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Abstract

Western immunoblot analysis of aqueous extracts of feces obtained from five giardiasis patients and from experimentally infected gerbils (Meriones unguiculatus) with rabbit antiserum to Giardia lamblia cysts has revealed antigens of three molecular weight groups. A stepladderlike, evenly-spaced set of strongly reactive antigens (darkest at a molecular weight [m.w.] of 55,000 to 70,000) appeared in the gerbil feces from day 4 (first experiment) or day 2 (second experiment) and lasted to about day 7 but disappeared completely by day 8 and did not reappear later. These antigenic bands were seen in gerbils infected with two isolates of G. lamblia. These bands were not revealed when antiserum to trophozoites was used as the probe, nor were they evident in specimens from the patients or in a preparation of sonicated cysts. A second group of antigens, represented by two to three low-m.w. bands of approximately 15,000 to 20,000, was evident in both the blots of gerbil feces after approximately day 8 and the specimens from the giardiasis patients. The third group of antigens revealed by blotting experiments was a high-m.w. band (approximately 110,000) which appeared on a number of days (beginning of day 8 of gerbil infection), but this band was not seen in the human specimens. A clear band corresponding to the previously reported GSA-65 antigen was not seen in either the gerbil or the human samples. Some low- and high-m.w. bands were also detected by antitrophozoite serum in the gerbil samples, but these were weak and unimpressive compared with those visualized using anticyst serum. A monoclonal antibody-based antigen capture enzyme-linked immunosorbent assay revealed that Giardia spp.-specific stool antigen rose suddenly at day 3 of gerbil infection, at the time when fecal cyst numbers began to rise rapidly.

Keywords

lamblia, feces, giardia, gerbil, identification, human, western, infected, antigens, specific, immunoblotting

Disciplines

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Identification of *Giardia lamblia*-Specific Antigens in Infected Human and Gerbil Feces by Western Immunoblotting

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Western immunoblot analysis of aqueous extracts of feces obtained from five giardiasis patients and from experimentally infected gerbils (*Meriones unguiculatus*) with rabbit antiserum to *Giardia lamblia* cysts has revealed antigens of three molecular weight groups. A stepladderlike, evenly-spaced set of strongly reactive antigens (darkest at a molecular weight [m.w.] of 55,000 to 70,000) appeared in the gerbil feces from day 4 (first experiment) or day 2 (second experiment) and lasted to about day 7 but disappeared completely by day 8 and did not reappear later. These antigenic bands were seen in gerbils infected with two isolates of *G. lamblia*. These bands were not revealed when antiserum to trophozoites was used as the probe, nor were they evident in specimens from the patients or in a preparation of sonicated cysts. A second group of antigens, represented by two to three low-m.w. bands of approximately 15,000 to 20,000, was evident in both the blots of gerbil feces after approximately day 8 and the specimens from the giardiasis patients. The third group of antigens revealed by blotting experiments was a high-m.w. band (approximately 110,000) which appeared on a number of days (beginning of day 8 of gerbil infection), but this band was not seen in the human specimens. A clear band corresponding to the previously reported GSA-65 antigen was not seen in either the gerbil or the human samples. Some low- and high-m.w. bands were also detected by antitrophozoite serum in the gerbil samples, but these were weak and unimpressive compared with those visualized using anticyst serum. A monoclonal antibody-based antigen capture enzyme-linked immunosorbent assay revealed that *Giardia* spp.-specific stool antigen rose suddenly at day 3 of gerbil infection, at the time when fecal cyst numbers began to rise rapidly.

Human infection with the protozoan parasite *Giardia lamblia* results in the excretion of cell-free, parasite-specific antigens which can be detected by immunochemical methods, including the enzyme-linked immunosorbent assay (ELISA) (5, 8, 10, 14, 15, 17) and counter-immunoelectrophoresis (3 11, 18). In 1988 (15), we reported the development and testing of a sensitive and highly specific antigen capture ELISA for water-soluble *Giardia* spp.-specific stool antigens (GSAs) based on the use of rabbit and mouse antibodies prepared against whole cysts of this parasite, and more recently (14), we reported the development of a monoclonal antibody-based antigen capture ELISA. Both of these assays were equally effective with formalinized stools and unfixed stools. By using the monoclonal antibody-based method, simple aqueous stool extracts could be diluted to a 1:60 or 1:600 final dilution for routine analysis, and sometimes antigen could be detected after 1:600,000 dilution of stool (14). The antigen(s) detected by the monoclonal antibody was shown by the monoclonal antibody-based ELISA to be present in abundance in cysts but not in trophozoites grown axenically in vitro, and immunofluorescence experiments indicated that the antigen is present in the cyst wall. The antigen was purified from cysts by immunoaffinity chromatography, and it was shown to be resistant to boiling and to periodate treatment by using ELISA reactivity as a criterion for antigenic integrity.

