# A COMPARATIVE STUDY OF SERUM ANTIBODY SPECIFICITIES AND ANTIGENIC DIFFERENCES AMONG STRAINS AS CONTRIBUTING FACTORS TO CHRONIC INFECTION WITH *GIARDIA LAMBLIA* IN HUMANS

by

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B. S., Missouri Western State College, 1983

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A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology
Microbiology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1985

Approved by:

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Giardia lamblia, (G. intestinalis, G. duodenalis), a single-celled protozoan parasite of man and some lower mammals, is rapidly gaining notoriety as a major cause of morbidity throughout the world. The parasite is distributed in both warm and temperate climates, with a prevalence estimated as high as 30% among the human population in some areas (76). Animal reservoirs constitute a significant source of possible infection, as cross-transmission among species is not uncommon (6, 28, 29, 14); 75% of the dogs in Colorado are infected (82). Giardiasis is the most frequently-documented cause of waterborne epidemic diarrhea in the United States (12).

G. lamblia is a member of the class Mastigophora (81) and order Diplomonadida (69). The infective form of G. lamblia is the cystic stage passed in the feces of the host. The cyst is ovoidal, 8 to 12 μm in length and 6 to 10 μm in width. Mature cysts are tetra-nucleate, indicating that they contain a double trophozoite enclosed in a cyst wall approximately 0.3 μm thick (69). Upon being ingested within contaminated water or food, the organism undergoes excystation in the acid environment of the host's stomach.

The freed, bilaterally symmetrical trophozoites colonize the upper small intestine where they divide longitudinally every five hours, at times invading the gall bladder, bile ducts, circulation, and lymphatics (26). Recently, giardiasis has been associated with proctitis and vaginitis, suggesting that the trophozoites may also inha-

bit the urogenital tract and be sexually transmitted (66). Perhaps the oddest location from which the trophozoites have been recovered is the alveoli of the lungs. In a reported case study, a patient was believed to have regurgitated G. lamblia from his gastrointestinal tract into pulmonary tissue, where it was recovered in bronchoalveolar lavage fluid (75).

The trophozoite appears as a flattened tear drop 10 to 12 μm long with an anteriorly-placed ventral adhesive disc. The trophozoites are binucleate; however, it is not known whether the nuclei have differing functions (85). Four pairs of flagella provide locomotion and means of adherence to the intestinal mucosa; beating ventral flagella expel fluid from the cavity under the ventral disc, producing adhesion (52).

The trophozoites are aerotolerant anaerobes that respire in the presence of $O_2$ by a flavin, iron-sulfur protein-mediated electron transport system. Endogenous respiration is stimulated by glucose but not by other carbohydrates or Kreb's cycle intermediates. G. lamblia lacks mitochondria, golgi, and microbodies, but contains lysosomes which can accumulate ferritin. ATP generation is by substrate level phosphorylation (38).

Encystment, possibly induced by a change in the pH or redox potential of the intestinal environment or by immune interaction (50), is believed to occur in the lower ileum or colon, where the cysts pass out in the feces. An infec-

ted individual can shed 1 x $10^6$ cysts/g of stool, while an infected muskrat can shed 2.5 x $10^6$ cysts/day. However, only 10 cysts need be ingested to establish an infection (57). The cysts are rapidly destroyed by drying, but may survive in wet conditions and remain infective two months or more (26). The cysts are highly resistant to conventional water purification methods, such as filtration (40), chlorination (33, 59), ultraviolet irradiation (58), ozone (84), and saturated iodine or tincture of iodine (32). A new resin-$I_5$, Pentacide (43), effectively inactivates cysts after a recommended five minute waiting period following passage of water through the resin. Inactivation is detected by the inability of the cysts to excyst in HCl at pH 2. Pentacide is commercially available for small scale use, but its application to large-volume water purification is still some years away (Louis Fina, personal communication). Thus, municipal water supplies will most likely continue to facilitate the spread of giardiasis as they have done in the past (68).

In the United States, 40% of giardiasis cases result from drinking contaminated water. The remaining 60% are the result of person-to-person contact, chiefly among children in day care centers and among male homosexuals (66, 82). There were 80 outbreaks of giardiasis in the U.S. from 1965 to 1983, the first documented case occurring in Aspen, Colorado among a group of skiers. Because giardiasis is not a reportable disease, exact quantitation of the individuals infected is difficult. From 1971 to 1980, six infec-

tions were voluntarily reported to the CDC as fatal (9). Rendtorff demonstrated that 86% of infected human volunteers spontaneously cleared the parasite after a short-lived diarrheal illness (57). Others remained asymptomatic carriers and cyst-shedders for years. The acute stage of giardiasis occurs one to three weeks after infection, and is characterized by the sudden onset of explosive, watery, foul diarrhea, flatulence, nausea, and anorexia. Malaise, chills, low-grade fever, and cramps are frequent complaints. The acute stage usually lasts only three or four days but symptoms can be prolonged for years in some patients (36, 74). Several allergic conditions have been reported in association with giardiasis including bronchial asthma, urticaria, arthritis, and uveitis (26).

Variable degrees of success against giardiasis have been claimed for various drugs. Wolfe claimed a cure rate of 95% with quinacrine (atabrine, mepacrine), an inhibitor of flavin-catalyzed electron transport (86). Quinacrine is the most commonly-prescribed treatment in the U.S. (26), but has the potential for serious side effects including severe skin rash leading to exfoliative dermatitis, vomiting, fever, and toxic psychosis (86). Boreham et al. found tinidazole to be the most effective drug against G. lamblia trophozoites in vitro, followed by furazolidone, metronidazole, and quinacrine (8). The side effects of tinidazole are fewer and milder than those of the other three commonly-used drugs and has a cure rate of 93% when

given in a single oral dose of 2.0 g (26). Furazolidone has caused mammary neoplasia in rats; quinacrine, metronidazole, and furazolidone all have the potential for teratogenicity and should not be administered during pregnancy (36).

