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Monoclonal Antibody-Based Enzyme Immunoassay for *Giardia lamblia* Antigen in Human Stool

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A visually readable monoclonal antibody-based antigen-capture enzyme immunoassay for the detection of *Giardia lamblia* antigen in human stool specimens was developed and found to be 97% (30 of 31 stool specimens) sensitive for formalinized stools and 82% (49 of 60 stool specimens) sensitive for unfixed stool specimens by visual reading. The storage of specimens in 10% Formalin resulted in increased absorbance in 20 of 26 *G. lamblia*-positive specimens tested as both formalinized and unfixed specimens; the increase averaged 1,336%. The assay was specific for antigens of this organism and for antigens derived from the cyst, as opposed to the trophozoite, stage. The assay could detect the antigens of five cysts per well, but could not detect antigen in in vitro-cultured trophozoites. A mouse monoclonal antibody of the immunoglobulin G1 (IgG1) subclass, which was prepared against cysts of *G. lamblia*, was used as the solid-phase capture antibody. The antibody was reactive with the cyst wall, as determined by immunofluorescence. Polyclonal rabbit anti-cyst IgG was used as the secondary antibody, and peroxidase-labeled goat anti-rabbit IgG was used as the tertiary antibody in the assay format. Maximal capture of antigen from stool specimens occurred by 30 min. Optimal dilution of specimens was in the range of 1:60 to 1:600. Preliminary characterization of affinity-purified antigen recognized by the monoclonal antibody showed that it is heat stable (100°C, 12 min) and resistant to sodium periodate treatment and that it may exist in multiple molecular weights from 45,000 to 110,000.

Giardia lamblia is a common cause of gastrointestinal illness worldwide (2, 12, 27) and is transmitted by direct contact and also through cyst-contaminated drinking water (5, 12). The disease giardiasis may exist in acute or chronic forms, with both forms being almost equally common (3, 26). Cyst excretion rates are highly variable in patients with either form of the disease, and sometimes the parasite cannot be found in the stools of patients with giardiasis even with repeated microscopic examination (6, 26). Because of this, diagnosis by microscopy is not entirely reliable (26); and there is a need for a rapid, stable, and highly sensitive assay for the detection of cell-free giardial antigens in stool specimens. Antigen-detection assays involving counterimmunoelectrophoresis (4, 15, 24) and antigen-capture enzyme-linked immunosorbent assay (ELISA) have previously been reported (9, 11, 20, 23). Our previously reported polyclonal antibody-based assay (20) was 92% sensitive; prior fixation of stool specimens in 10% Formalin was found to have no detrimental effect on the ability of that assay to detect the antigen. For the first time, I report here an enzyme immunoassay based on the capture of water-soluble giardial antigens in stool specimens with a solid-phase mouse monoclonal antibody, followed by sandwiching with rabbit anti-cyst polyclonal antibody.

MATERIALS AND METHODS

Monoclonal antibody production. Female BALB/c mice were immunized with cysts of the H-2 isolate of *G. lamblia* from a human source. Cysts were isolated and purified from the feces of experimentally infected, dexamethasone-immunosuppressed gerbils as described previously (20) and stored at 4°C in distilled water with antibiotics for up to 10 days before use. Mice received biweekly intraperitoneal injections of cysts suspended in distilled water (1×10^6 to 2×10^6 cysts per mouse per injection) for a total of four injections. Ten days after the last intraperitoneal boost, the mice were boosted by injection of 2×10^6 cysts in sterile normal saline into the tail vein. Four days later, mice were sacrificed and their spleen cells were fused with cells of the NS-1 (P3-NS-1-Ag 4.1) mouse myeloma cell line by using 40% (wt/vol) polyethylene glycol (molecular weight, 1,500) in RPMI 1640 medium without serum. Then, the resulting hybridoma cells were selected for and grown at 37°C in 96-well culture plates by using medium with 15% fetal bovine serum and hypoxanthine-aminopterine-thymidine supplement by previously established methods (8). Hybridomas secreting antibody to *Giardia* cysts were detected by indirect immunofluorescence by using air-dried, methanol-fixed spots of cyst-laden human feces obtained from a patient; the human feces was placed at the bottom of 96-well polystyrene microtiter plates (no. 3915; Falcon Plastics, Becton Dickinson and Co., Lincoln Park, N.J.). The secondary antibody used was fluorescein-labeled goat anti-mouse immunoglobulins antibody (Cooper Biomedical, Malvern, Pa.), which was used at a 1:80 dilution in 0.0175 M phosphate-buffered saline (PBS; pH 7.4). Plates were inverted for fluorescence microscopy and were viewed by using long-distance ×10 and ×40 objectives. The positive hybridoma cells were cloned three times by limiting dilution in 96-well culture plates by using mouse thymocytes as feeder cells. A stable immunoglobulin G1 (IgG1)-secreting hybridoma cell line, called 5-3C, which proved to be effective in stool antigen capture, was thus produced. Its antibody was reactive with the cyst wall of *G. lamblia*, as determined by immunofluorescence. Ascites fluid was produced in male BALB/c mice that were pretreated with pristane (Sigma Chemical Co., St. Louis, Mo.) by injecting them with 10^7 cells from a log-phase culture. Antibody was purified from ascites fluid by precipitation with 45% saturated ammonium sulfate, dialysis of the precipitate against 0.01 M Tris hydrochloride buffer (pH 8.0; exclusion dialysis tubing with a molecular weight of 12,000 to 14,000), followed by ion-exchange chromatography with a DEAE-Sephacel

