Lectin Activation in *Giardia lamblia* by Host Protease: A Novel Host-Parasite Interaction

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A lectin in *Giardia lamblia* was activated by secretions from the human duodenum, the environment where the parasite lives. Incubation of the secretions with trypsin inhibitors prevented the appearance of lectin activity, implicating proteases as the activating agent. Accordingly, lectin activation was also produced by crystalline trypsin and Pronase; other proteases tested were ineffective. When activated, the lectin agglutinated intestinal cells to which the parasite adheres in vivo. The lectin was most specific to mannose-6-phosphate and apparently was bound to the plasma membrane. Activation of a parasite lectin by a host protease represents a novel mechanism of host-parasite interaction and may contribute to the affinity of *Giardia lamblia* to the infection site.

GIARDIA LAMBLIA, A FLAGELLATED protozoan that thrives in the human intestine, is a significant cause of diarrheal disease worldwide. Infection is initiated by ingestion of the cyst form, followed by excystation and colonization of the gut mucosa by *Giardia* trophozoites. The parasite displays distinct tissue tropism in that infection is restricted to the small intestine where *Giardia* attach to the mucosal surface, exerting pathological effects.

The molecular basis of the specific adherence of *Giardia* to host cells is unknown. Attachment of the parasite to the substratum is thought to be mediated by the ventral suction disk (*1*). However, this mechanism may not account for the selective colonization of the proximal small intestine. Therefore, recognition and adherence must be mediated by specific host and parasite surface membrane determinants. Membrane-bound lectins are believed to mediate several specific cell-cell interactions, including those between parasite and host cells (*2*). Since the heavily glycosylated microvillus membrane (*3*) is the preferred attachment site of *Giardia* trophozoites, a parasite surface lectin may play a role in the recognition process involved in the host-parasite interaction. In the experiments reported here, we studied the activation of a parasite lectin by a proteolytic activity that naturally occurs at the infection site.

Giardia lamblia trophozoites (Portland I strain), grown axenically in trypticase, yeast extract, and bovine serum (*4*) at 37°C for 72 hours, were harvested and washed with 0.01*M* sodium phosphate–buffered saline (PBS) (pH 7.2), lysed by sonication, and assayed for hemagglutination. These preparations were completely devoid of detectable lectin activity. However, when incubated with low concentrations of trypsin for a few seconds, the digested lysates exhibited a potent agglutinating activity for rabbit erythrocytes (Fig. 1A). No hemagglutinating activity was generated when trypsin was inactivated with soybean trypsin inhibitor or phenylmethylsulfonyl fluoride (PMSF) before incubation with the *Giardia* lysates (Fig. 1A) or when untreated lysates were assayed with trypsinized erythrocytes. Rabbit erythrocytes treated with trypsin or neuraminidase were agglutinated by the activated lectin to the same extent as untreated cells. Sheep, mouse, and human ABO erythrocytes were not agglutinated by the lectin, regardless of whether the cells were treated with proteases or neuraminidase. However, enterocytes isolated from mouse small intestine (*5*) were agglutinated by the activated lectin (Fig. 2A) but not by nontrypsinized lectin (Fig. 2B). The enterocyte agglutination was mediated by the sugar-binding site of the lectin, since it was blocked by a specific sugar inhibitor (Fig. 2B). This restricted cell-agglutinating activity of the *G. lamblia* lectin is not unique, since most conventional lectins are also selective in their agglutinating pattern (*6*); nevertheless, the interaction may be biologically significant since *G. lamblia* adheres to enterocytes of man (*1*), mice (*7*), and other mammals.

The kinetics of appearance of hemagglutinating activity are consistent with a process of limited proteolysis (*8*), inasmuch as the activated hemagglutinin was not readily destroyed by the protease (Fig. 1A). Thus, at a concentration of 0.5 μg of trypsin per 10^6 lysed organisms in 1.0 ml at 23°C, hemagglutinating activity appeared within seconds. Titers reached maximum values in about 10 minutes, remained constant for approximately 2 hours, and then declined slowly (Fig. 1A). In a dose-response experiment in which the time of trypsin digestion was held constant (2 hours), lectin activity was induced by a trypsin concentration as low as 7×10^{-7} mg/ml or as high as 1 mg/ml.