The mechanism of disease production in giardiasis is still debatable. Toxins have been implicated in a number of diarrheal illnesses, including amoebiasis caused by a related protozoan, Entamoeba histolytica (41). Smith et al. isolated a strain of G. lamblia from a patient who had been symptomatic for giardiasis 2.5 years before finally being cured by a combination of quinacrine and metronidazole drug therapies. The researchers used this strain, WB, in a series of assays to determine whether G. lamblia produces an enterotoxin. The tests included the rabbit ileal loop and rabbit skin capillary permeability factor assays to test trophozoites or culture filtrates for activity analogous to cholera toxin or E. coli heat labile toxin. In addition, a ganglioside $G_{M1}$-ELISA for cholera toxin was employed to probe trophozoite proteins for cross-reacting antigens that might have been biologically inactive. The infant mouse assay was used to test for rapid-acting toxin activity like that of E. coli heat stable toxin. All tests were negative (72).

Abnormalities of the mucosa in giardiasis have been linked to disaccharidase deficiencies, increased intraepithelial lymphocyte counts, and malabsorption (25). Electron microscopy has shown damage to the microvillus surface of

epithelial cells beneath Giardia trophozoites in vivo in both animal (50) and human (64) giardiasis. This damage to the surface epithelium provides the basis for functional abnormalities that occur because the cell membrane of the microvilli contain digestive enzymes and transport mechanisms for monosaccharides and amino-acid absorption (87).

In vitro cultures of G. lamblia trophozoites and the human embryonic pulmonary fibroblast line ICP-14 provide some clue as to how damage to the microvilli comes about. When G. lamblia trophozoites, but not acellular filtrates of medium used to grow the trophozoites, are cultured with ICP-14 cells, the mammalian cells are completely destroyed within 96 hours. When viewed by electron microscopy it becomes apparent that the cytoplasms of the trophozoite and mammalian cell become continuous. It has been proposed that membrane-bound enzymes within the trophozoite are released upon contact with the mammalian cells (55), causing fusion of the trophozoite and mammalian cell membranes.

The immune reaction to Giardia has also been implicated as the cause of brush border damage. In the murine model Owen (51) found that the ratio of villus length to crypt depth in the jejunum of nude mice infected with G. muris decreased significantly when the mice were reconstituted with lymphocytes from normal animals. Owen suggested that an immunological reaction leads to a more rapid extrusion of epithelial cells at the villus tips and compensatory

proliferation of columnar cells within crypts. Gillon has gone on to propose that the nature of this immunological reaction is the production of "enteropathic lymphokines" by T cells which act directly on crypt cells to stimulate mitosis (25). Migration of less mature columnar cells to the villus surface could account for the reduced intestinal brush border enzymes found in giardiasis. These suggestions were supported when Ferguson et al. visualized by autoradiography doubling of the crypt cell production rate with rapid transit of enterocytes up the villi (19).

As an alternative to enterotoxin production, a reduction of brush border enzymes could account for the diarrhea found in 96% (67) of all symptomatic giardiasis cases. Lactase deficiency can lead to osmotic diarrhea after ingestion of milk. Altered enterocyte sodium transport may result in secretion of sodium and water, causing a secretory diarrhea (19).

Stool examination is the most routinely used technique in identifying patients of giardiasis. However, efficiency is low, and the procedure fails to detect trophozoites or cysts in 30%-50% of cases (11). Other diagnostic procedures include duodenal aspiration for trophozoite identification, and the swallowed nylon string method (Enterotest). In less than 5% of cases, jejunal biopsy is necessary when cysts and trophozoites are not observed in stools or duodenal aspirates.

Since 1976 several serological tests have been described for the diagnosis of giardiasis. Ridley and

Ridley (60) used G. lamblia cysts as antigen in an indirect immunofluorescence test, but autofluorescence of the cysts and the unavailability of a reliable cyst source limited the benefits of the technique. In 1978 Vinayak (77) performed gel immunodiffusion using cysts as antigen, but the cysts lost their antigenicity within three weeks even when stored at -10 C. In addition, not all batches of antigen behaved similarly; only two out of seven cyst preparations were satisfactory in the gel tests. Visvesvara et al. (78, 79) by-passed the problems of Ridley and Ridley when they developed an indirect immunofluorescence test using axenically grown G. lamblia trophozoites as antigen. Trophozoites also served as antigen in an enzyme-linked immunosorbant assay performed by Smith et al. in which 81% of 59 symptomatic patients and 12% of 17 persons uninfected by G. lamblia at the time of the study had circulating IgG to G. lamblia (70).

In 1982 Craft and Nelson (11) attempted to diagnose giardiasis by counterimmunoelectrophoresis of feces containing Giardia cysts. Antisera were made in rabbits to either cysts alone, or to trophozoites and cysts. These antisera were tested against feces of giardiasis patients or controls, and against axenically grown trophozoites. Oddly, the serum from the rabbit immunized with both trophozoites and cysts, as well as the rabbit immunized with cysts alone, failed to yield a precipitin band when electrophoresed against trophozoites. Both types of

antisera reacted with patient feces, but not with control feces.

The most recent diagnostic procedure to employ fecal specimens as antigen was described by Ungar et al. in 1984 (76). The researchers immunized goats and rabbits with axenic trophozoites and collected immune sera from the animals. The sera were used in a double-antibody ELISA to determine the assay's ability to detect known numbers of trophozoites or cysts. The ELISA was able to detect between 37 and 375 trophozoites, and between 12 and 125 purified cysts. Fecal specimens diluted to the point where no cysts were seen by microscopy remained positive by ELISA, suggesting that soluble breakdown products of trophozoites or cysts were contained within the feces. Results indicated a sensitivity of 92% and a specificity of 98%.