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As another approach to the identification and characterization of the *Giardia* stool antigens which may prove to have diagnostic utility, we have attempted to identify GSAs through Western immunoblotting by using aqueous extracts made from gerbil (*Meriones unguiculatus*) feces collected before and at selected time intervals following experimental infection with defined strains of *G. lamblia* and by using extracts made from the feces of *G. lamblia*-infected human patients and of uninfected controls. The gerbil has previously been shown to be susceptible to infection with most isolates of *G. lamblia* and to serve as a useful model for human infection (1, 4, 19, 20). Also, separate immunoblots were made using polyclonal anticyst and antitrophozoite rabbit antibodies in order to see whether the two types of antisera might reveal different GSAs.

MATERIALS AND METHODS

Culture and isolates of *G. lamblia*. The H-2 and H-3 isolates of *G. lamblia*, in experimentally infected gerbils, were obtained through Charles Hibler, formerly of Colorado State University, Boulder. These isolates originated from two human giardiasis patients in Colorado. The authors adapted these isolates to in vitro culture in TYI-S-33 medium (6, 15). The isolates were not cloned prior to use.

Gerbils and experimental infections. Female gerbils (*M. unguiculatus*, the Mongolian gerbil or jird) were obtained from Tumblebrook Farms, West Brookfield, Mass. Six-to-eight-week-old animals were experimentally infected by inoculation of approximately 250,000 log-phase trophozoites (suspended in approximately 0.25 ml of the TYI-S-33 culture medium in which they had been growing) into the stomachs

of the gerbils by gavage by using fine polypropylene tubing and a 1-ml tuberculin syringe, after treating the gerbils with methoxyflurane inhalant anesthesia. The trophozoites were detached from the borosilicate tubes in which they were grown by chilling the tubes in ice for 12 min and then inverting the tubes 20 times. Gerbils were maintained on a diet of Purina rodent chow 5001 and water ad libitum.

Infected gerbils were employed in two experiments, each of which utilized three gerbils. In the first experiment, lasting 8 days, gerbils were infected on day 0 with trophozoites of the H-2 isolate and were then housed together, except for a period of 4 to 5 h each day when they were placed individually in separate mouse cages over a screen of 0.25-in. (~0.6-cm)-mesh hardware cloth so that their fecal pellets could be collected. In this experiment, the collected feces of each gerbil were kept separate from the others; aqueous fecal extracts (made as described below) were also prepared separately. Feces were collected from late morning to late afternoon each day for approximately 5 h. The gerbils were provided with water and food during this period. Sufficient distilled water was maintained on the cage bottom below the screen to keep fecal pellets from drying during collection. Fecal pellets were scraped from the bottom of each feces collection cage into a 15-ml conical plastic disposable centrifuge tube by using a wooden tongue depressor, and the feces were gently pressed to the bottom of the tube with a thin wooden applicator stick. A volume of distilled water approximately twice that of the packed feces was added, and the feces and water were mixed thoroughly but gently with a wooden applicator stick. The mixture was centrifuged at approximately 900 × *g* for 7 min, and the supernatant aqueous extract was removed and frozen at −10°C. The volume of supernatant removed was immediately replaced by an equal volume of 10% Formalin made up in 0.175 M phosphate-buffered saline (PBS), pH 7.4, and the formalinized fecal pellets were gently mixed and then stored at 4°C for several weeks, after which the cyst concentrations in the formalinized feces of one of the gerbils were determined microscopically as follows. The fecal suspension was gently mixed with a thin wooden applicator, and then two drops were placed, by using a 9-in. (~23-cm) glass Pasteur pipette, onto a glass microscope slide (1 by 3 in. [~2.5 by ~7.6 cm]) and covered with a 25-mm^2 cover glass. The suspension was examined unstained at ×400 magnification by light microscopy; the cysts present in 20 fields of view (five fields in four directions radiating from the center of the cover glass) were enumerated and averaged.

