# Growth Inhibition of the Intestinal Parasite *Giardia lamblia* by a Dietary Lectin Is Associated with Arrest of the Cell Cycle

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## Abstract

***Giardia lamblia*, a cause of diarrheal disease throughout the world, is a protozoan parasite that thrives in the small intestine. It is shown here that wheat germ agglutinin (WGA), a naturally occurring lectin widely consumed in normal human diets, reversibly inhibits the growth of *G. lamblia* trophozoites in vitro, and reduces infection by *G. muris* in the adult mouse model of giardiasis. The inhibitory effect was dose related, not associated with cytotoxicity and reversed by *N*-acetyl-D-glucosamine in accordance with the known specificity of the lectin and in agreement with the presence of GlcNAc residues on the surface membrane of *G. lamblia* trophozoites. Cell cycle analysis revealed that parasites grown in the presence of WGA are arrested in the *G2/M* phase, providing an explanation for the lectin-induced inhibition of cell proliferation. Comparison of electrophoretic profiles by lectin blot analysis revealed both glycoprotein induction and suppression in growth-arrested organisms. Our findings raise the possibility that blocking trophozoite growth with naturally occurring dietary lectins may influence the course of giardiasis. In addition, the study of cell cycle arrest by WGA may provide a model to study the regulation of cell division in lower eukaryotes. (*J. Clin. Invest.* 1994. 94:2283–2288.) Key words: *G. lamblia* • wheat germ agglutinin • growth inhibition • cell cycle arrest**

## Introduction

*Giardia lamblia* is one of the most common protozoan parasites of the human intestinal tract, infecting 2–15% of the population in various parts of the world (1). In developing countries giardiasis is among the 10 most common infections affecting humans (2) and is widely prevalent in children, in whom it is a significant cause of diarrhea and malnutrition (3, 4). Infection is initiated by ingestion of the cyst form, followed by excystation and colonization of the proximal small intestine by the trophozoite form. The latter attach to enterocytes, multiply, and exert their pathogenic effects by mechanisms that remain largely unknown at the molecular level. Ultimately some trophozoites develop into cysts which are excreted in the feces and serve to propagate infection to the next host.

Cell surface glycoconjugates of eukaryotes have been postulated to play an important role in a variety of biological functions such as the maintenance and regulation of growth, differentiation, and cellular adhesiveness (5–7). The nature and properties of carbohydrate residues of glycoproteins on the plasma membrane of diverse cell types have been assessed using lectins, a class of sugar binding proteins of nonimmune origin (8). The presence of such lectin receptors on the surface membrane of *Giardia lamblia* has been shown previously (9, 10). Of a variety of lectins tested, only wheat germ agglutinin (WGA),[1] tomato lectin, and succinylated wheat germ agglutinin (S-WGA), bound specifically to carbohydrate determinants on the surface of *Giardia* trophozoites. As part of our goal to understand the mechanisms governing *Giardia lamblia*–host cell interaction, we have previously confirmed, on the basis of gas chromatography/mass spectrometry, that *N*-acetyl-D-glucosamine (GlcNAc) is a major sugar of trophozoite cell surface glycoproteins, and serves as the parasite receptor for WGA (11).

To investigate the functional role that such lectin receptors may play in the process of infection, we studied the influence of exogenous lectins on the growth of *G. lamblia* in vitro and *G. muris* in vivo.

## Methods

*Materials.* WGA, biotinylated-WGA, PHA, lima bean agglutinin (LBA), were from Sigma Chemical Co. (St. Louis, MO). Vectastain ABC kit was from Vector Laboratories, Inc. (Burlingame, CA). Concanavalin A (Con A) was from Miles-Yeda LTD (Israel). Soybean agglutinin (SBA) was from Calbiochem-Behring Corp. (La Jolla, CA). LBA was purified by affinity chromatography on hog gastric mucin (A+H substance) immobilized on Sepharose 4B-CL-200 (12). Aaptos papillata agglutinin was purified according to a previously described method (13). Succinylated WGA was prepared by treatment of WGA with succinic anhydride as described earlier (14).