Not only have these serological tests aided in the diagnosis of giardiasis, they have also firmly established that a humoral response to the parasite does occur. Rendorff (57) showed that most infected human volunteers become resistant to reinfection, suggesting that circulating immunoglobulins in at least some cases confer immunity. Animal models support these findings; for example, mongolian gerbils infected with G. lamblia are resistant to secondary infection for up to 8 months after clearance of the parasite (6). In addition, the incidence of giardiasis in hypogammaglobulinemic patients is much higher than that in persons with normal immune function (2, 83). Murine studies suggest that recrudescence of occult giardial infe-

ctions is an important cause of morbidity when immunosuppressive therapy is given to patients in areas where giardiasis is endemic (47).

Both humoral and cellular immune mechanisms are important in the prevention and elimination of infection by Giardia. In immunologically competent mice, Giardia are cleared from stools within 28 days of oral infection with 1000 cysts (62). In nude and neonatally thymectomized mice infection persists up to 6 months (74) unless the mice are reconstituted with cells from normal mice. Immune lymphocytes are even more efficient in restoring immunity than naive cells (63). Normal mice usually resist reinfection (61), but thymus-deficient mice do not except after reconstitution (63).

Macrophages from individuals not previously exposed to G. lamblia are capable of nonspecific cytotoxicity against G. lamblia trophozoites, as demonstrated through cold target inhibition studies in which cytotoxicity for $^{3}$[H]-G. lamblia was inhibited equally by unlabeled G. lamblia and Trichomonas vaginalis trophozoites in vitro (73). Phagocytosis by macrophages from immune and nonimmune animals can be enhanced by the opsonizing effect of immune whole serum or purified IgG (56). [IgG is also involved in antibody-dependent cellular cytotoxicity in conjunction with peripheral granulocytes (16)]. Heat-inactivated serum is capable of opsonization, suggesting that complement is not involved. Electron micrographs have shown G. muris

engulfed by macrophages in intestinal dome cells surrounded by lymphocytes, apparently awaiting antigen presentation (53).

G. lamblia trophozoites are susceptible to the lethal effect of both normal and immune human sera. In a study conducted by Hill et al. (30) sera from patients without a history of giardiasis and with no detectable antibody by indirect immunofluorescence killed from 8% to 76% of trophozoites in vitro. Less than 10% of parasites incubated in phosphate buffered saline alone were killed, but 16 sera killed from 10% to 25% and six sera killed from 25% to 75%. One serum with an anti-G. lamblia antibody titer of 1:128 killed greater than 98% of the parasites. Kill appeared to be complement-dependent since heat-inactivated serum failed to kill the trophozoites.

IgA is the primary immunoglobulin class of plasma cells in the intestine and the predominant immunoglobulin in secretions. Because Giardia is a luminal parasite, a local secretory response by the host would be expected. However, the existence and importance of a secretory IgA response in humans and animals with giardiasis remains controversial. If a secretory IgA response occurs, it could enhance clearance of the parasite from the intestine by blocking attachment of the trophozoites to receptors on intestinal epithelium, by inhibiting motility, or by assisting phagocytosis by macrophages and monocytes. In rats inoculated with live G. lamblia trophozoites directly into the intestine, secretory IgA antibodies were first detected

in bile 3 days after inoculation, remained elevated through 12 days, and then returned to normal levels (39). The demonstration by indirect immunofluorescence of heavy chains, secretory component, and light chains attached to G. lamblia trophozoites indicates that the antibody detected was indeed secretory IgA. The presence of potentially protective IgA molecules directed at trophozoite surface antigens was substantiated by the use of colloidal gold immunocytochemistry in conjunction with scanning electron microscopy. Specific secretory IgA antibodies to G. lamblia trophozoites were demonstrated on their flagella and both dorsal and ventral surfaces including the adhesive disc.

Other studies in murine giardiasis using sonicated trophozoite antigen and solid phase radioimmunoassay have shown the presence of specific IgA antibodies in intestinal washes from infected mice in addition to specific serum IgG and IgA antibodies (3). Milk from immune female mice shown to contain specific IgA and IgG by immunofluorescence protected suckling neonates from infection (4), but a possible role for cells within the milk could not be ruled out. In contrast, killing of G. lamblia trophozoites by normal human milk was shown to be secretory IgA-independent since full activity was retained after passage of milk over a column of sheep antibodies to S-IgA coupled to Sepharose 4B, a treatment which decreased the S-IgA content to below that detectable by ELISA (23). Neither IgG nor IgM was

detected in milk by radial immunodiffusion. Thus, the killing capability of normal human milk must be attributed to factors other than antibodies.

Recent evidence points to bile-salt stimulated lipase (BSL) in human milk as the mediator of kill (24): 1)killing and BSL enzyme activity co-chromatographed on Sepharose 6B and Sephadex G-100; 2)both killing and enzyme activity are inhibited by heat and by treatment with diisopropyl fluorophosphate, a specific inhibitor of BSL; and 3)milk from cows and goats lack both BSL and kill activity. BSL is activated by bile salts to cleave fatty acids from milk triglycerides. Certain free fatty acids and monoglycerides are toxic to G. lamblia, which may account for the killing of trophozoites.

From the preceding discussion it is clear that many host defenses, both generalized and immune, are activated by the presence of G. lamblia trophozoites within the small intestine. Why then, are some individuals capable of spontaneously eliminating the parasite or evading it completely, while others become chronically infected as asymptomatic carriers or patients of active disease? As previously discussed, persons with humoral immune deficiencies are more likely to become infected with G. lamblia, indicating the importance of an adequate antibody response. Giardiasis is also more common in persons with blood type A (35). It is possible that some component of the trophozoite surface is antigenically related to the blood group A antigen, reducing the efficiency of

antibodies to recognize the parasite as foreign.