The concentrations of GSAs were measured in the previously frozen aqueous fecal eluate of the gerbils in the first experiment by using the monoclonal antibody 5-3C-based antigen capture ELISA described previously (14), except that a capture antibody protein concentration of 10 μg/ml of carbonate coating buffer was employed. The aqueous gerbil fecal eluates were diluted 10-fold to give a 1:30 final dilution in PBS-T (PBS with 0.05% Tween 20, vol/vol). The monoclonal antibody-based ELISA utilizes the mouse anticyst monoclonal antibody as the capture antibody and rabbit anticyst polyclonal immunoglobulin G (IgG) as the secondary antibody (14).

In the second gerbil experiment, three gerbils were inoculated as on day 0 with culture-derived trophozoites of the H-3 isolate. The gerbils were housed together, as described above. Fecal pellets were collected from the gerbils each day, as described above, except that the gerbils were kept together as a group so that their feces were combined on each day. Fecal pellets were collected almost every day until 30 days postinoculation. In this experiment, fecal pellets were stored frozen at −10°C until the end of the experiment, at which time the collected fecal pellets from each day were thawed and extracted with approximately 2 volumes of distilled water. The fecal suspensions were homogenized and centrifuged by the method described above. GSA content of each aqueous extract was determined by the entirely polyclonal antibody anticyst antigen capture ELISA method (15) after diluting a portion of each extract 10-fold in PBS-T as described above. The ELISA system utilizes rabbit anticyst IgG as the capture antibody and mouse anticyst serum as the secondary antibody (15). Each extract was assayed in duplicate. Antigen capture was allowed to occur overnight at 4°C. Cyst excretion levels were not monitored in this experiment. Gerbils were sacrificed on day 30, and the duodenal scrapings were checked microscopically to determine whether the animals remained infected. Duodenal scrapings were made with a scalpel at approximately 2 to 4 cm below the pyloric sphincter, and wet mounts of the scrapings were prepared in saline with 1% D-glucose on microscope slides. One wet mount from each animal was examined at ×100 and ×400 magnifications.

Human specimens. Five fresh (nonformalinized) stool specimens were obtained from different patients with clinical giardiasis in Seattle, Wash., with the assistance of clinical staff at a major medical center there. The specimens were obtained prior to chemotherapeutic intervention; however, the duration of infection in each patient was not known. These specimens were part of a larger stool bank accumulated over several years by H.H.S. The specimens were selected for this study largely on the basis of two factors: (i) the high GSA content of aqueous extracts of each specimen, as determined by the monoclonal antibody-based ELISA by using aqueous stool extracts diluted to a 1:60 final dilution in PBS-T; and (ii) the high parasite content of each, as reported by the clinical laboratory personnel. All five specimens had shown moderate to high relative levels of *Giardia* cysts but not trophozoites, as determined by examination (by trained clinic staff) of direct, unstained wet mounts of the specimens with phase microscopy at ×100 and ×400 magnifications. The ELISA absorbance values of the specimens (determined at a 1:60 final dilution, as previously described) (14) were as follows: specimen 1 (numbers correspond to patients; see Fig. 6), 1.0; 2, 1.395; 3, 0.411; 4, 0.601; and 5, 0.308. Five specimens were also obtained from five paid student volunteers who claimed to have had no history of giardiasis and not to be experiencing giardiasislike symptoms at the time of passing the stool specimen. The latter specimens were tested for the presence of GSAs by the monoclonal antibody-based ELISA and were all found to be negative. Aqueous eluates were made of each of the 10 stools by adding two parts of distilled water to one part of stool in a centrifuge tube, mixing, and centrifuging as done with the gerbil feces. These eluates were stored frozen at −10°C.

SDS-polyacrylamide gel electrophoresis and Western blotting. The human and gerbil aqueous fecal eluates were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under reducing conditions (7) and then transferred to nitrocellulose by Western blotting (16) as follows. Equal volumes of the initial aqueous fecal eluates and of 2× sample buffer (2% SDS, 125 mM Tris [pH 8.0], 20% glycerol, 2% 2-mercaptoethanol, and 0.001% bromphenol blue) were added to a capped 1.5-ml polypropylene vial, heated in boiling water for 2 min, and then cooled on ice. Forty microliters of each solubilized sample and of similarly solubilized high-molecular-weight standards (Sigma Chemi-