*Parasites.* Trophozoites of the WB strain of *G. lamblia* were cloned in semisolid agarose medium as previously described (15). Trophozoites of the Portland 1 strain, WB strain, and WB-M clone were axenically cultivated in TYI-S-33 medium supplemented with 10% adult bovine serum and bovine bile (16). Parasites in late-log phase were harvested by chilling the tubes on ice for 15 min, pelleted at 500 *g* for 10 min, washed once in fresh culture medium and used in growth inhibition experiments.

*G. muris* was obtained as a gift from Dr. Edward Jarroll, (Cleveland State University, Cleveland, OH) and maintained in CF-1 Swiss mice

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1. *Abbreviations used in this paper:* Con A, Concanavalin A; GlcNAc, *N*-acetyl-D-glucosamine; LBA, lima bean agglutinin; SBA, soybean agglutinin; S-WGA, succinylated-WGA; WGA, wheat germ agglutinin.

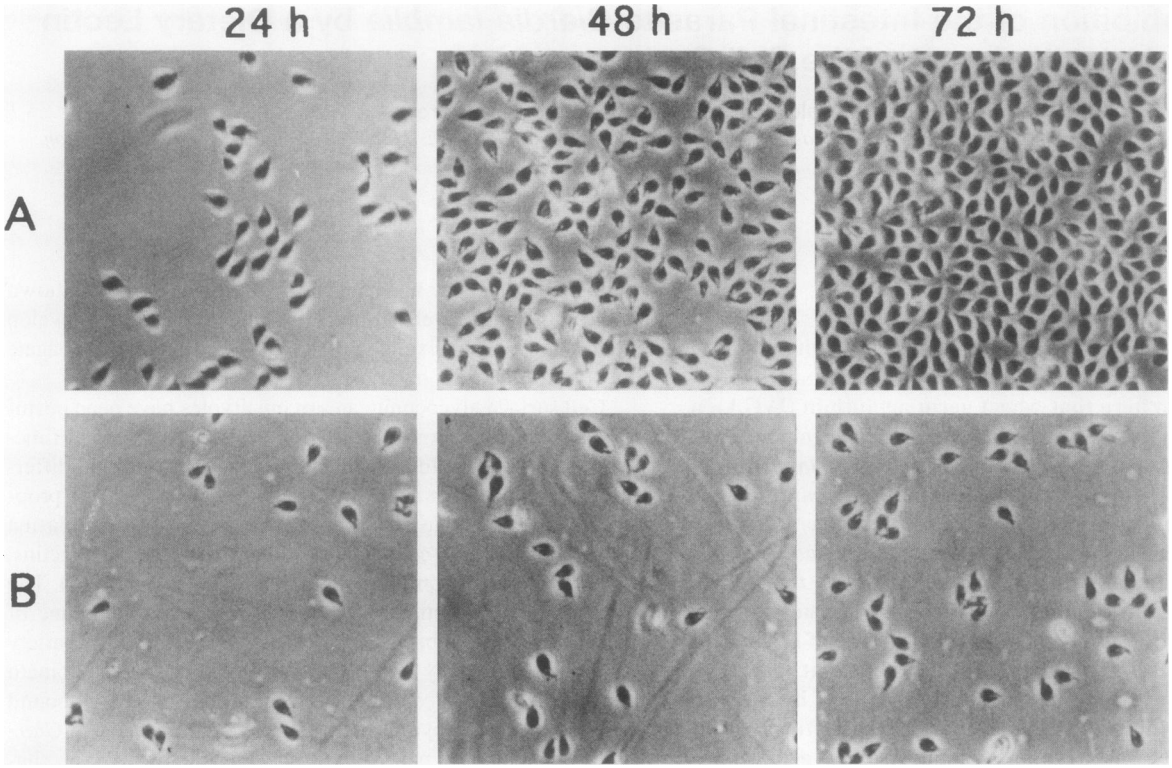


*Figure 1.* Effect of WGA on *G. lamblia* growth in vitro. (*A*) Trophozoites attached to the culture tube walls 24–72 h after the start of the culture, in the absence of WGA. (*B*) Trophozoites in the presence of 100 $\mu g/ml^{-1}$ WGA for 24–72 h.

(17). Cysts were isolated from stool samples of infected mice as described (17), and used within 24 h.