Activation of the lectin was protease-specific since many other proteolytic enzymes, such as ficin, subtilisin, pepsin, chymotrypsin, and papain, were ineffective (Fig. 1A). Only Pronase was as effective as trypsin in

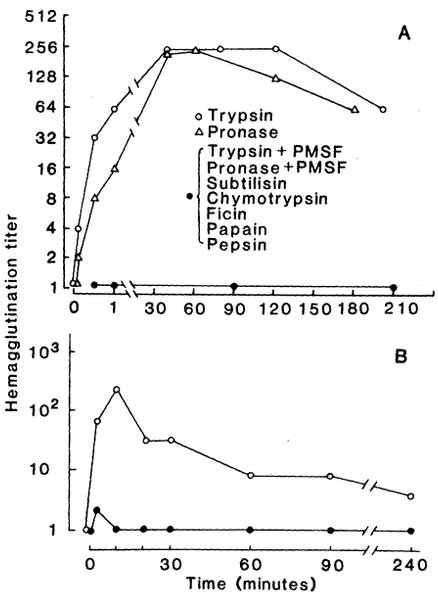

Fig. 1. Induction of hemagglutination activity in *G. lamblia* by proteases. Parasites were grown (*4*), washed three times in PBS, sonicated on ice for 90 seconds (Branson model 185 cell disruptor), and filtered through a 0.45-μm nitrocellulose membrane (Millipore). Lysates corresponding to 10^6 trophozoites per milliliter were each incubated at 23°C with the following enzymes (Sigma) at the indicated final concentrations (A): trypsin (0.5 μg/ml) in PBS, Pronase (0.5 μg/ml) in PBS, subtilisin (25 and 500 μg/ml) in PBS, chymotrypsin (25 and 500 μg/ml), ficin (25 and 500 μg/ml) in PBS containing 1 m*M* EDTA and 5 m*M* cystein, and pepsin (25 and 500 μg/ml) in 0.1*M* glycine HCl (*p*H 3.5). At the times shown an aliquot was withdrawn from the reaction mixture and protease activity was stopped by the following inhibitors at the final concentration indicated: trypsin, Pronase, subtilisin, and chymotrypsin (serine proteases) by 5 m*M* PMSF (Sigma); ficin and papain (thiol proteases) by 20 m*M* iodoacetate; and pepsin by 10 μ*M* pepstatin. Trypsin and Pronase incubated with 5 m*M* PMSF before reaction with *G. lamblia* lysates were also included. Lysates were also incubated with human duodenal fluid (B). Duodenal fluid obtained from a patient undergoing endoscopy was assayed for trypsin activity (*8*). *Giardia lamblia* lysate was incubated with duodenal fluid containing the equivalent of 5 μg of trypsin per milliliter of fluid. Protease activity was inhibited by 5 m*M* PMSF at the time indicated and the digested lysate was assayed for hemagglutination (○). Incubation of the fluid with 5 m*M* PMSF before reaction with the lysate served as a control (●). Lectin activity was determined with rabbit erythrocytes (*6*).

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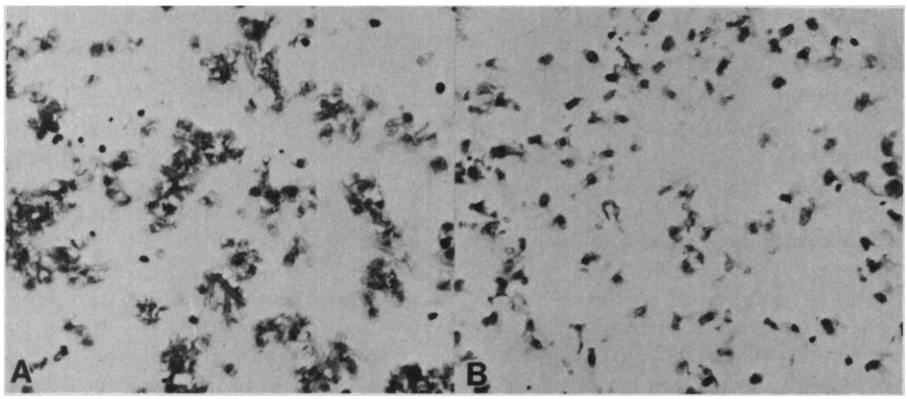