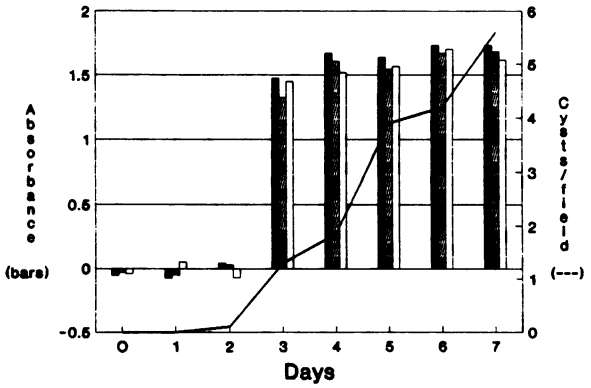

FIG. 1. Levels of GSA, expressed as absorbance (determined by monoclonal antibody-based capture ELISA) (vertical bars) and as number of cysts in feces (mean number of cysts per ×400 microscope field) (line) after experimental infection with H-2 isolate *G. lamblia*. Fecal cyst levels were determined in only one gerbil, and ELISAs were performed on extracts from three gerbils.

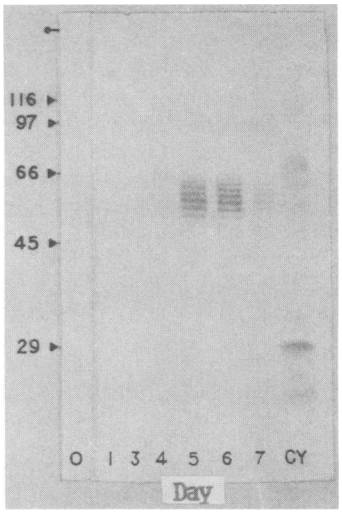

FIG. 2. Western blot analysis of aqueous fecal extracts from gerbils in first experiment, from 0 to 7 days postinoculation with H-2 *G. lamblia* trophozoites. Location of antigens was revealed with rabbit anticyst IgG. A sonicated cyst preparation was run in the lane at far right, marked CY.

cal Co., St. Louis, Mo.) was then applied to starting lanes of the 4% acrylamide stacking gel and electrophoresed through the stacking gel at 20 mA and then through either a 10% (gerbil specimens) or 12% (human specimens) running gel (160 by 180 by 1.5 mm) at 40 mA by methods described by Laemmli (7). Protein in the aqueous eluates was not measured. (Electrophoresis under nonreducing conditions or with nondenatured samples was not attempted.) The electrophoretically separated proteins were transferred to nitrocellulose (0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) by the method of Towbin et al. (16) at 100 mA for 16 to 18 h. After briefly washing the nitrocellulose membrane with PBS-T, the lanes containing the transferred protein standards were cut out and the proteins were stained briefly with 0.1% fast green in methanol-glacial acetic acid-water (5:1:4), destained in the solvent without stain, and air dried. The rest of each nitrocellulose membrane was first washed briefly in PBS and was then incubated for 1 h at room temperature in a solution of 5% nonfat dry milk (generic) in PBS to block nonspecific binding sites. Immunoreactive giardial antigens were then visualized by incubating the nitrocellulose membranes for 2 h at room temperature with rabbit anticyst IgG prepared against cysts derived from experimentally infected immunosuppressed gerbils as previously described (15), with rabbit IgG against cultured WB strain (ATCC 30957) *G. lamblia* trophozoites (previously cloned in our laboratories), or with the IgG of preimmune, normal rabbit serum (as a control). For this purpose, IgGs were diluted to 8 μg of protein per ml in PBS-T. IgG was isolated by the caprylic acid method (13), and protein was estimated by the Bradford method (2). After being briefly washed three times in PBS-T, the blots were incubated for 1 h at room temperature in peroxidase-conjugated, affinity-purified goat anti-rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), diluted 1:1,000 in PBS-T, washed twice in PBS-T, washed once in PBS, washed once in distilled water, and then developed with 4-chloro-1-naphthol substrate. Molecular weights were estimated by reference to the stained molecular weight markers.