*Growth Inhibition assays.* The effect of various lectins on growth of *G. lamblia* trophozoites in vitro was determined by a modification of a previously described method (18). Briefly, cultures were initiated by addition of $2.5 \times 10^4$ trophozoites in 0.1 ml of medium to vials (15 × 45 mm) containing 3.9 ml of medium containing one of the following lectins: Con A, PHA, SBA, S-WGA, Aaptos papillata agglutinin, LBA 100 $\mu g/ml^{-1}$ (100 $\mu g/ml^{-1}$), WGA (10, 50, 100 $\mu g/ml^{-1}$) and WGA (100 $\mu g/ml^{-1}$) plus GlcNAc (2 $\mu g/ml^{-1}$). The vials were incubated at 37°C for different time intervals, chilled on ice to detach trophozoites, centrifuged at 500 *g* for 10 min and the pellet resuspended in 1 ml of PBS (20 mM sodium phosphate, pH 7.2, containing 150 mM NaCl). The total number of organisms per vial was counted and compared to that of parallel untreated cultures.

Reversibility of WGA inhibitory effect on *Giardia* growth was assessed by growing trophozoites in the presence of 100 $\mu g/ml^{-1}$ WGA. After 48 h at 37°C the vials were chilled and cells pelleted by centrifugation. The medium was removed and equal numbers of trophozoites were resuspended in fresh complete TYI-S-33 medium with or without 100 $\mu g/ml^{-1}$ WGA. Parasites were then incubated at 37°C for 72 h, harvested and counted.

To assess attachment of *G. lamblia* to the substratum, $1 \times 10^5$ trophozoites were inoculated into 4 ml medium containing WGA at different concentrations and incubated at 37°C. At different periods of time, vials were inverted three times at 37°C and an aliquot withdrawn to determine the number of unattached parasites. Vials were then chilled on ice and an aliquot withdrawn to determine the total number of organisms (attached plus unattached).

*Cell culture.* The rat intestinal epithelial cell line IEC-6, (American Type Culture Collection CRL 1592, Rockville, MD), was maintained in RPMI 1640 supplemented with 5% FCS. IEC-6 cells were plated in 24-well microplates 48 h before the attachment assay, and used at confluence.

*G. lamblia attachment assay.* The effect of WGA on the attachment of *G. lamblia* to IEC-6 cells was assessed as previously described (19). Briefly, trophozoites were grown in the presence or absence of 100 $\mu g/ml^{-1}$ WGA for 72 h or 1 h at 37°C. 24 h before harvesting, parasites were labeled with 25 $\mu$Ci [$^3$H]thymidine, specific activity, 84.8 Ci/mmol (DuPont-NEN, Boston, MA). After 72 h of incubation, organisms were harvested, washed three times in RPMI 1640, and resuspended in RPMI 1640, supplemented with 2% FCS, and 0.1% L-cysteine. [$^3$H]thymidine labeled-trophozoites ($2.5 \times 10^6$ organisms in 1 ml) were added to each well of confluent IEC-6 cells and incubated at 37°C. At various periods of time, medium containing unbound trophozoites was aspirated and the monolayer washed three times with warm PBS. Monolayers were lysed by 0.5 M KOH and bound trophozoites were determined by scintillation counting.

*Animal model.* 3-wk-old female Swiss Albino (CF-1) mice (Taconic, Germantown, NY) were determined to be free of *G. muris* infection before use by examination of fecal samples on three alternate days. Mice were infected by intraesophageal administration of $10^3$ cysts in 0.2 ml PBS. Starting the previous day or on the day of infection groups of 3–4 mice were given daily intraesophageal administration of 100 $\mu g$ of WGA in 0.2 ml of PBS for 2 wk. Control animals received Con A, 100 $\mu g$/d or PBS alone. Cyst excretion was quantitated on alternate days by placing mice in separate cages and collecting the feces excreted over a 2 h period. Cysts were isolated on a sucrose cushion as described earlier (17) washed in distilled water and counted. Animals were sacrificed on day 14 by cervical dislocation, the small intestine was removed and trophozoites isolated and counted as described (20).

