D-GlcNAc residues in these glycoproteins. These results are consistent with those of lectin histochemistry and confirm the finding that D-GlcNAc is the major carbohydrate moiety expressed on the trophozoite surface as determined by lectin binding. This finding of a single predominant saccharide moiety on the cell surface has not been reported in any other eukaryote. Analysis of carbohydrate residues in trophozoites by gas–liquid chromatography confirms the finding that D-GlcNAc is a major saccharide moiety in *G. lamblia* glycoproteins (Ward, H. D., G. T. Keusch, and M. E. A. Pereira, manuscript in preparation). In N-linked glycoproteins, D-GlcNAc is present in the core region of oligosaccharides and may also be added onto complex type oligosaccharides together with D-Gal, L-Fuc, and sialic acid during processing in the Golgi complex (23). Recently D-

GlcNAc has been reported to be present as a monosaccharide in an O-glycosidic linkage on certain mammalian glycoproteins (24). The finding that D-GlcNAc residues are the only detectable saccharide moieties on trophozoite-surface glycoproteins raises the possibility that these residues may be present in an O-glycosidic linkage in *Giardia* as well. Although pathways of protein glycosylation have been studied in some parasitic protozoa (25), very little is known about oligosaccharide synthesis and processing in *Giardia.* In fact, organelles such as Golgi bodies, which are known sites of oligosaccharide processing in higher eukaryotes, have not been identified as such in *Giardia* (2, 26, 27).

In this study we have identified β-linked D-GlcNAc as the only saccharide moiety on the trophozoite surface as detected by studies of lectin binding and glycosidase digestion. We have also shown, that in contrast to the cyst, chitin is not present on the trophozoite surface suggesting that chitin synthesis is developmentally regulated. Furthermore distinct subsets of trophozoites were identified based on WGA reactivity, and may represent varying stages of differentiation from trophozoite to cyst. The findings in this study may therefore be of importance in elucidating glycoconjugate structure and function in this parasite as well as of significance in defining the mechanisms underlying encystation. Ultimately, the goal would be to interrupt the life cycle of this parasite and thus control transmission of the disease.

Summary

Lectins and glycosidases of known sugar specificity were used as probes to analyze the surface carbohydrate moieties of *G. lamblia* trophozoites and in particular to determine whether chitin or oligomeric D-GlcNAc is present in the trophozoite form of the parasite as well as on the cyst. Of 13 lectins with varying sugar specificity, only D-GlcNAc–specific lectins bound specifically to the trophozoite surface as determined by light microscopy and EM. A striking finding was the identification of two distinct subsets of trophozoites, distinguished by reactivity with WGA and detected by light microscopy and EM as well as by flow cytometry. Unlike the cyst wall, the trophozoite D-GlcNAc residues were resistant to chitinase treatment. In contrast *N*-acetyl-β-D-glucosaminidase abolished WGA binding suggesting that the lectin binds to terminal β-linked D-GlcNAc residues. These residues were identified as being present on surface glycoproteins by Western blotting of parasite membrane proteins using WGA as a probe. This study identifies D-GlcNAc as the only saccharide moiety detectable by lectin binding on the surface of *G. lamblia* trophozoites and demonstrates that in contrast to the cyst, chitin is not present in the trophozoite. In addition two distinct subsets of trophozoites were identified based on reactivity with WGA and may represent varying stages of differentiation from trophozoite to cyst.

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