RESULTS

When three gerbils were infected experimentally with *G. lamblia* trophozoites by gavage and then the GSA levels and cyst excretion levels were monitored (Fig. 1), it was found that the level of monoclonal antibody-specific GSA rose suddenly and dramatically at the time point (3 days postinoculation) at which cyst levels in the feces had risen to an average of 1.3 cysts per ×400 field. At this time, the average of the GSA levels in the gerbils had risen to 83% of the maximum levels seen at days 6 and 7. Cyst excretion was detectable but very low on the previous day, but at that time GSA were undetectable. GSA excretion seemed to peak by day 6, at which time cyst levels were still rising quickly. (It is not known when cyst excretion levels reach a maximum in gerbils infected with the H-2 isolate, although general cyst-shedding characteristics of gerbils experimentally infected with *G. lamblia* cysts have been described by Faubert et al. [4]).

When the aqueous, pooled extracts of the collected gerbil feces were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting by using rabbit anticyst IgG as the antibody probe (Fig. 2), a stepladderlike series of evenly spaced antigenic bands appeared on days 4 to 7 in the molecular mass range of 45 to 66 kilodaltons (kDa), became darkest on days 5 and 6, but appeared to fade in intensity by day 7 (the bands were weak but visible on day 4 but do not show up in the figure). The bands appeared darkest at a molecular mass of 55 to 65 kDa. These bands, however, did not correlate with bands observed in a sonicated preparation of H-2 isolate *G. lamblia* cysts which was run simultaneously (Fig. 2), nor did they correlate with bands seen when in vitro-cultivated trophozoite antigen was Western blotted against anticyst antibodies (not shown).

Water-soluble antigen from feces collected over a 30-day period from a second group of gerbils, infected this time with the H-3 isolate, revealed a similar set of stepladder bands on days 2 through 6 in the molecular mass range of 45 to 70 kDa when analyzed by Western blotting with anticyst antibodies (Fig. 3) (traces of the bands could be seen on day 6 but do not show up in the figure). These bands were darkest in the blots in the mass range of approximately 55 to 70 kDa. By day 8 postinoculation, this unique set of antigenic bands was no longer evident and did not reappear later during the 30-day

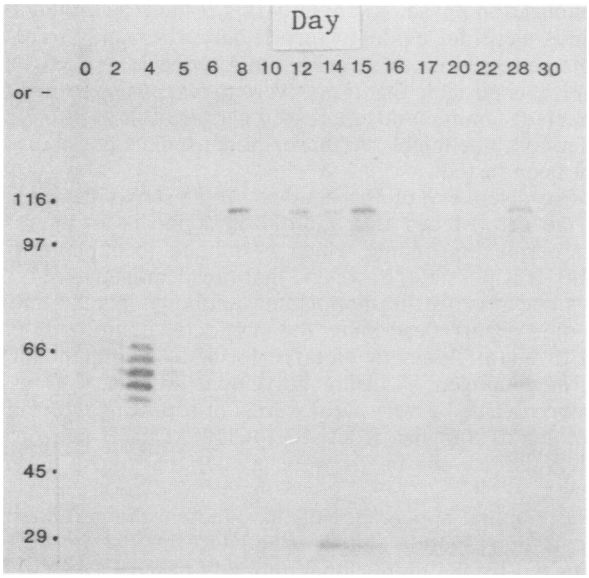

FIG. 3. Western blot analysis of *G. lamblia* cyst antigens in combined aqueous fecal extracts of three gerbils in the second experiment taken on days 0 to 30 postinoculation with H-3 isolate *G. lamblia* trophozoites. Antigen locations were revealed with rabbit anticyst IgG.

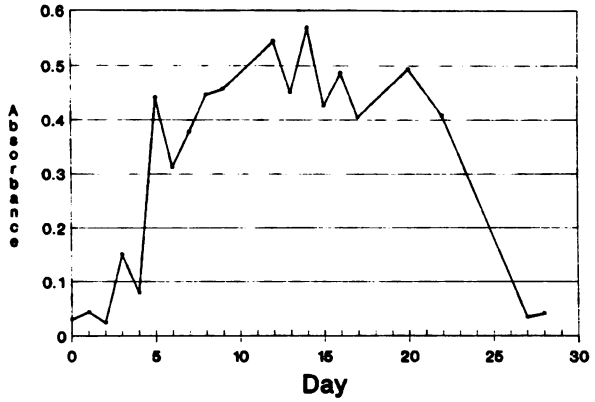

FIG. 5. Levels of GSAs (as absorbance) in combined aqueous fecal extracts of three gerbils in the second experiment over 28 days of infection, as determined by polyclonal antibody-based antigen capture ELISA.

experiment. However, on day 8 postinoculation, two days after the stepladder bands had disappeared, an antigenic band at approximately 110 kDa appeared and was evident in the blots on days 8, 12, 14, 15, and 28. In addition, on day 10 a small-molecular-mass antigenic band below 29 kDa showed up and was present in the blots on every day thereafter until days 28 and 30.