*Cell cycle analysis.* Cultures were initiated by addition of $2.5 \times 10^4$ trophozoites to tubes containing TYI-S-33 medium, supplemented with different lectins at 100 $\mu g/ml^{-1}$. After 24, 48, and 72 h of culture, trophozoites were harvested, washed, and resuspended in 2 ml PBS. Cells were fixed by slow addition of 5 ml 95% ethanol while vortexing at low speed. Fixed cells were kept at room temperature for 30 min and then stored at 4°C. Before staining, the sample was centrifuged (500 *g*,

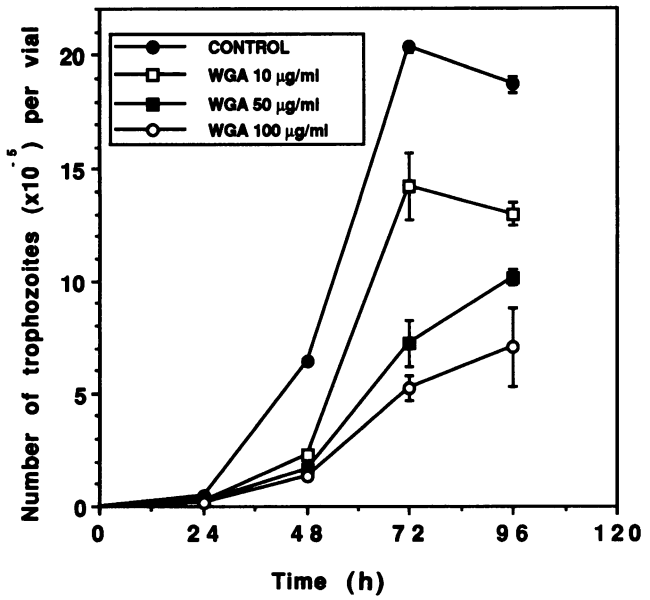


*Figure 2.* Inhibition of *G. lamblia* growth by WGA in vitro. Trophozoites were grown in the absence (*closed circles*) or presence of 10 μg/ml (*open squares*), 50 μg/ml (*closed squares*), or 100 μg/ml (*open circles*) of WGA , and cells numbers determined at 24 h intervals. The results represent the mean of duplicate determinations±SD of a representative experiment.

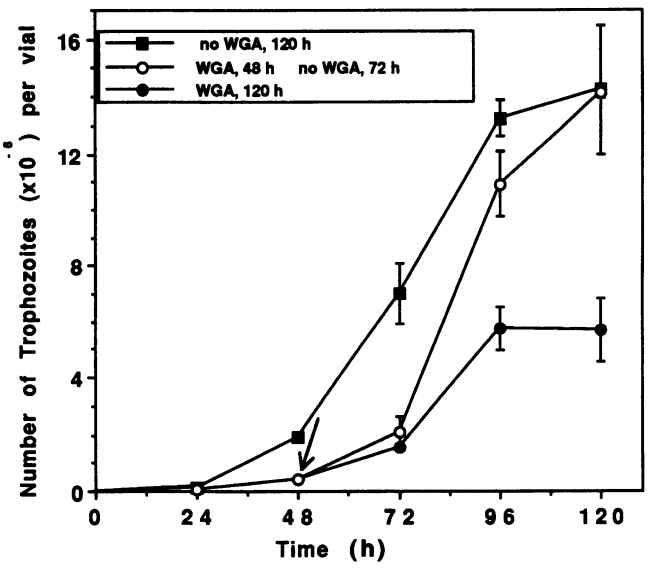


*Figure 3.* Reversibility of WGA inhibitory effect on *G. lamblia* trophozoite growth. Trophozoites were inoculated into culture tubes supplemented with (*closed circles, open circles*) or without (*closed squares*) WGA. After 48 h (*arrow*) trophozoites were harvested and equal numbers resuspended in fresh culture medium with or without 100 $\mu g/ml^{-1}$ WGA. Parasites were then further incubated for an additional 72 h and counted. The results represent the mean of duplicate determinations±SD of a representative experiment.

5 min), the fixative removed by aspiration, and cells processed for cell cycle analysis as described (21). Flow cytometry analysis was performed using a Coulter Electronics Epics-Profile flow cytometer.