Antigenic differences among strains of G. lamblia may contribute to the variation of host responses to the parasite, some strains perhaps being more immunogenic than others. That antigenic variation occurs among strains was well illustrated in a study conducted by Smith et al. (71). Four trophozoite strains from widely differing geographical locations were analyzed by SDS-PAGE, immunoelectrophoresis, crossed immunoelectrophoresis, and ELISA. SDS-PAGE and IEP patterns were similar for all four strains. However, crossed immunoelectrophoresis revealed that the Oregon strain, which has been in culture 6 years longer than the other strains, lacked a set of anodic antigens and a neutral antigen that were present in strains from Afghanistan, Puerto Rico, and Ecuador. Other minor differences were noted. When the researchers used trophozoites of each strain as antigen in an ELISA against 10 human sera of various titers, differences were again detected.

In a related study Nash et al. further examined the host immune response to excretory-secretory products of the Oregon (P-1) and Afghanistan (WB) strains (48). The surface antigens of the trophozoites were radiolabeled by either the IODOGEN or lactoperoxidase technique. The excretory-secretory products free in the culture medium were then immunoprecipitated at various times post-label with either rabbit anti-WB or goat anti-P-1 antisera and excess Staph A. Acrylamide gel electrophoresis of the precipi-

tated labeled components showed that although anti-WB serum precipititated P-1 antigens and vice versa, the antisera preferentially precipitated their homologous excretory-secretory antigens.

Differences in serum antibody specificities toward antigens of a single G. muris strain have been observed in BALB/c mice which spontaneously eliminate the parasite and C3H/He mice which are chronically infected (16, 17, 46). Both strains of mice injected with trophozoites in adjuvant and challenged orally with cysts develop serum antibodies to numerous trophozoite proteins. No difference in IgG and IgA titers to whole trophozoite extracts measured by RIA are detected between the two strains. However, amounts of antibodies of certain specificities do differ in sera from the two host types as detected by immunoprecipitation analysis of radiolabeled antigens. BALB/c IgG and IgA preferentially immunoprecipitate an 83,000 MW $^{125}$I-labeled surface protein, while C3H/He IgG and IgA recognize a surface protein of about 55,000 MW. A group of four proteins of about 30,000 MW biosynthetically labeled with $^{35}$S-methionine are immunoprecipitated 100 times more efficiently by BALB/c antisera than by C3H/He antisera (17).

Evidence suggests that the 30,000 MW group of proteins may be exoantigens. A 30,000 MW component is also radiolabeled by the surface-labeling agent IODOGEN and found free within medium in which trophozoites are cultured short-term. The internally-labeled 30,000 MW protein and IODOGEN-labeled surface protein appear identical in size

and charge heterogeneity on 2-D gels. Purified 30,000 MW protein in complete Freund's adjuvant provides some protection against infection in BALB/c but not C3H/He mice. However, other data suggests that responsiveness to the 30,000 MW protein is not invariably associated with resistance to chronic infection since 2 out of 4 chronically-infected (BALB/c x C3H/He)$F_2$ mice recognized the component.

In short, it is known that antigenic differences occur among trophozoite strains of G. lamblia and that human sera react in variable fashion to the contrasting antigens. Differences in serum antibody specificities of mice to G. muris antigens may contribute to chronicity of infection. Logically, subsequent studies should focus on whether the combination of strain variation and differences in antibody specificities contribute to persistant giardial infections in humans. To this end, antibody specificities to two strains of G. lamblia, Portland-1 and KSU-1, have been examined herein through Western blot analysis and radioimmunoprecipitation techniques. Trophozoite antigens were reacted against human sera from both a chronically infected asymptomatic carrier and from a giardiasis patient who was subsequently able to clear the parasite without drug treatment. In addition, rabbit antiserum made separately to trophozoites or cysts was reacted with the parasites to gain some knowledge of antigens shared among, or unique to, these two developmental forms of G. lamblia.

## MATERIALS AND METHODS

Trophozoite strains. Our laboratory obtained the Portland-1 strain of Giardia lamblia, axenized by E. A. Meyer (45), from the laboratories of G. S. Visvesvara at the Centers for Disease Control, Atlanta, Ga., and from L. S. Diamond at the National Institutes of Health, Bethesda, Md. The KSU-1 strain was axenized by G. L. Marchin, Kansas State University, from an asymptomatic student by the method of Bingham and Meyer (7). Axenization consisted of exposing cysts purified from feces to an aqueous HCl solution at pH 2.0 to induce excystation, followed by culturing the trophozoites in Diamond's TPS-1 medium (trypticase-panmede-serum) according to Visvesvara (80). Cultured organisms were either maintained continuously in borosilicate 15-ml screwcap test tubes at 37 C or were preserved at -70 C by the method of Lyman and Marchin (42).

To determine whether strain differences were apparent simply by observing the trophozoites' grow rates, a growth curve for each strain was constructed. At various times post-inoculation into new medium, 0.9 ml trophozoite suspension in TPS-1 was mixed with 0.1 ml 1% formalin and the organisms in 10 fields of a Neubauer hemacytometer were enumerated at 100X.

Cysts. Cysts used for rabbit immunizations were donated by the same asymptomatic student from which the

KSU-1 trophozoite strain was axenized. The student developed symptoms of giardiasis soon after providing our laboratory with sufficient cysts for immunization and preliminary gel immunodiffusion tests, and received atabrine treatment to eradicate the parasite. For subsequent immunodiffusion and immunoblotting experiments, cysts were obtained from Mary Hall at Irwin Army Hospital, Fort Riley, Kansas.