Duplicate blots of the same aqueous fecal extracts, probed with polyclonal antitrophozoite antibody, revealed a small-molecular-mass band at approximately 20 kDa on days 14 through 17 (Fig. 4). Thin bands were also seen at approximately 116 kDa on days 14 to 17 and at a large molecular mass (approximate mass was not determined) on days 6 and 15 to 28. A dark, diffuse band appeared at a molecular mass higher than 116 kDa on days 15 and 16. The stepladder bands at 45 to 70 kDa, seen previously when anticyst antibody was used as the probe, were not evident when antitrophozoite antibody was used, at any time during the 30-day period.

Control blots performed by using preimmune rabbit IgG revealed no bands whatsoever in the gerbil fecal extracts in each of the two experiments.

When levels of water-soluble GSAs were monitored in aqueous fecal extracts of these gerbils on days 0 through 28 by using polyclonal antibody anticyst antigen capture ELISA (Fig. 5), GSAs were found to appear at day 3, to dip slightly on day 4, and then to rise to high levels suddenly on day 5, an increase similar to that noted above on day 3 in the first experiment. Stool antigen levels remained high until after day 22; by day 28, stool antigen was no longer detectable. When the gerbils were sacrificed on day 30, trophozoites were detectable in only one of the gerbils, but only very few (three) were seen. It was apparent that after one month of infection, the gerbils had eliminated or nearly eliminated the infection, presumably through immune mechanisms.

Western blots of the aqueous fecal extracts from five human giardiasis patients were performed using anticyst rabbit IgG as the probe (Fig. 6, patients 1 through 5), but these did not show either the stepladder bands of 45 to 70 kDa or the 110-kDa band which was evident in the gerbil experiments. Instead, in the human fecal blots, two to three bands at a molecular mass range of approximately 15 to 20 kDa were seen. These bands were clearly in approximately the same molecular mass region as the small-molecular-mass bands seen in the 30-day gerbil experiment (Fig. 3 and 4). Control blots performed with preimmune rabbit IgG revealed no bands.

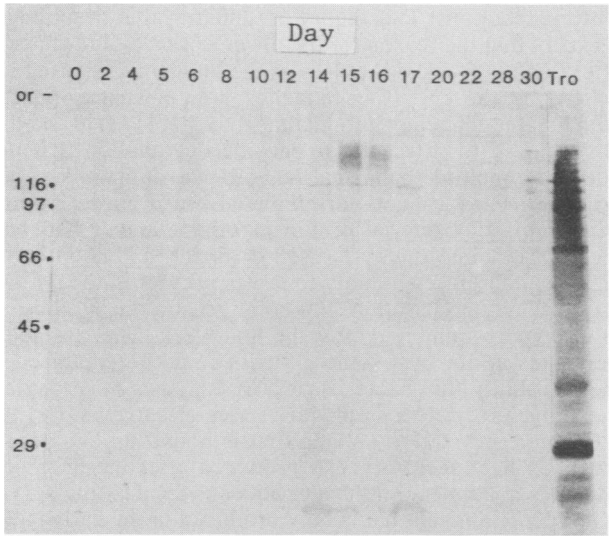

FIG. 4. Western blot analysis of *G. lamblia* trophozoite antigens in combined aqueous fecal extracts of three gerbils in the second experiment taken on days 0 to 30. Antigen locations were revealed with rabbit IgG against cultured WB strain trophozoites (Tro).

DISCUSSION

By using Western blotting with aqueous fecal extracts from human patients and from infected gerbils, it has been possible to identify water soluble, *Giardia* spp.-specific, fecal antigens with potential diagnostic application. The gerbil has been studied before as an animal model of human giardiasis (1, 4, 19, 20), and the present study confirms that this animal is indeed valuable for this purpose.