*Analysis of carbohydrate residues on G. lamblia glycoproteins by lectin blot analysis. Giardia* trophozoites were grown in the presence or absence of 100 $\mu g/ml^{-1}$ WGA for 72 h at 37°C. Organisms were harvested, washed three times in PBS, and lysed with 1% Triton X-100 in PBS containing 2 mM PMSF overnight at 4°C. Lysates were centrifuged at 175 *g* for 5 min to pellet nuclei and the supernatant boiled with sample buffer before electrophoresis on a 7% SDS-polyacrylamide gel (10). Separated proteins were electrotransferred to nitrocellulose and probed with 1 μg/ml of biotinylated WGA, using the avidin-biotin alkaline phosphatase technique as described earlier (11).

## Results and Discussion

Under standard culture conditions (37°C in TYI-S-33 medium supplemented with 10% bovine serum) trophozoites of *G. lamblia* multiply after attaching to the substratum, grow to high cell densities and reach stationary phase within 72 h (Fig. 1 *A*). In the presence of exogenously added WGA, however, growth was markedly reduced (Fig. 1 *B*). Growth-arrested organisms appeared normal by light microscopy and remained so upon long term culture. Dose-response and time course experiments (Fig. 2) showed that the degree of growth inhibition was dependent upon the concentration of WGA with the maximal effect seen at 72 h after start of the culture. The growth inhibition was specific for WGA since other lectins with varying sugar specificity (8), including Con A, SBA, PHA, and LBA and *Aaptos papillata* lectin, were all ineffective (data not shown). Furthermore, addition of GlcNAc to the WGA containing cultures prevented the inhibitory effect (data not shown), in agreement with the known saccharide specificity of WGA, and consistent with an effect mediated by the sugar-binding site of the lectin, and the presence of terminal GlcNAc residues on the trophozoite surface (11).

There are several possible mechanisms by which WGA could inhibit *Giardia* growth. One explanation is that the lectin could be cytotoxic to the parasite, as it is to a number of mammalian cell lines (22). This was not the case since the inhibitory effect was reversed by removing the medium containing WGA and replacing it with fresh medium (Fig. 3). If WGA were cytotoxic for *G. lamblia*, one would expect the effect to be irreversible once the parasite was incubated with the lectin for prolonged periods of time. In addition, S-WGA, a derivative of WGA which is nontoxic to mammalian cells (23), inhibited the growth of *Giardia* trophozoites as efficiently as the native lectin. Moreover, successive cultivation of *Giardia* in the presence of inhibitory concentrations of WGA (100 $\mu g/ml^{-1}$) failed to select parasites resistant to WGA, as observed with mammalian cells where resistant cell lines emerge when grown with lectins at concentrations that produce cytotoxicity (22).

A second possibility is that WGA could agglutinate trophozoites and, in so doing, prevent them from attaching to the substratum and thus from multiplying. However, at the concentration of WGA used in this study, i.e., 100 μg/ml we found that only 10.6% of trophozoites were agglutinated by the lectin (data not shown) even though it binds to trophozoite glycoproteins as assayed by fluorescence microscopy (9), light and electron microscopy (10), and FACS® analysis (11). The basis for this lack of cell agglutination despite the presence of numerous WGA-binding sites on the trophozoite surface glycoconjugates is not clear. One possible explanation is that the structural arrangement of GlcNAc residues prevents the appropriate cross-linking of adjacent organisms. Although less likely, it is also possible that the characteristic vigorous movement of the tro-