Cyst purification. Cysts were purified from feces by the method of Sauch (65). Briefly, distilled water containing 0.01% Tween 20 (TDW) was added to feces to create a slurry. The slurry was layered atop an equal volume of 1.0 M sucrose in TDW and centrifuged 15 minutes at 1500 rpm in an IEC centrifuge. The interface was collected and washed in TDW 3 times for 10 min, keeping the pellet and discarding the supernatant of each wash.

A Percoll gradient, specific gravity 1.01 to 1.03 g/ml, was prepared in a Buchler two-chambered mixing device. The pellet, resuspended in a small volume of TDW, was overlaid onto the gradient and was left standing at room temperature for 2 h, after which 5-ml fractions were collected using a drip-out device. The fractions were examined for the presence of cysts by light microscopy, and those fractions containing the most cysts and the least amount of fecal debris were pooled and washed in TDW. Cysts were stored in 10 ml TDW at 4 C until used.

Antigen preparation for rabbit immunization. Portland-

1 trophozoites were grown to maximum cell density, approximately 2 x $10^6$/ml. The trophozoites, which grow as monolayers on the walls of the culture tubes, were shocked off the tube walls by immersion in an icewater bath for 10 minutes. 50 ml of the suspended trophozoites were centrifuged at 2000 rpm in an IEC centrifuge for 10 minutes and were washed 3 times in PBS, pH 7.2. The final washed pellet was resuspended in 1 ml physiological saline and sonicated with a Sonifier Cell Disruptor, Model 185W. The protein concentration of the suspension was estimated at 4.8 mg/ml by reading the $A_{280}$ in a Gilford Spectrophotometer.

Purified KSU-1 cysts were pelleted by centrifugation at 2000 x g in a JA-20 rotor in a Beckman Model J-21 refrigerated centrifuge. The pellet was resuspended in 1 ml distilled water and sonicated by five 30-sec bursts with the Sonifier. This suspension was spun again and the supernatant, containing soluble cyst antigen, was assayed for protein concentration by reading the $A_{280}$. The protein content of the soluble supernatant was approximately 6 mg/ml. The supernatant was then recombined with the cyst pellet for injection into a rabbit.

Rabbit immunization and antiserum collection. Young New Zealand white rabbits were immunized with either Portland-1 trophozoites or KSU-1 cysts prepared as described above. 0.2 ml antigen suspension (trophozoite or cyst) was emulsified in 0.8 ml Freund's complete adjuvant and injected subcutaneously into four sites on the back of

a rabbit. The procedure was repeated twice again at one-week intervals. Two weeks after the last injection in Freund's adjuvant the rabbit was boosted by injection into the ear vein with 0.2 ml antigen without adjuvant. Sera were collected aseptically at weekly intervals, beginning 3 weeks after the first injection, by bleeding from the lateral ear vein into sterile centrifuge tubes. The tubes were left at room temperature 4 h, refrigerated overnight, and then centrifuged at 2000 rpm in the IEC centrifuge 15 min. The sera were aspirated off the erythrocyte pellets and stored at -20 C until used. Immunization was preceded by the collection of preimmune sera.

Antisera from human giardiasis patients. Serum from the asymptomatic student who donated the KSU-1 strain was obtained. In addition, a human convalescent serum shown to be reactive with G. lamblia trophozoite antigens by means of an indirect immunofluorescence test were obtained from Dr. Visvesvara at the CDC.

Gel immunodiffusion analysis of antisera. Our laboratory first attempted to detect G. lamblia-specific antibodies by the immunodiffusion technique of Vinayak (77). Vinayak prepared slides by overlaying them with 1.0% noble agar in PBS. However, we have found that resolution of precipitin bands can be improved by substituting purified agarose and Tris-borate buffer for the noble agar and PBS respectively.

1% agarose (Type III: High EEO, Sigma Chemical Co., St.

Louis, Mo.) was heated to boiling in Tris-borate buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA in high quality glass-distilled water). The solution was cooled to 55 C and applied to Standard GelBond Film (FMC Corp., Rockland, Maine). After solidification, a pattern was punched in the gel and the well plugs removed by aspiration. Purified trophozoite and cyst antigens were resuspended in PBS to concentrations of approximately 4-6 mg/ml. Antisera were used undiluted. Ten µl of each sample were applied to Ouchterlony wells and the gels incubated 3 to 5 days at room temperature in humidity chambers.

After development of precipitin arcs, the gels were washed in a rocker bath 48 h at room temperature. The wash solution was composed of 5 parts borate solution (0.1 M boric acid, 0.047 M sodium borate, and 0.075 M NaCl, pH 8.4) and 95 parts aqueous saline (0.15 M NaCl, pH 7). The washed gels were stained 20-30 min with 0.1% Coomassie blue R-250, and then rocked in destain until their appearance was suitable. The destain contained 20 parts distilled water, 3 parts glacial acetic acid, and 20 parts absolute methanol. The composition of the Coomassie stain was identical to that of the destain, except that 0.2% aqueous Coomassie stock was substituted for the distilled water. The gels were washed in water 15 min and then left to dry at room temperature.

SDS-PAGE. SDS-PAGE was performed according to Laemmli (37) using a Bio-Rad Model 360 mini vertical slab-cell

apparatus. Purified trophozoite and cyst proteins were solubilized in electrophoresis sample buffer containing 2% SDS, 5% mercaptoethanol, and 10% glycerol in 0.5 M Tris-HCl, pH 6.8 by sonicating the organisms, subjecting them to 3 rounds of rapid freeze-thaw, and boiling 30 sec. Thirteen µg of protein, determined by using protein assay reagents from Bio-Rad, were applied to a 12% polyacrylamide gel and electrophoresed at 6 mA constant current for approximately 4 h or until the tracking dye reached the bottom of the gel. The gels were either stained with Coomassie brilliant blue R-250 or subjected to immunoblot analysis.