column (1.8 by 18 cm; Sigma) and elution with a linear gradient of 0 to 300 mM NaCl in 0.01 M Tris hydrochloride buffer (pH 8.0). The antibody peak was detected by using A_{280} and by indirect immunofluorescence with *Giardia* cysts. The antibody was dialyzed back into 0.01 M Tris hydrochloride buffer (pH 8.0) and reconcentrated by using a chamber (Diaflo; Amicon Corp., Danvers, Mass.) with nitrogen gas pressure and a membrane (YM-30; 30,000-molecular-weight exclusion; Diaflo; Amicon). It was then stored at a concentration of 4.7 mg of protein per ml at 4°C, with 0.002% chlorhexidine (Hibitane; Sigma) used as a preservative. The antibody isotype was determined by double diffusion in 1% agarose gel by using anti-mouse antibody-isotype antibodies (Sigma). Protein concentrations of antibodies and antigens were measured by the method of Bradford (1).

Polyclonal antiserum. Rabbit serum to *G. lamblia* cysts was prepared in New Zealand White rabbits as described previously (20). Briefly, cysts were obtained from immunosuppressed gerbils that were experimentally infected with the H-2 and H-3 isolates of *G. lamblia* (isolates were obtained from Charles Hibler, formerly of Colorado State University, Fort Collins, and adapted to culture in TYI-S-33 medium by H. H. Stibbs). A total of approximately 55×10^6 cysts, representing approximately 2.2 mg of protein, were injected into each rabbit during the course of immunization. IgG was isolated from serum by the caprylic acid method (18). IgG was stored at a concentration of 0.6 mg of protein per ml at −20°C until use.

Patient specimens. Stool specimens from 60 patients with symptomatic giardiasis were provided by local clinical laboratories (see Acknowledgments). Each specimen was examined by trained clinical staff and found to contain *G. lamblia* cysts, trophozoites, or both. This was determined by microscopy on direct wet mounts of stool specimens or on stool concentrates made by the ethyl acetate-Formalin method (28). These specimens were microscopically positive for this parasite only. However, in most cases Schaudinn-fixed, stained wet mounts were not prepared or examined; therefore, the possible presence of trophozoites of other protozoan parasites, such as *Dientamoeba fragilis*, in these specimens could not be ruled out. Seven specimens containing *Entamoeba histolytica* cysts or trophozoites, four specimens containing *Cryptosporidium* oocysts, one specimen containing *Enteromonas hominis*, and seven specimens containing *Blastocystis hominis* were also provided and tested. Twenty-six *G. lamblia*-negative control specimens were also obtained from paid student volunteers; each of these specimens was examined once microscopically at magnifications of ×100 and ×400 as a direct smear or a smear made after concentration by the ethyl acetate-Formalin technique (27). Each was found to be negative for parasites. Each volunteer claimed to have experienced no giardiasislike symptoms and to have never been previously diagnosed as *G. lamblia* positive. However, because *Giardia* infection is often asymptomatic, and because detection of *Giardia* infection often requires examination of three or more stool specimens over a period of several weeks or more (26), I could not exclude the possibility that some of the controls might have been positive for *G. lamblia*. A portion of 26 of the *G. lamblia*-positive specimens and of 20 of the *G. lamblia*-negative control specimens was also taken and stored in 10% Formalin in order to evaluate the effects of formaldehyde on the stability and detectability of the *Giardia* antigens by enzyme immunoassay. In addition, five other formalinized specimens (not tested as unfixed) were also tested, giving a total of 31 formalinized *G. lamblia*-positive specimens tested in all. Finally, 119 formalinized stool specimens (formalinized by the patient at the time of defecation in prepared, take-home parasite specimen kits that were commercially available) were submitted to three large medical laboratories in Seattle, Wash. (see Acknowledgments), for ova and parasite examination and were obtained from these laboratories after they were coded by laboratory staff. These stool specimens were tested in a double-blind format by the enzyme immunoassay described below. In the clinical laboratories, these specimens were all examined for parasites after they were concentrated by the ethyl acetate-Formalin concentration method (28).