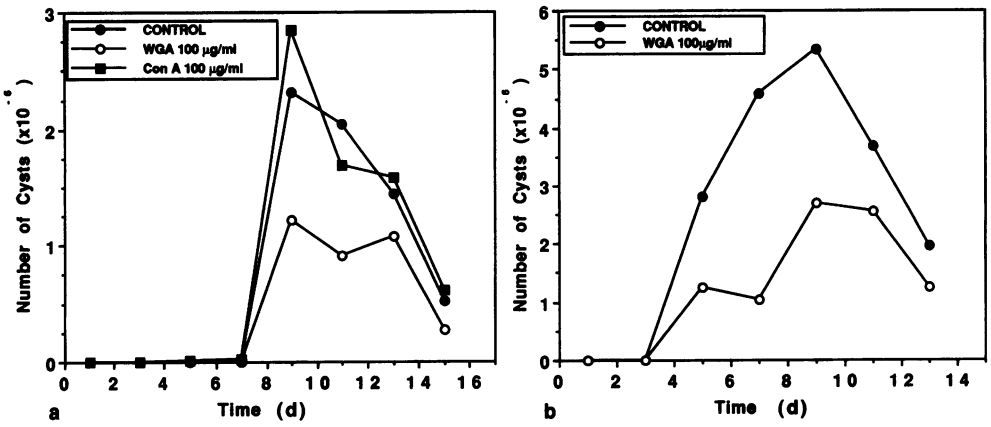


*Figure 4.* Effect of WGA on the course of *G. muris* infection. Mice, were infected with $1 \times 10^3$ cysts of *G. muris* . Starting one day before (Fig. 5 *a*) or on the day of infection (Fig. 5 *b*), groups of 3 to 4 mice received the indicated amounts of WGA (*open circles*) daily. Control animals were treated with PBS (*closed circles*) alone or Con A (*closed squares*). Fecal cyst output was determined on alternate days. The results are expressed as the mean number of cysts excreted/g feces in groups of 3–4 mice. Data show mean of two independent experiments.

phozoites may overcome the active cross-linking of cells by WGA.

The third possibility, and one that we favor, is that WGA interferes with the function of surface glycoproteins involved in *Giardia* attachment to the substratum, as is the case with other cell types (5). In accordance with this hypothesis, we found that the proportion of unattached cells in the culture medium increased with the time of incubation and concentration of lectin; ~45% of the trophozoites remained in suspension after 8 h of incubation in the presence of 50 $\mu g/ml^{-1}$ WGA, compared to 5% for the control parasites grown for the same time in the absence of the lectin. Unattached parasites remained viable because they grew normally when washed and transferred to normal medium (data not shown). The decrease in attachment to the substratum may relate to a change in the overall charge density at the cell surface after lectin binding, mechanical interference with the inert glass surface, or interference with putative contractile events occurring at the parasite surface during focal contact (24). We also found that trophozoites grown in the presence of an inhibitory dose of WGA for either 1 or 72 h, attach with 50% less efficiency to rat intestinal epithelial cell monolayers in vitro (binding of control trophozoites grown for 72 h at 37°C in the absence of WGA, $5.96 \pm 0.028 \times 10^{-2}$ cpm, trophozoites grown in the presence of WGA 100 µg/ml, for 1 h at 37°C, $1.81 \pm 0.240 \times 10^{-2}$ cpm, trophozoites grown in the presence of WGA 100 µg/ml, for 72 h at 37°C, $2.41 \pm 0.5 \times 10^{-2}$ cpm). Since the extent of inhibition of attachment was independent of the length of lectin exposure, it suggests that interference with binding to the substratum precedes growth arrest.

Since *Giardia* colonize the small intestine and attach to enterocytes in vivo, we next examined the effect of dietary WGA on the course of *G. muris* infection in adult mice (17). Fig. 4 shows that cyst excretion in mice treated with WGA was reduced by 50% after 5–9 d of infection, while the number of intestinal trophozoites was reduced by 30% compared with control animals (data not shown). As was the case with *Giardia* growth in vitro, Con A had no effect on the fecal cyst output (Fig. 4) or the intestinal trophozoite count (not shown). The decrease in trophozoite numbers could be due to the inhibitory effect of WGA on growth and multiplication of this form of the parasite as occurs in vitro. However, since WGA also binds specifically to cyst walls (11) it is possible that this lectin may decrease infection in vivo by inhibiting excystation. The concomitant decrease in cyst excretion could be a direct consequence of reduced trophozoite numbers. However we have recently shown that in addition to its effect on trophozoite growth, WGA also inhibits encystation in vitro as well (Ward, H., A. Kane, E. Ortega-Barria, G. T. Keusch, and M. E. A. Pereira, manuscript in preparation).