Immunoblot analysis of cyst and trophozoite antigens. Proteins separated by SDS-PAGE were transferred to nitrocellulose according to Partanen et al. (54). Transfer was carried out in a Hoefer Transphor Cell Model TE 50 in 25 mM Tris-HCl buffer, pH 8.3, containing 192 mM glycine and 20% v/v methanol. The nitrocellulose was incubated in PBS-Tween 20 blocking buffer (4 mM $KH_2PO_4$, 16 mM $Na_2HPO_4$, 115 mM NaCl, pH 7.3, with 0.05% Tween 20), as suggested by Batteiger (5), for 1 h in plastic microscope slide boxes. The blocker was replaced with 20 ml of relevant antiserum diluted 1:100 in PBS-Tween 20. The samples were incubated at 37 C in a Gyratory Water Bath Shaker, Model G76 (New Brunswick Scientific, New Brunswick, N.J.) for 45 min, after which the sera were poured off and the nitrocellulose strips washed 3 times for 15 min each in 20 ml PBS-Tween 20. The washes and all subsequent steps were performed at

room temperature.

Goat antibodies conjugated to horseradish peroxidase made against rabbit IgG (Bio-Rad) or human IgG (Sigma) were diluted 1:3000 and 1:1000 respectively, as suggested by the manufacturers, in PBS-Tween 20. The nitrocellulose strips were incubated 45 min in 20 ml of the appropriate conjugate solution, washed as before, and then developed with 20 ml Bio-Rad color development reagent (4-chloro-1-naphthol) for up to 30 min. Color development was stopped by flushing the blots with water.

Radioiodination of trophozoite surface antigens. Preferential labeling of surface antigens was accomplished by an adaptation of the chloramine T method described by Frost and Bourgaux (20). Trophozoite cultures were grown to late log phase in TPS-1 medium, shocked off the culture tube walls by immersion into an ice bath, and pelleted by centrifugation. The pelleted organisms were washed 3 times in PBS, pH 7.2. $10^8$ washed trophozoites of each strain were suspended in approximately 200 µl PBS. 20 µl 1 M phosphate buffer, pH 7.4; 500 µCi $Na^{125}I$; and 20 µl chloramine T reagent were added sequentially and the reaction allowed to proceed 3 min. The iodination was stopped by the addition of 100 µl (100 mg/ml) cold tyrosine. The labeled trophozoites were washed in 10 ml PBNaI (PBS, with NaI substituted for NaCl) 10 times, until the radioactivity in the wash supernatant remained low and constant. Nearly all trophozoites retained normal morphology throughout the labeling and washing procedures, and many remained motile

when viewed by light microscopy.

In early experiments, trophozoites were labeled by the lactoperoxidase-glucose oxidase method used by Einfeld and Stibbs (15). Five x $10^7$ washed organisms were resuspended in 2 ml PBS with 10 mM glucose (G-PBS). Added sequentially were 10 µg lactoperoxidase (LPO), 1 µg glucose oxidase, and 300 µCi $Na^{125}I$. After incubation for 20 min on ice, the trophozoites were washed four times in G-PBS and extracted into PBS-Triton X-100 as in the chloramine T procedure.

Antigens were extracted from the radiolabeled trophozoites by agitating the parasites at 4 C overnight in PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The Triton extract was centrifuged at 20,000 x g for 30 min at 4 C in a Sorvall Superspeed RC2-B centrifuge (Dupont Co., Wilmington, Del.) fitted with an SS-34 rotor and the supernatant was used for radioimmunoprecipitation experiments.

Radioimmunoprecipitation. 45 µl of the Triton extract supernatant were reacted with 5 µl of normal or immune serum for 6 h at 4 C with constant gentle agitation. Immune complexes were precipitated by the method of Kessler (34). 50-µl volumes of a 10% suspension of Staphylococcus aureus cells bearing protein A (Pansorbin, Calbiochem, LaJolla, Cal.) were agitated constantly with the complexes in 1.5-ml microfuge tubes and precipitation was allowed to proceed overnight at 4 C. The mixture was microfuged for 4 min in a Brinkmann Centrifuge (Eppendorf), Model 5412 and

the supernatants removed by aspiration. The bacterial pellets with the bound immune complexes were washed 4 times in 1 ml PBS containing 0.5% Triton X-100. The final pellets were boiled 5 min in 40 µl SDS sample buffer, and the supernatants were counted in a Model 1275 Minigamma counter (LKB Wallac). Approximately 1500 cpm of each sample were subjected to SDS-PAGE as previously described.

Autoradiography. Autoradiographs of dried gels were obtained by exposure to Kodak XAR-5 film plus an intensifying screen for 15 days at room temperature.

## RESULTS

Trophozoite growth curves. From the growth curve illustrated in Fig. 1, generation times for KSU-1 and Portland-1 trophozoites in TPS-1 medium were calculated as 16 and 13 hours, respectively. Weisehahn et al. calculated a doubling time of 15 hours for the Portland-1 strain by autoradiographic analysis of nuclear replication (85). In their study, trophozoites were labeled for various times with [$^3$H]-thymidine and grains incorporated over each of the two nuclei were counted. However, our simple light microscopy method is sufficient to show that growth rates differ between the two Giardia strains.

Double immunodiffusion gels. The results of immunodiffusion tests are illustrated in Fig. 2-4. It is important to note that the cysts used as antigen in the gels are of the KSU-1 strain, representing the last cyst donation made by our asymptomatic carrier before receiving atabrine treatment. To control for interactions which might occur between antisera and fecal contaminants, a fecal sample known to be cyst-free and purified in the same manner as the cyst sample was applied to the gels. Preimmune serum from the rabbit immunized with cysts failed to form precipitin bands with any of the antigens (Fig. 2). The figure illustrates that rabbit antiserum made to KSU-1 cysts reacted only with cysts when the protein concentration of the antigens was approximately 40 µg/well. When the antigen concentration

Figure 1.