Stool eluate preparation. Aqueous eluates were made of a portion of each stool specimen by adding approximately 2 parts of distilled water to 1 part of stool in a 15-ml polypropylene centrifuge tube. They were stirred well with a thin, wooden applicator stick and then centrifuged at 900 × *g* for 10 min, after which the supernatant was aspirated and saved and the pellet was discarded. The supernatant was stored at −20°C. Formalinized eluates were prepared by adding 2 parts of 10% Formalin in 0.0175 M PBS (pH 7.4) to 1 part of stool in a 15-ml centrifuge tube and mixing as described above. Then, the formalinized stool specimens were stored uncentrifuged at 4°C; gravity-induced sedimentation left a clear supernatant in these specimens. Stool specimens were stored at 4°C for 1 to 7 days prior to elution by either method. All eluates were diluted 1:20 with PBS–Tween 20 (0.05%) (PBS-T) to a final dilution of approximately 1:60 for the enzyme immunoassay.

Monoclonal antibody-based sandwich ELISA. The antigen-capture ELISA was performed as follows. Flat-bottom, 96-well polystyrene plates (no. 3915; Falcon Plastics, Becton-Dickinson and Co.) were coated overnight at 4°C with 50 μl of purified monoclonal antibody at a concentration of 5 μg of protein per ml in 0.05 M carbonate buffer (pH 9.5). (Optimal concentrations of all antibodies were determined by checkerboard titration.) Afterward, the plates were rinsed twice with distilled water, and nonspecific binding sites were blocked by filling all wells with a solution of 5% nonfat dry milk in PBS and incubating them for 30 min at 37°C. Plates were rinsed again with distilled water, air dried for 1 h, and stored tightly wrapped in plastic wrap at 4°C for 1 to 2 days. Stool specimens were assayed in duplicate. A total of 50 μl of each stool eluate, diluted 1:60 in PBS-T, was added to each of four adjacent wells located in two adjacent columns, and the plates were wrapped in plastic wrap and incubated at 37°C for 2 h. Plates were then rinsed four times with PBS-T; and then 50 μl of rabbit anti-cyst IgG, at a concentration of 2 μg of protein per ml, was added to odd-numbered columns of wells, while preimmune rabbit IgG, at the same concentration, was added to even-numbered columns. Plates were incubated for 1 h at 37°C and rinsed as described above; and 50 μl of horseradish peroxidase-labeled, affinity-purified, goat anti-rabbit IgG (heavy and light chains; Boeringer Mannheim Biochemicals, Indianapolis, Ind.) diluted 1:1,000 in PBS-T–1% bovine serum albumin was added to all wells. Plates were incubated for 30 min at 37°C, rinsed as described above, and then developed by adding 50 μl of a solution of enzyme substrate (40 mg of *o*-phenylenediamine in 100 ml of PBS containing 400 μl of 3% hydrogen peroxide) to all wells. The plates were then incubated for 10 min at ambient temperature in the dark. The chromogenic reaction was stopped by adding 50 μl of 2.5 N H_2SO_4 to all wells. Visible reactions were recorded immediately, and then the plates were scanned spectrophotometrically at 492 nm by using an automatic ELISA plate reader

(Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.). The adjusted absorbance value for each specimen was calculated as the average of the difference in absorbances between the two wells that received immune secondary antibody minus those of the two wells that received preimmune secondary antibody. The statistical cutoff point for positive values was taken as the mean adjusted absorbance of the *G. lamblia*-negative control specimens plus three times the standard deviation of these values.

Optimal duration of the antigen-capture step. Five nonformalinized *G. lamblia*-positive stool eluates diluted 1:60 in PBS-T were assayed by using the following five different time periods of antigen capture: 1, 5, 15, and 30 min and 2 h.

Optimal stool dilution. Nine *G. lamblia*-positive unfixed stool specimens were tested at various dilutions, from 1:30 to 1:600,000. All other conditions of the assay remained the same.

Attempted use of other secondary antibodies. Polyclonal rabbit IgG against in vitro-cultured *G. lamblia* trophozoites, rabbit IgG against cysts of *Giardia muris*, rabbit IgG against the previously described GSA 65 antigen of *G. lamblia* (15), and peroxidase-labeled 5-3C monoclonal antibody were all tested as secondary antibodies in the assay after monoclonal antibody capture of antigen from *G. lamblia*-positive stool specimens as described above. The purified monoclonal antibody was conjugated to horseradish peroxidase by the periodate method of Nakane and Kawaoi described previously (21). The conjugated antibody was tested by direct ELISA with plates coated with a sonicated preparation of cysts to confirm that the antibody retained activity for the antigen.