Inhibition of cell growth can be associated with a specific arrest of replication at some stage in the cell cycle (25). The rate of cell division in most eukaryotic cells is generally regulated at a point in the cell cycle before the initiation of DNA synthesis (26, 27). When conditions are unfavorable, cells become arrested at the *G1* phase and cannot complete the division cycle as shown with mouse 3T3 fibroblasts, whose proliferation was inhibited by succinyl-Con A (28, 29). However, under certain conditions a small number of cells within a cell population are arrested in the *G2* phase; this arrest is usually irreversible (26, 30, 31). It was therefore of interest to determine the point in the cell cycle at which growth of *G. lamblia* is inhibited by WGA. Fig. 5 *a* shows that ~32% of normal growing control, trophozoite cultures, are at the *G2/M* phase of the cell cycle at 72 h of culture. In comparison, when trophozoites are grown for the same time interval in the presence of WGA, ~80% of the cells are arrested in this phase. Time course experiments show that at 24 h of growth with WGA 76.7% of the trophozoites were already arrested at *G2/M* as compared with 30.5% of the control trophozoites (data not shown). This results in a low level of cell proliferation (Fig. 5 *b*), and is specific for WGA since neither Con A nor LBA influenced cell cycle progression (Fig. 5, *c* and *d*). The finding of an arrest point at *G2/M* suggests the existence of a major regulatory control point at that phase which determines the timing of mitosis in *G. lamblia,* as has been shown in other organisms (32, 34). Further support for this view comes from the recent observation that treatment of *G. lamblia* trophozoites with the anti-protozoan drugs metronidazole and furazolidone causes a moderate increase of *G2/M* phase organisms (35). However, both these drugs unlike WGA, are lethal to the parasite. Interestingly, hydroxyurea, which usually blocks mammalian cells in *G1/S* (34) also arrested trophozoites in *G2/M* (35). The high proportion of *Giardia* trophozoites arrested by WGA at *G2/M* is of interest since only a small number of the total population of other eukaryotic cells are blocked at some point in *G2* (27, 28, 31). Although several physical and chemical agents can induce arrest of cells in *G2,* unlike the impact of WGA on *Giardia*, their effects are not readily reversible (26, 30, 31).

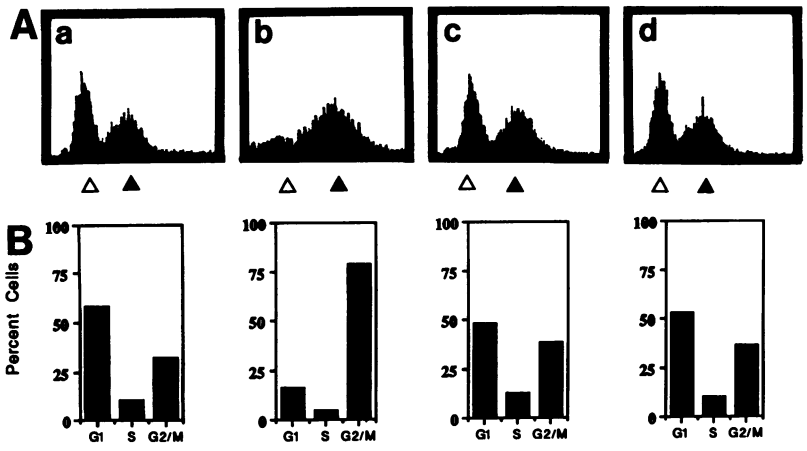


*Figure 5.* Cell cycle arrest in *G. lamblia*. (*A*) Flow-cytometric profiles of *G. lamblia* trophozoites growth (*a*) in the absence of lectins. (*b*) with 100 $\mu g/ml^{-1}$ WGA. (*c*) with 100 $\mu g/ml^{-1}$ Con A. (*d*) with 100 $\mu g/ml^{-1}$ LBA (Sigma Chemical Co.). The DNA content of the cell is represented on the abscissa and the number of cells falling into each category on the ordinate. Open and closed triangles mark 2N and 4N DNA content, respectively. (*B*) Histogram representation of data in *A*.

How does WGA reversibly block the cell cycle? One possibility is that the changes observed in *G. lamblia* adhesion after exposure to WGA influence the regulatory signals that control proliferation during the phases of the parasite cell cycle (36). Another explanation is that binding and cross-linking of trophozoite surface glycoconjugates by WGA produces activation and autophosphorylation of cell surface receptors with intrinsic protein tyrosine-kinase activities, as has been shown in other eukaryotic cells (37).