Growth curves for G. lamblia trophozoites grown axenically in TPS-1 medium. The generation time for the KSU-1 strain was calculated as 16.01 hours, and that of the Portland-1 strain as 12.96 hours, by regression analysis of the points plotted on the graph. The correlation coefficients for KSU-1 and Portland-1 growth data were +0.9446 and +0.9676, respectively.

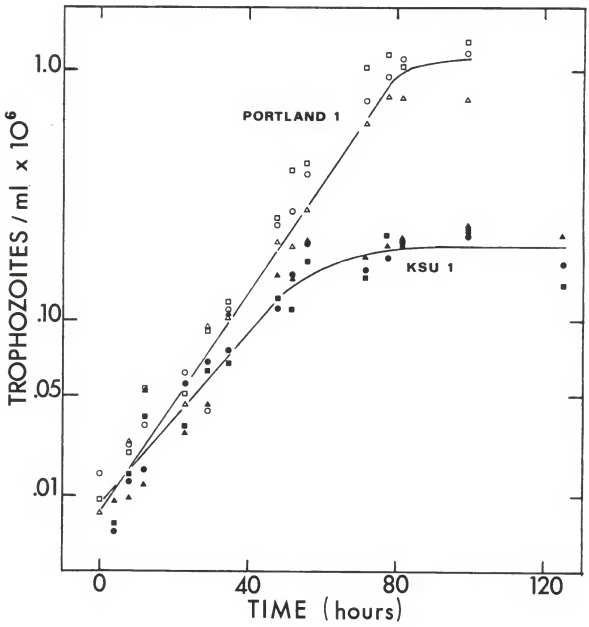
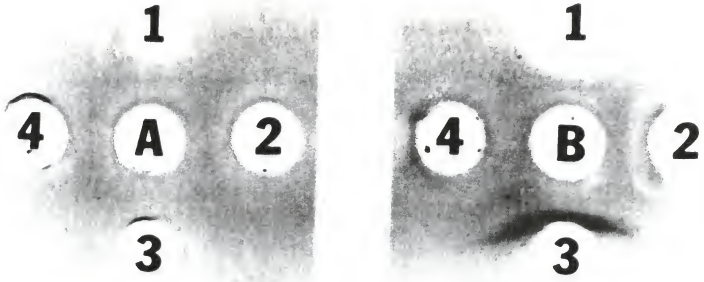

1.0
.10
.05
.01
TROPHOZOITES / ml x $10^6$
PORTLAND 1
KSU 1
0
40
80
120
TIME (hours)

Figure 2.

Double diffusion tests using rabbit antiserum to KSU-1 cysts. Antigen concentrations were 40 µg/well. (A), preimmune rabbit serum. (B), rabbit antiserum to KSU-1 cysts. (1), KSU-1 trophozoites. (2), cyst-free fecal sample. (3), KSU-1 cysts. (4), Portland-1 trophozoites.


1
4
A
2
3
1
4
B
2
3

was increased to 60 µg/well, the anti-cyst antiserum reacted slightly with KSU-1 trophozoites, but not with the Portland-1 trophozoites (Fig. 3). Neither normal human serum nor antiserum from the asymptomatic G. lamblia carrier formed precipitin bands with trophozoites of either strain.

Antiserum made to Portland-1 trophozoites reacted with antigens of both strains, although more antigenic determinants appear to be recognized within the homologous versus the heterologous system (Fig. 4). Some minor reactivity was noted between the rabbit antisera (both preimmune and immune) and the cyst and cyst-free fecal samples. Because the preimmune serum failed to form precipitin bands with the trophozoites, we feel that the reactivity to fecal samples indicates the presence of natural antibodies in the rabbit antiserum to enteric contaminants rather than prior infection with Giardia.

SDS-PAGE. Numerous protein bands with molecular weights ranging from below 10,000 to approximately 96,000 were discernable when trophozoite preparations were electrophoresed through a 12% polyacrylamide gel and stained with Coomassie blue (Fig. 5). The overall SDS-PAGE patterns for the two strains were quite similar, but minor differences were detected. When four strains of G. lamblia were electrophoresed through a 5 to 15% gradient gel, Smith et al. (71) detected 26 bands with molecular weights ranging from 10,000 to 140,000. They, too, found subtle differences in electrophoretic mobility patterns among strains.

Figure 3.

Double diffusion tests using rabbit antiserum to KSU-1 cysts. The antigen concentration was increased from that in Fig. 2 to 60 µg/well. (A), KSU-1 trophozoites. (B), Portland-1 trophozoites. (1), rabbit antiserum to KSU-1 cysts. (2), rabbit antiserum to Portland-1 trophozoites. (3), human asymptomatic antiserum. (4), normal human serum.

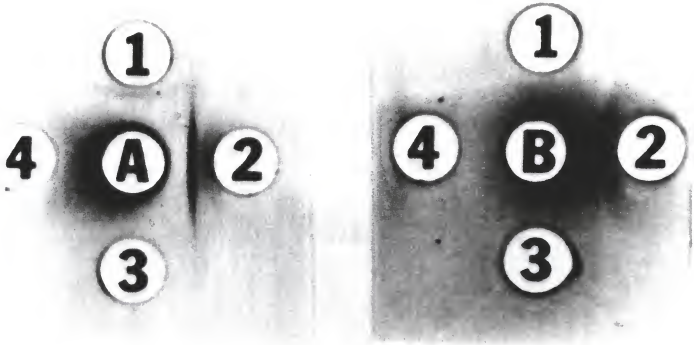
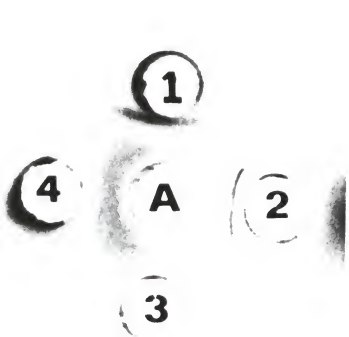

1
4
A
2
3
1
4
B
2
3

Figure 4.