Immunoaffinity isolation of the *Giardia* antigen. Purified monoclonal antibody 5-3C was used to isolate by immunoaffinity the *Giardia* antigen for which the antibody was specific from a sonicated preparation of *G. lamblia* cysts. Antibody was linked to a 0.8-ml (wet volume) column of Sepharose 6MB beads (Pharmacia Fine Chemicals, Piscataway, N.J.) by using coupling buffer (0.1 M bicarbonate buffer [pH 9.3] with 0.5 M NaCl) for 4 h at ambient temperature. The column was rinsed sequentially with coupling buffer–0.5 M NaCl–PBS–2.5 M NaI in 0.01 M phosphate buffer (pH 8.0)–PBS and then with 0.2 M glycine in 0.01 M phosphate buffer (pH 8.0). It was incubated overnight at 4°C with the glycine solution to block excess linking sites on the gel. Approximately 30×10^6 cysts of *G. lamblia* (H-2 isolate) purified from the feces of infected gerbils were suspended in 1.5 ml of PBS, freeze-thawed twice, and then sonically disrupted while they were immersed in ice (10 sonic bursts of 15-s duration each) by using a sonifier equipped with a microprobe tip. The resulting sonic extract was checked microscopically to confirm that >95% of the cysts were disrupted. Tween 20 was then added to 0.05% (vol/vol), and the sonic extract was centrifuged at $900 \times g$ for 15 min. The soluble sonic extract fraction was applied to the affinity column and allowed to incubate with it at ambient temperature for 1 h, after which it was allowed to elute by gravity. The eluate was passed through the column 15 additional times over a period of 15 min. The column was then rinsed twice with PBS-T, twice with 0.5 M NaCl, and twice with PBS again. Bound antigen was eluted with 3.5 ml of 2.5 M NaI in 0.01 M phosphate buffer (pH 8.0). This solution was passed through the column four times and was dialyzed overnight at 4°C against PBS containing 0.05% NaN_3. The antigen solution was concentrated to 1.3 ml of PBS by using a conical membrane (Centriflo CF-25; Amicon). The titer of this eluate obtained by the monoclonal antibody-based enzyme immunoassay was determined to be 1:1,000.

Preliminary physicochemical characterization. The affinity-purified antigen was subjected to heat and periodate treatment in order to assess physical stability and possible carbohydrate structure and was studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blotting (immunoblotting) against both the monoclonal antibody and the polyclonal anti-cyst IgG in order to determine its molecular weight. Heat treatment consisted of submersing an aliquot of the affinity-purified antigen in boiling water for 12 min; 1:60 eluates of a *G. lamblia*-positive and a *G. lamblia*-negative stool specimen were also treated in the same manner. Unboiled antigen and specimens served as controls. The antigen was incubated at 37°C for 14 h with 0.05 M sodium *m*-periodate (Sigma) in 0.02 N acetate buffer (pH 4.5); buffer only was added to a control vial of antigen. The reaction was terminated by adding 0.5 volume of 0.5 M $NaBH_4$. After 45 min at ambient temperature, the samples were diluted fivefold with PBS and then tested at 10-fold dilutions.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting analysis of the antigen, both the purified antigen and a sonicated cyst preparation were electrophoresed in a discontinuous 10% polyacrylamide gel by standard methods by using approximately 40 μg of protein per lane (10). Antigens were transferred electrophoretically to nitrocellulose (22). Strips were then cut out; blocked with 5% nonfat dry milk in PBS; and incubated with monoclonal antibody 5-3C, polyclonal rabbit anti-cyst IgG, or preimmune rabbit IgG. Purified monoclonal antibody was used at 5 μg/ml, and rabbit anti-cyst or preimmune IgG was used at 5 μg of protein per ml in PBS-T. A blot of purified antigen against monospecific rabbit IgG to the previously reported GSA 65 antigen of *G. lamblia* (15) was also performed (IgG was used at 5 μg/ml). Peroxidase-conjugated secondary antibody was either goat anti-mouse immunoglobulins (Cooper Biomedical) or goat anti-rabbit IgG (heavy and light chains; Boeringer Mannheim Biochemicals) diluted 1:1,000 in PBS-T–1% bovine serum albumin.

Stage specificity and intracellular location of the antigen. To determine the intracellular locations of the antigen recognized by the monoclonal antibody, indirect immunofluorescence was performed by using cysts that were present in unfixed human stool specimens, trophozoites of the H-2 isolate from an in vitro culture in TYI-S-33 medium, and trophozoites harvested from the small intestine of infected gerbils. These preparations were air dried as spots onto acetone-cleaned glass microscope slides and fixed in acetone. Monoclonal antibody 5-3C, which was diluted to 10 μg/ml in PBS, was applied to each spot for 45 min at 37°C in a humid chamber; after two brief rinses in PBS, fluorescein-labeled goat anti-mouse immunoglobulin antibody (Cooper Biomedical) diluted 1:80 in PBS–1% bovine serum albumin was added, and the slides were incubated for another 45 min. Slides were viewed by epifluorescence (excitation wavelength, 450 to 490 nm; emission wavelength, >520 nm) at ×400 and ×1,000 magnifications. The ability of the enzyme immunoassay to detect antigen in sonicated (in PBS) preparations of *G. lamblia* cysts and of culture-derived trophozoites was determined. Antigens were assayed at 10-fold dilutions in PBS-T, beginning at the equivalent of 500 cells per well and going down to 5 cells per well. Sonicated preparations of *G. muris* cysts (isolated from a hamster at Case Western Reserve University, Cleveland, Ohio [16]), which were harvested from experimentally infected Swiss