Although the mechanisms by which WGA causes growth inhibition and cell cycle arrest remain poorly understood, we have observed that exposure to WGA is associated with profound changes in the expression of specific trophozoite glycoproteins. It is clear from Fig. 6 that biotinylated-WGA binds to different trophozoite glycoproteins in both control and growth arrested parasites. However, trophozoites grown in the presence of inhibitory doses of WGA consistently induced a 215-kD band, whereas a 190-kD glycoprotein showed significant reduction as compared to control trophozoites. In addition, a 150- and a 22-kD glycoprotein which were only weakly expressed in normal parasites, were strongly expressed during growth arrest. A 35- and a 30-kD glycoproteins synthesized by control trophozoites are not produced by growth arrested *Giardia*. Comparison of the kinetics of the synthesis of these induced and suppressed glycoproteins indicates that they are analogous to the inhibition kinetics, suggesting that these changes may represent a molecular mechanism by which binding of WGA to *G. lamblia* and its effects are regulated (Ortega-Barria, E., and M. E. A. Pereira, manuscript in preparation).

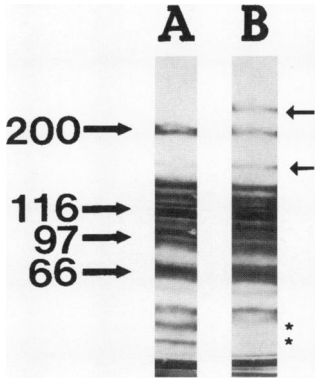


*Figure 6.* Effect of WGA on induction and suppression of *G. lamblia* glycoproteins. Trophozoites were grown in the absence (lane *A*) or in the presence (lane *B*) of 100 $\mu g/ml^{-1}$ WGA for 72 h at 37°C. Parasite lysates were subjected to electrophoresis on a 7% SDS-PAGE, electrotransferred to nitrocellulose and probed with biotinylated-WGA. Glycoproteins induced by WGA (*arrows*) included bands of ~215, 150, and 22 kD. Glycoproteins suppressed during growth arrest (*) included bands of 30 and 35 kD. Molecular mass markers (left, $\times 10^{-3}$) are indicated.

The presence of WGA-reactive sites on the surface membrane of *Giardia lamblia* raises the question of their chemical nature. Earlier, using lectin binding and glycosidase digestion assays as probes for the study of the cell surface, we concluded that GlcNAc is the saccharide moiety recognized by WGA on the surface of *Giardia* trophozoites (11). Although one study failed to demonstrate the presence of GlcNAc in *Giardia* (38), we have obtained direct evidence for GlcNAc in *Giardia* trophozoites and cysts by labeling *Giardia* surface glycoproteins with UDP–[$^3$H]galactose using bovine milk galactosyl transferase, as well as by chemical analysis using gas chromatography/mass spectrometry (11). Regardless of the nature of the WGA receptors, the evidence presented above indicates that WGA is an inhibitor of *G. lamblia* growth in vitro by arresting the parasite at the *G2/M* phase of the cell cycle, that it blocks trophozoite attachment to the substratum, and that it reduces *G. muris* infection in mice. Since WGA reduces *Giardia* growth in cultures and in vivo in mice, it is possible that the lectin may have a similar effect in human giardiasis. To be of practical value, this would necessitate that the ingested agglutinin remain active in the gastrointestinal tract as a sugar binding molecule, and that it not be toxic. Although structural alterations in the mucosa have been observed after injection of lectins into the lumen of ligated segments of the small intestine of rats (39), no side effects were reported by human volunteers who were fed wheat germ (containing the equivalent of 200 mg of active agglutinin) daily for 4 d, in an attempt to characterize the ability of dietary WGA to traverse the human intestinal tract (40). Moreover, biologically intact WGA was present in the feces from these individuals. WGA is a natural component of the human diet and is present in many commonly ingested cereals (41). When we determined the lectin content of these cereals, assessed by means of hemagglutination assays using purified WGA as a standard, the amounts ranged from 13 to 53 $\mu g$ WGA/g cereal.

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