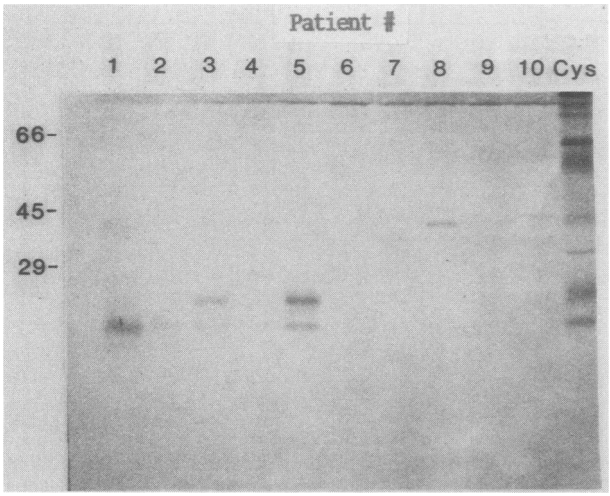

FIG. 6. Western blot analysis of *G. lamblia* cyst antigens present in aqueous extracts of nonfixed stools of five giardiasis patients (1 to 5) and of five uninfected control volunteers (6 to 10). Cys, A sonicated cyst antigen preparation.

When the results obtained with gerbil and human fecal extracts were compared, the one antigenic similarity appeared to be the small-molecular-mass antigenic bands at approximately 15 to 20 kDa detected by anticyst antibodies. Antitrophozoite antibodies also detected antigen at this approximate molecular mass in the gerbil samples, but only on days 14 to 17. In the 30-day gerbil experiment, this band was not evident until after the early, colonization (the equivalent of prepatent) stage of infection, but then it persisted during the mature stage of infection until the infection was apparently resolved by the host. The five *G. lamblia*-positive specimens of human origin came from patients with patent giardiasis. This suggests that the small-molecular-mass antigens might prove to be diagnostically important. It would be worthwhile to attempt to prepare monospecific antiserum to these electrophoretically isolated antigens in order to see if these antibodies can detect these antigens in stool extracts from giardiasis patients by using ELISA. It is probable, although unproven here, that this antigen(s) is one, perhaps one of many, that is detected in the polyclonal antibody-based ELISA of ours (15). However, it is virtually certain that it is not the antigen to which the monoclonal antibody developed by H.H.S. is directed since, as we have shown previously (14), the monoclonal antibody-specific antigen, after immunoaffinity purification from cysts, is retained by an Amicon CF-25 CentriFlo cone, which retains >25,000-molecular-weight molecules. Of course, it is possible that the low-molecular-weight antigens represent dissociated subunits of the monoclonal antibody-recognized antigen. Reiner et al. (9) have observed heterodisperse antigens in the 21 to 39 kDa mass range in *G. lamblia* induced to encyst in vitro; if it is assumed that some of their low molecular weight antigens are identical to some of those observed here, then it seems probable that this antigen or group of antigens is one that is associated with parasite encystation and is resistant enough to enzymes and physical conditions present in feces to pass from the host in an antigenically reactive and water-soluble form.

In blots performed with the human specimens, there was no trace of the 45- to 70-kDa stepladder bands that had been seen early in the gerbil infections; naturally, it had not been possible to study the human infections during the prepatent, colonization phase, so possibly this is the reason why these bands were not evident. Surprisingly, the bands were also not seen in blots made with sonicated cyst antigen. However, it is possible that if one were to test cysts purified from infected, nonimmunosuppressed gerbils at days 4 to 7 postinfection, one might see these antigens. This possibility has not been tested.

The discovery of this unique set of stepladder antigens in the gerbil feces after giardial infection is an interesting yet perplexing finding which is difficult to explain at this time. It is possible, however, that these antigens are related in some way to the monoclonal antibody-specific antigen. In our report describing the monoclonal antibody-based ELISA (14), Western blots performed on immunoaffinity-purified antigen by using polyclonal anticyst IgG as the probe revealed a very weak series of repeating, stepladder-like bands ranging from 45 to 110 kDa. However, the intervals between bands were greater than those observed here.

The temporal appearance of the bands in the gerbil experiments corresponds almost exactly to the rise in GSA, as detected by the monoclonal antibody-based ELISA (Fig. 1 and 2). But the fading and eventual disappearance of the bands after the first week of infection is puzzling. Reiner et al. (9) did not observe bands like these with in vitro-encysting *G. lamblia*.