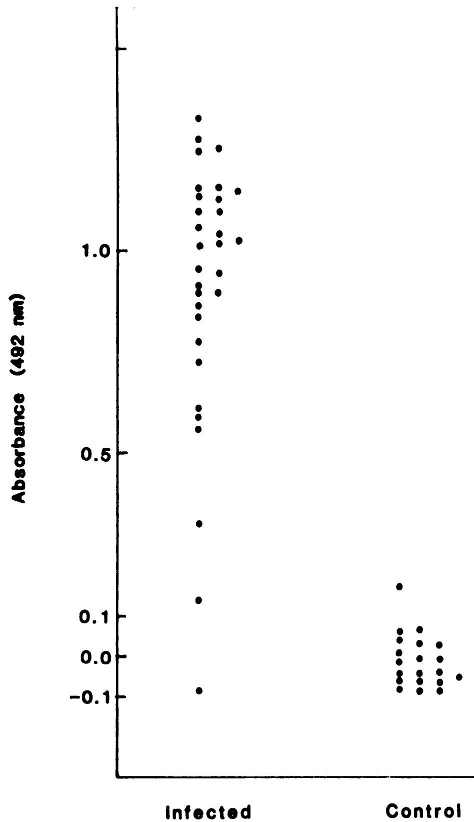

FIG. 1. ELISA results obtained with 31 formalinized, *G. lamblia*-positive specimens and 20 formalinized, *G. lamblia*-negative specimens. Values represent the means of two determinations.

Webster mice, and of in vitro-cultured *Pentatrichomonas hominis* (Diamond strain; American Type Culture Collection, Rockville, Md.) trophozoites were also tested in the assay.

RESULTS

The enzyme immunoassay gave positive visual readings with 30 of the 31 formalinized *G. lamblia*-positive specimens, giving the assay a sensitivity of 96.8%. Statistically, according to the absorbance, 29 of the 31 readings were positive. The adjusted absorbance values are shown in Fig. 1. The mean of the *G. lamblia*-positive samples was 0.905; that of the controls was −0.0163. The absorbance cutoff point for positive samples was 0.176 (calculated as described above). Of the 20 *G. lamblia*-negative control specimens 1 weak visual false-positive specimen was obtained; this specimen was not positive statistically by its absorbance. With unfixed specimens, the sensitivity was lower; in this case, the assay gave positive visual and spectrophotometric readings with only 49 of 60 (81.6%) of the *G. lamblia*-positive stool specimens (Fig. 2). All negative control specimens were visually negative, but one was statistically positive by its absorbance.

A total of 26 *G. lamblia*-positive and 20 *G. lamblia*-negative specimens were tested both as unfixed and as formalinized samples. In the group of specimens containing *G. lamblia*, 25 of 26 of the formalinized specimens proved to be positive by ELISA, while only 18 of 26 of the unfixed specimens were positive by ELISA (Fig. 3). Overall, 20

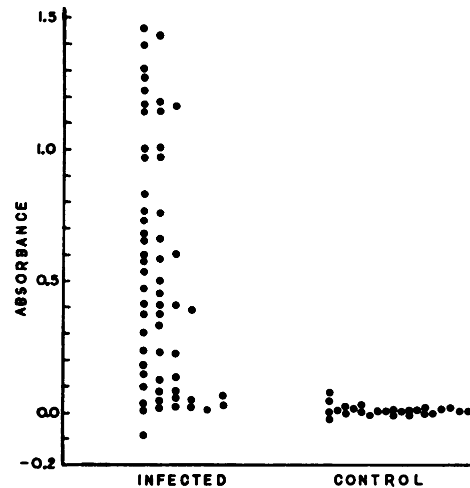

FIG. 2. ELISA results obtained with 60 unfixed, *G. lamblia*-positive specimens and 26 unfixed, *G. lamblia*-negative specimens. Each value is the mean of two determinations.

specimens showed higher absorbances as formalinized samples; the average increase in absorbance was 1,336%. Six samples had lower values as formalinized specimens; of these, one was negative as a fixed or unfixed specimen, and the other five only decreased by an average of 23% and remained strongly positive. Of eight *G. lamblia*-positive specimens that gave negative readings as unfixed specimens, seven converted to strong positive readings (four with adjusted absorbances over 1.0) when tested after Formalin fixation. Four of these eight specimens were also negative by the polyclonal antibody-based ELISA reported previously (20). Statistically, by the McNemar test for correlated proportions (13), the use of Formalin resulted in significantly increased sensitivity ($P < 0.01$) in this group of 26 specimens. In general, enhancement by Formalin was greatest with specimens that were only weakly positive in the unfixed condition. Formalin had no effect on the ELISA readings of the 20 *G. lamblia*-negative specimens.