Fig. 2. Agglutination of mouse enterocytes by *G. lamblia* lectin. CF-1 mice were killed by cervical dislocation and epithelial cells from the small intestine were isolated (*5*). The cell suspension (100 μl) was incubated with 100 μl of lectin or control solutions for 60 minutes at 4°C and then a slide preparation of an aliquot was stained with Diff-Quick (American Scientific Products) and observed under a light microscope at ×135. (A) Enterocytes incubated with *G. lamblia* lectin (prepared as described in the legend to Fig. 1; hemagglutination titer, 1:8000 against rabbit erythrocytes). (B) Enterocytes mixed with *G. lamblia* lectin incubated with bacterial lipopolysaccharide (LPS) of *S. arizona* (0.5 mg/ml). Incubation of enterocytes with untrypsinized *G. lamblia* lysates resulted in a similar pattern.

the activation process (Fig. 1A). The inability of most proteases to induce lectin activity was not due to enzyme inactivation by inhibitors that might be present in *Giardia* lysates, since the specific activity of those proteases for various substrates was not affected by the presence of parasite lysate. In fact, the lectin was probably destroyed by the nonactivating proteases, as judged by the inability of trypsin or Pronase to produce hemagglutination in crude lysates that had been treated with ficin or subtilisin.

Since trypsin is a digestive enzyme normally present in the proximal small intestine of mammals, attempts were made to determine whether the *Giardia* lectin could also be activated by the natural parasite habitat. Human duodenal fluid did indeed produce lectin activity in *G. lamblia* in a manner analogous to that of crystalline trypsin (Fig. 1B). This activation was blocked by incubation of the duodenal fluid with specific trypsin inhibitors.

The sugar specificity of the activated lectin was determined by inhibition of hemagglutination with simple sugars, oligosaccharides, polysaccharides, and glycoproteins (Table 1). A lectin (or antibody) combining site is considered to be most specific for the hapten or macromolecule that inhibits at the lowest concentration (*9*). The best inhibitor of lectin hemagglutination was mannose-6-phosphate (Man-6-P), which was seven times more active than the unphosphorylated hexose. The role of phosphate in increasing the inhibitory power of mannose appears to be specific, since other phosphorylated sugars such as glucose and galactose-6-phosphates and mannose-α-1-phosphate were not inhibitory at concentrations at least five times higher than that of Man-6-P. *N*-acetyl-D-galactosamine, *N*-acetyl-D-mannosamine, and D-mannosamine also inhibited the lectin but were considerably less potent than Man-6-P. Among polysaccharides the lipopolysaccharides (LPS's) of *Salmonella arizona* and *Salmonella weslaco* showed exquisite inhibition of lectin activity, while other LPS's failed to bind to the lectin, suggesting that the lectin recognizes a specific sugar structure in the LPS side chain but neither in the core polysaccharides nor lipid A. The sugar determinant recognized by the lectin on the *S. weslaco* or *S. arizona* LPS has not been determined.