There are several possible theories as to the identity of the stepladder antigens and explanations for their appearance and disappearance. (i) The stepladder antigens represent a cyst-specific antigen that in its native state gives no band on Western blotting but that is released by the encysting parasite, perhaps as an excess component in cyst wall construction. In the early, colonization stage of infection, the antigen is partially degraded by enzymes, perhaps glycosidases or proteases, originating in bacterial flora, in intestinal epithelial cells, or in the parasite itself. Repeated subunits are removed from the antigen by this enzyme action, yielding products with a regular array of molecular weights. The enzymes are active or abundant or both during the early colonization phase of infection but become absent or inactive after the first week. At that time the native antigen ceases to be degraded and also ceases to give a positive blotting result. (ii) This theory is similar to that described in i, except that the reason for the disappearance of the antigen after the first week is that less of the antigen is released and wasted by the encysting parasites, i.e., it is incorporated with greater efficiency into increasingly stable cysts and thus not exposed to extracellular enzyme degradation that produced the multiple bands earlier. (iii) The antigen is of host origin, perhaps a mucus or epithelial brush border component, or is of bacterial or fungal origin, and it had been incorporated into the cysts used originally for rabbit immunization; therefore antibody to it was present in the anticyst IgG antibody preparation. For some reason the synthesis of this antigen is inhibited after the first week. The absence of the bands in our cyst sonic extracts could be explained by the possibility that bacterial or host enzymes must degrade the antigen to yield a stepladder series of antigens. (iv) It is a *Giardia* encystation antigen that is bound up and precipitated by host secretory IgA produced after the first week and does not appear in the later aqueous fecal extracts. (v) It is a *Giardia* antigen that is only produced in the early stages (or only as a heterodisperse molecule in early stages) of animal infection (but not by the parasite during encystation induced artificially in vitro), and it cross-reacts with or is present as a related antigen of a single molecular weight

found in mature cysts. (vi) The infecting isolates were mixtures of strains, one of which produced this peculiar antigen, but the growth of this one strain was competitively inhibited by the presence of one or more faster-growing strains, and thus the production of this antigen ceased after the first week. (vii) The bands represent molecules produced as a part of a nonspecific intestinal (and not necessarily antibody) response to colonization by an intestinal pathogen and are not limited to giardial infection; antibodies to these host molecules might have been produced in the original rabbit antiserum because the molecules could have been adsorbed onto or present within the cysts used as the immunogen.

It is interesting to note that a single band at 65 kDa, which could have corresponded to the GSA-65 antigen reported by Rosoff and Stibbs in 1986 (11, 12), was not visible in either the gerbil or the human specimen blots. The possibility exists that the GSA-65 antigen is the source of the 45- to 70-kDa stepladder bands seen in the gerbil experiments, since the former antigen was shown to be at least partially composed of carbohydrate. The previous reports indicated that no problems existed in Western blotting of this antigen, so it is difficult to understand why this antigen would fade in intensity after the first week. The five human specimens all had high titers of GSAs by our monoclonal and polyclonal ELISA (see Materials and Methods) but also showed no sign of a GSA 65-kDa molecule. This casts some doubt not upon the existence of such an antigen but upon the previously reported apparent molecular weight (11, 12). It is possible that the real GSA-65 antigen, which is the antigen reportedly detected by a commercially available ELISA kit for diagnosis of *G. lamblia* infecton (10), does not give a Western blotting band and may have a molecular weight very different from 65,000. The band seen in the original reports describing this antigen might have resulted from the presence of a contaminant in the immunoprecipitate used as the antigen and the presence of a cross-reactive contaminant in cyst and trophozoite cell preparations, although this has not been proven.

In conclusion, the use of Western blotting together with the use of the gerbil model for *G. lamblia* infection has provided several new clues to antigens that may have diagnostic utility or important roles in the transformation of trophozoites into cysts or both. Three antigens or groups of antigens have been identified through the use of the gerbil as an experimental model. One of these antigens, with a molecular mass of 15 to 20 kDa, was also seen in five specimens from giardiasis patients. However, because of the shortcomings associated with Western blotting (i.e., antigenic denaturation by SDS, enzymatic degradation, and variability in affinity of macromolecules for nitrocellulose), we recognize the possibility that other potentially important *G. lamblia*-derived molecules exist in the stool during infection. For example, water-insoluble antigenic products of this parasite might exist in the stool but would not have been detected by our methods. The relationship of the stool antigens identified here to the 5-3C monoclonal antibody-specific antigen (14) and to the GSA-65 antigen has yet to be established. The origins and chemical nature of the fascinating stepladder bands also deserve further attention.

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