In the double-blind study performed on 119 consecutively submitted, formalinized stool specimens, the assay yielded 12 visually positive readings. Ova and parasite exam results from the clinics showed a total of nine positive readings, all

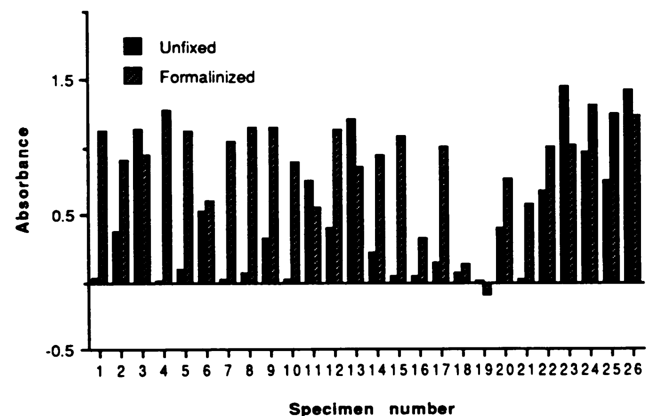

FIG. 3. Adjusted A_{492} values of 1:60 dilutions in PBS-T of both aqueous and formalinized eluates of *G. lamblia*-positive stool specimens from 26 patients.

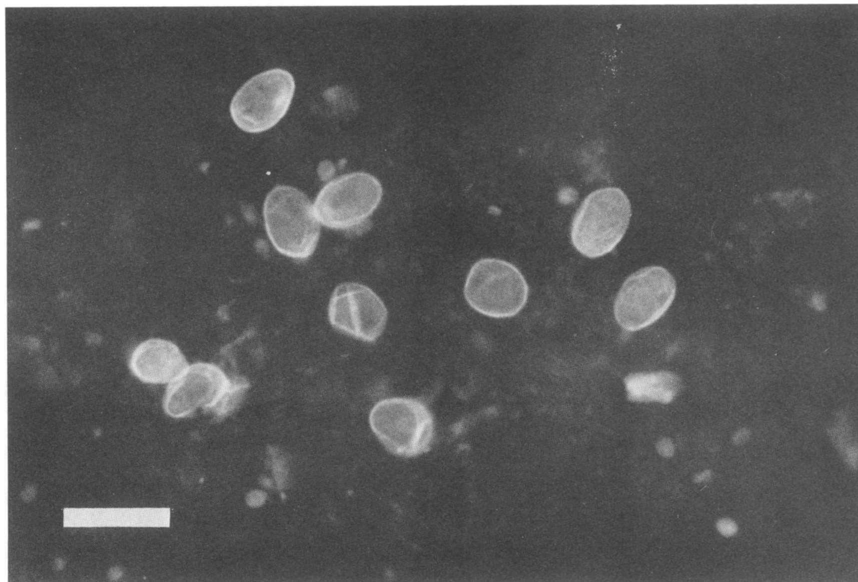

FIG. 4. Immunofluorescence photograph showing the binding of monoclonal antibody 5-3C to cysts of *G. lamblia* present in a smear of feces from a patient with giardiasis. Bar, 20 μm.

of which were detected by this assay. The assay also detected *Giardia* antigens in three ova and parasite-negative specimens, as well. Unfortunately, follow-up specimens from these three patients were not available.

All of the human specimens containing parasites other than *G. lamblia* were negative in the assay.

In the stool dilution experiments with nine different stool specimens, two stool specimens gave a positive visual result even at a 1:600,000 dilution. In three cases a 1:600 dilution gave a stronger result than the 1:60 dilution did, and in one of these the 1:60 dilution was negative, while the 1:600 and 1:6,000 dilutions gave strong positive results. In three stool specimens, the 1:60 dilution was clearly better than higher dilutions, but in most cases 1:300 and 1:600 dilutions were as strong as the 1:60 dilution. It appears that testing of stool specimens at 1:60 and 1:600 dilutions gives optimal results.