Table 1. Monosaccharide and LPS inhibition of hemagglutination of rabbit erythrocytes by *G. lamblia* lectin. *Giardia lamblia* was prepared as described in the text. Sugar inhibition was performed by incubating equal volumes of crude lectin (titer, 1:4) with different sugar concentrations, followed by the standard hemagglutination assay. The following saccharides did not inhibit the *G. lamblia* lectin at the highest concentration tested and indicated: α_1-acid glycoprotein (2 mg/ml); ovomucoid (10 mg/ml); pneumococcus polysaccharide (2 mg/ml) types IV, IX, and XIX; D-glucose (1000 m*M*); sucrose (300 m*M*); lactose (300 m*M*); α-methyl-D-mannoside (300 m*M*); D-galactose (800 m*M*); D-galactose-6-phosphate (100 m*M*); fructose-6-phosphate (100 m*M*); D-glucose-6-phosphate (100 m*M*); and LPS's (2 mg/ml) of *Salmonella typhosa, Salmonella typhimurium, Salmonella minnesota, Salmonella synechococcus, Vibrio cholera, Serratia marcescens*, and *Escherichia coli*. Values are means for two experiments.

Inhibitor	Minimum concentration required to inhibit *G. lamblia* lectin	Relative inhibitory power compared to mannose
D-Mannose-6-phosphate	20.5 m*M*	7.0
D-Mannosamine	65.0 m*M*	2.2
N-Acetyl-D-galactosamine	100.0 m*M*	1.5
N-Acetyl-D-mannosamine	113.0 m*M*	1.3
D-Mannose	145.0 m*M*	1.0
Mannose-α-1-phosphate	>100.0 m*M*	<1.0
LPS of *Salmonella arizona*	0.03 mg/ml	
LPS of *Salmonella weslaco*	0.06 mg/ml	

The *Giardia* lectin is apparently bound to membranes—some of it to the cell surface. When intact, freshly harvested *G. lamblia* trophozoites (10^6 cells per milliliter) were treated with trypsin (1 mg/ml, 15 minutes at 23°C), which was then removed by washing extensively with PBS containing protease inhibitor (soybean trypsin inhibitor), lectin activity could be detected in the parasites after sonication (titer against rabbit erythrocytes, 1:4). Parasite lysates that had not been incubated with trypsin were, as usual, devoid of hemagglutinating activity but, on trypsinization, they produced a lectin titer of 1:64. These experiments suggest that most lectin is intracellular (inaccessible to trypsin in live parasites). However, a small proportion of lectin is on the plasma membrane (accessible to trypsin in live parasites), which may be enough to mediate cell attachment of the parasite to the intestinal mucosa. If that is the case, it is not without precedent, since the mammalian hepatic lectin, which is believed to exert its biological function at the plasma membrane, is primarily intracellular, and only about 5 percent is located at this site (*10*). Furthermore, all lectin activity of crude lysates was pelleted after centrifugation at 100,000*g* for 2 hours and always emerged in the void volume of a Sepharose 4B column (50 by 1 cm) equilibrated with PBS. The sedimented lectin was also readily soluble and stable for several weeks in nonionic detergents.

The lectin was partially purified with either the specific LPS of *S. weslaco* coupled to Sepharose (*11*) and elution with specific haptens or blue Sepharose (*12*) and elution with water. Biosynthetically labeled [^{35}S]-methionine lectin purified on blue Sepharose and subjected to electrophoresis on a sodium dodecyl sulfate (SDS)–polyacrylamide gel (*13*) followed by autoradiography consistently produced one major band of 56K. This band was destroyed by subtilisin (1:100 by weight, 10 minutes at 37°C) but not by further trypsin digestion (1:100 by

weight, 10 minutes at 37°C). The purified lectin exhibited hemagglutinating activity at a minimum concentration of 10 μg/ml. This activity was inhibited by Man-6-P and by the other sugars shown in Table 1. These preliminary results, therefore, suggest that the lectin has a subunit of 56K.

Lectin activation by limited proteolysis is, as far as we know, a novel phenomenon in lectin research. It can be explained in two ways. One is by a high association constant between lectin and endogenous glycoconjugates, which should produce a concentration of free hemagglutinin below the detection limit of the hemagglutination assay. Trypsinization of the lectin would result in hemagglutination activity because of preferential digestion of the ligand, which, once digested, could display a lesser affinity to the lectin, with a consequent increase in the concentration of free hemagglutinin that would now be detected by hemagglutination (*14*). The other explanation for the lectin activation is that the *Giardia* lectin is in the form of an inactive hemagglutinin (prolectin), which, on proteolysis, will generate active lectin by exposing more than one sugar-binding site to the environment.