When five stool specimens at a dilution of 1:60 were incubated in antibody-armed wells for various periods of time to assess the rate of antigen capture, it was found that the average absorbance was highest (1.353) after 30 min. Individually, two samples peaked at 30 min, two peaked at 2 h, and one peaked at 15 min. The average absorbance at 2 h (1.165) was 13.9%, that at 15 min (1.183) was 13%, that at 5 min (1.164) was 14%, and that at 1 min (1.038) was only 23% lower than that obtained at 30 min. Therefore, 77% of maximal capture seemed to occur in the first minute of incubation.

Experiments with other types of secondary antibodies in the assay revealed that no positive results whatsoever could be obtained by substituting rabbit IgG to *G. lamblia* trophozoites, to *G. muris* cysts, or to the GSA 65 antigen or peroxidase-labeled monoclonal antibody for the rabbit anti-cyst IgG in the assay.

Indirect immunofluorescence experiments showed that the monoclonal antibody bound strongly to an antigen located in or adjacent to the cyst wall of the parasite (Fig. 4). No internal binding in the cyst was evident. *G. muris* cysts also showed a positive reaction with the antibody. No reactivity was observed with in vitro-derived trophozoites; however, in trophozoites obtained from the small intestine of infected gerbils, some binding to unidentified structures, possibly vesicles, located in the cytoplasm posterior from the nuclei was observed (data not shown).

The assay could detect the antigens of as few as five cysts per well (average absorbance with antigens of five cysts per well, 0.362; average absorbance with no cysts added, −0.004). Lower numbers of cysts per well were not tested. The test could not detect antigen in sonicated trophozoites at 500 cysts per well, nor could it detect antigen in sonicated preparations of *P. hominis* or *G. muris* cysts.

Physicochemical characterization studies on the antigen recognized by the monoclonal antibody revealed that the antigen, whether isolated by immunoaffinity chromatography from cysts or as present in whole aqueous stool eluate, was completely resistant to heat treatment. The affinity-isolated antigen was also completely unaffected by periodate treatment. In Western blotting (immunoblotting) experiments, the blotting of cyst sonic extract antigens against the monoclonal antibody gave a sharp but weak band between the origin and the heaviest molecular weight marker, which was 205,000. Blotting of the purified antigen against the monoclonal antibody showed nothing whatsoever, but blotting of it against polyclonal rabbit anti-cyst IgG gave a series of five more-or-less regularly spaced, stepladderlike bands between molecular weights of 45,000 and 110,000. Blotting of purified antigen against anti-GSA 65 IgG gave a strong band at a molecular weight of 65,000 and a sharp band midway between the origin and the 205,000-molecular-weight marker. Blotting of cyst sonic extract antigens against the anti-GSA IgG produced bands at molecular weights of approximately 65,000, 115,000, and 150,000 to 160,000 and a sharp, dark band just below the origin. Blotting with preimmune rabbit IgG gave no bands.

DISCUSSION

The development of a sensitive, monoclonal antibody-based antigen-capture assay for a cell-free, water-soluble antigen of *G. lamblia* has been described. This is the first published report of a monoclonal antibody-based ELISA for

an antigen of *G. lamblia*. The assay was significantly more sensitive with stool specimens that were stored in 10% Formalin than it was with unfixed stool specimens. Maximal capture of the antigen occurred rapidly; 1 min of capture resulted in 76% of the maximum absorbance, which was attained after 30 min of antigen capture. Results obtained in the double-blind study with 119 specimens suggest that the assay is at least as sensitive as microscopic examination of ova and parasites.

From stool dilution studies, it appears that the testing of stool specimens at two or more 10-fold dilutions, beginning at 1:60 or 1:100, would provide optimal sensitivity, since at the higher concentrations of stool the antigen was sometimes not detectable. Nondetection of antigen at higher concentrations may have attributable to the presence of nonspecific blocking substances which were inactive at greater dilutions, by association of the giardial antigen with other molecules or with itself, or by the presence of immune complexes of the antigen. At present, however, there is no clear explanation for this phenomenon.

It is not clear why storage in Formalin enhanced the sensitivity of the assay. Perhaps because the formalinized stool specimens were stored in the Formalin without centrifugation, unlike the unfixed specimens, antigen may have leaked into solution from dead cysts in the stool specimen. It is also possible that formaldehyde denatured interfering compounds or dissociated preexisting antigen-antibody complexes that may have existed in the stool specimen.

The assay appeared to detect an antigen which is completely specific to the genus *Giardia*. No antigen was detected in stool specimens that contained other common intestinal protozoa. While one visual false-positive result occurred in a *G. lamblia*-negative control specimen, the absorbance of this particular sample was so markedly different from the mean and range of the other 19 control samples that it seems possible that this specimen was from a patient who had an asymptomatic *Giardia* infection.

It was surprising that the enzyme immunoassay could not detect antigen in sonicated *G. muris* cysts, since the monoclonal antibody was found to bind to whole cysts of this species by immunofluorescence. It is possible that the antigen is present in an insoluble form in *G. muris* cysts.

Studies on the affinity-purified antigen from cysts revealed that it is a heat- and periodate-insensitive molecule. Western blotting (immunoblotting) experiments with the monoclonal antibody produced a weak band well above a molecular weight of 205,000. It is not yet clear whether this band is meaningful. Sodium dodecyl sulfate might denature the antigenic epitope on the molecule and prevent the binding of the monoclonal antibody. Blotting against rabbit anti-cyst IgG produced a regularly spaced, stepladderlike series of reactive bands between molecular weights of 40,000 and 65,000. The appearance of these bands is strongly reminiscent of Western blots obtained by other investigators working with lipopolysaccharide cell wall antigens of bacteria (7, 14, 17). This, together with the heat stability of the antigen, suggests that the antigen may be a carbohydrate with multiple molecular weights. Ward et al. (25) have found that chitinase destroys the integrity of the *Giardia* cyst wall and that the cyst wall binds only lectins specific for *N*-acetylglucosamine. Chitin is a polymer of *N*-acetylglucosamine. Therefore, the cyst wall contains carbohydrate, and at least one of the important carbohydrates is chitin or a chitinlike molecule. Chitin, however, is very insoluble, unlike the cyst wall antigen detected by the monoclonal antibody used in this study. Whether this antigen binds lectins specific for *N*-acetylglucosamine and its relationship to chitin remain to be determined. The discovery that the monoclonal antibody reacts by immunofluorescence with an antigen that is present in the cyst wall of *G. muris* and other *Giardia* isolates from animals (H. H. Stibbs, manuscript in preparation) suggests that the antigen is an integral and perhaps essential part of the giardial cyst wall. This antigen may be related to the GSA 65 antigen which has been reported earlier (15); however, the two antigens, while they are heat stable, water soluble, and present predominantly in cysts, appear to differ in sensitivity to periodate and in molecular weight, as judged by Western blotting.

It might seem surprising that the monoclonal antibody, which binds to an antigen of cysts and not of trophozoites, is so effective at detecting infection since the cyst excretion rate is so variable in patients with *Giardia* infections. In theory, one would want a capture antibody that binds a stable trophozoite antigen, so that even with symptomatic patients whose stool specimens are repeatedly ova and parasite negative for cysts and trophozoites one could detect the presence of the trophozoite population in the small intestine. This study did not involve the latter type of patients. The group of *G. lamblia*-positive specimens selected for this study necessarily consisted of clinically positive specimens with detectable concentrations of parasites in the stool. All but two of these specimens were reported to have cysts. With the two specimens in which only trophozoites were found, the assay gave a positive result with one and a negative result with the other. Neither specimen was assayed as a formalinized specimen. It is possible that this assay might give relatively poor results with specimens that contain only trophozoites or that have no trophozoites or cysts. However, it is impossible to state that a stool specimen is cyst negative, since there unquestionably exists a level of cyst excretion below which even repeated microscopic examination yields a negative finding. Therefore, one can never conclude that there are no cysts in the stool, only that no cysts were found microscopically. Finally, as we have suggested previously (20), even during periods of very low cyst excretion by a patient, it is conceivable that a fraction of the trophozoite population is attempting encystation and, in doing so, is producing cyst-specific antigens. The attempts at encystation may be aborted by immunological and physical factors, causing trophozoites or "trophocysts" (intermediate forms) to die and degenerate in the lower intestine, releasing their antigens into the lumenal contents of the gut. This hypothesis would help to explain why the monoclonal antibody assay has such a high sensitivity.

Potential use of the monoclonal antibody as both the primary capture antibody and as the secondary detector antibody would eliminate the need for a polyclonal antibody in the test format. Purification of enough cysts for immunization of rabbits is difficult, tedious, and somewhat hazardous because of the possible presence of bacterial and viral pathogens, in addition to *Giardia* cysts, in human stool specimens. The use of infected gerbils for cyst production eliminates most of the hazard, but animals and animal care are costly, and tedious purification of cysts is still necessary. Unfortunately, an attempt to use this monoclonal antibody as a secondary, peroxidase-labeled antibody after the capture of stool antigen in the usual way was not successful, indicating that the reactive epitope is not repeated on the molecule or that, if repeated, other sites are not available after one site has bound to the solid-phase capture antibody. Perhaps monoclonal antibodies to other epitopes on the molecule (assuming that these exist) can be made and used

as a secondary antibody in a two-site monoclonal antibody sandwich ELISA without the need for polyclonal antibody.

Numerous other formats of the assay are possible. I have experimented with a dot antigen-capture ELISA format on nitrocellulose and have found it to be potentially useful. The capture antibody could also be linked to plastic tubes and beads or to latex particles for other ELISA formats. The assay time can, no doubt, eventually be reduced to approximately 10 to 30 min for one sample, as in commercially available antigen-detection tests.

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