Since trypsin is abundant in the segment of the small intestine where *Giardia* thrives, it may be involved in the in situ activation of the parasite lectin. Once activated, the lectin may react with the small intestine mucosa to produce parasite attachment. This is in accordance with the observation that the activated lectin reacted with enterocytes in vitro. The activation of the *Giardia* lectin by a host protease could be similar to a mechanism in myxovirus, in which specific proteolytic cleavage of a surface glycoprotein is essential to render the virion infectious (*15*), and to a mechanism in *Trypanosoma cruzi*, in which proteolysis of surface membrane proteins appears to enhance infection of host cells in vitro (*16, 17*).

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Living *Nautilus* Embryos: Preliminary Observations

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***Nautilus*, long recognized as the most primitive living cephalopod, provides insight into molluscan evolution. Despite many attempts, embryos have not been observed until now. This report details the surface morphology and extraembryonic circulatory pattern. It was found that development, as in other extant cephalopods, is direct, without larval stages. There appears to be no embryonic protoconch associated with shell ontogeny.**

IN 1832 OWEN (*1*) DESCRIBED *NAUTILUS* and noted the importance of finding the embryos (*2–5*). Many questions concerning the phyletic relations in the Mollusca could be resolved by an understanding of the embryology of this most primitive living cephalopod (*6–10*). Among the many attempts to obtain embryos was that of Willey (*11, 12*) in the late 1800's. He described infertile egg capsules but did not obtain embryos. Haven (*13, 14*) kept adult animals in submerged cages for 1 year, but eggs were not laid. Mikami and Okutani (*15*) and Mikami *et al.* (*16*) described copulation and egg deposition in captive *Nautilus*, as did Carlson (*17*), but embryos did not develop. Until 4 March 1985 none of the egg capsules that we had opened contained living embryos, although one contained an embryonic shell. We report here egg deposition in *Nautilus* and preliminary observations of the living embryos.

Eleven adult *Nautilus* (seven females) obtained in July 1982 off Palau (7.2°N, 134.3°E) were maintained in two tanks. The flow from a saltwater well was 50 liter/hour and the temperature fluctuated from 15°C (daytime) to 24°C (nighttime), simulating natural conditions (*18*). After 5 months, egg laying began and was nearly continuous for 30 months. Egg capsules were separated from the adults (who tended to eat them) and placed in a tank at 21°C. By February 1984, 70 egg capsules had been examined, one of which contained a shell. Thirty-one more egg capsules laid between November 1984 and May 1985 were opened and five living embryos and one shell were found.

The egg capsules, deposited on vertical surfaces of the aquarium or on rocks, conformed to the contour of the substrate. The outer layer of the capsule was open to the sea by several channels, each a few millimeters in diameter. A teardrop-shaped inner capsule surrounded an ovate mass of semifluid yolk about 1.4 by 2 cm in diameter (Fig. 1A). Within the apex of the inner capsule was a space where the embryo developed (*12, 15*). Infertile eggs and blastoderm-stage embryos had a definite, semitransparent chorion that was not obvious in the older embryos, and there was whitish egg jelly between the chorion and the inner capsule. Two embryos were in the blastoderm stage (one 14 days old), one was in an early organogenetic stage, and two had shells covering the visceral mass, which is contained in the mantle cavity in the adult.

The blastoderm (Fig. 1B) was 6.7 mm in diameter and bulged outward from the contour of the yolk mass; it appeared to be constrained by the chorion. At its edge was a circle of large, white, triangular cells similar to the blastocones described for other cephalopod embryos (*6*). Inside this was a region of relatively uniform cells. Internal to this were bilaterally placed cells of several thicknesses, but it was not possible to identify organ primordia at ×100 magnification. Symmetrical folds were observed in the blastoderm, but these may have been caused by

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