Double diffusion tests using rabbit antiserum to Portland-1 trophozoites. (A), preimmune rabbit serum. (B), rabbit antiserum to Portland-1 trophozoites. (1), purified KSU-1 cysts. (2), cyst-free fecal sample. (3), KSU-1 trophozoites. (4), Portland-1 trophozoites.


1
4
A
2
3

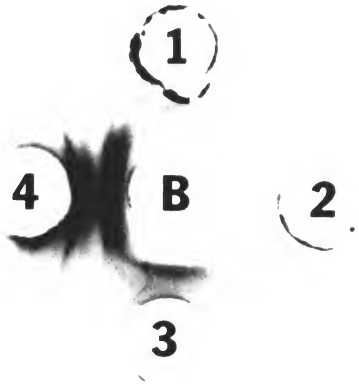
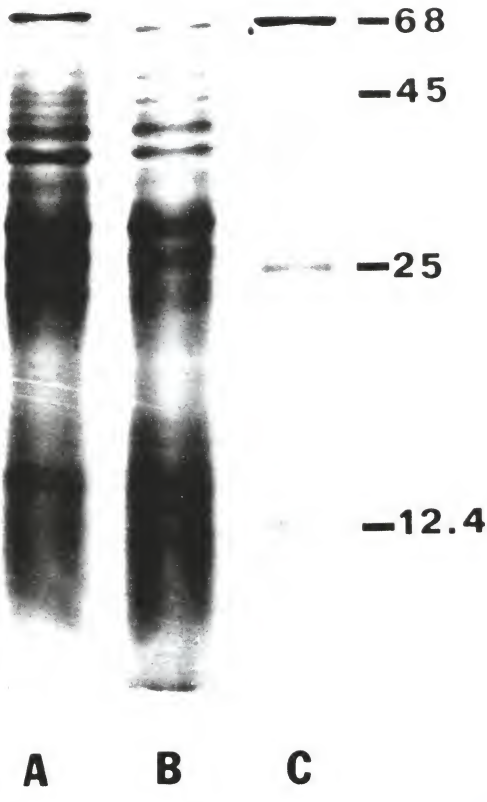

1
4
B
2
3

Figure 5.

SDS-PAGE gel of G. lamblia trophozoites stained with Coomassie blue. (A), KSU-1. (B), Portland-1. (C), molecular weight markers: bovine albumin, 68,000; ovalbumin, 45,000; chymotrypsin, 25,000; cytochrome C, 12,4000.


68
45
25
12.4
A
B
C

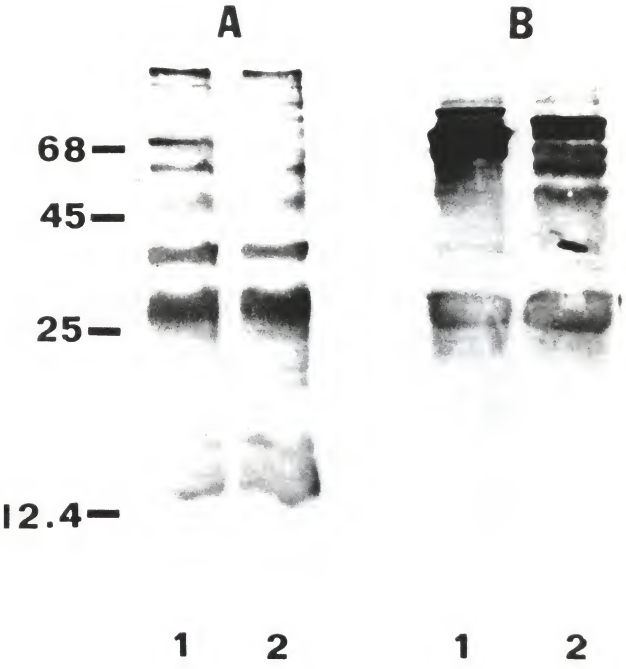
Immunoblot analysis. The immunoblot patterns of trophozoite proteins differentially recognized by rabbit anti-cyst and anti-trophozoite antisera are shown in Fig. 6. Preimmune rabbit sera did not react with G. lamblia antigens (data not shown).

We attempted to analyze those antigens that are unique to trophozoites by comparing the specificities of anti-cyst versus anti-trophozoite antibodies toward trophozoite antigens. It is reasonable to assume that trophozoite proteins recognized by anti-trophozoite antiserum and not by anti-cyst antiserum are unique to trophozoites. Conversely, trophozoite antigens more avidly recognized by anti-cyst antiserum may be present in enriched quantities within the cyst. A comparison of panels A and B in Fig. 6 indicates which protein species are most abundant in each of the two developmental forms.

Unfortunately, a reciprocal Western blot experiment using cysts as antigen in an effort to detect proteins unique to the cystic stage was unsuccessful. Our efforts were hampered by the unavailability of sufficiently large stool specimens for purification. Typically, the efficiency of cyst recovery in the purest fraction of a Percoll gradient was about 1%. When an entire stool was available, as from our asymptomatic donor, the yield was great enough to be usable. However, the specimens we infrequently obtained from an area hospital in transport vials were simply too small from which to collect cysts of sufficient purity for blot analysis. In addition, our

Figure 6.

Antigens recognized by rabbit IgG, detected by incubation with goat second antibody conjugated to horseradish peroxidase. (A), rabbit antiserum to Portland-1 trophozoites. (B), rabbit antiserum to KSU-1 cysts. (1), KSU-1 trophozoites. (2), Portland-1 trophozoites.


A
B
68
45
25
12.4
1
2
1
2

attempts to artificially induce the encystment of our axenic trophozoite strains by altering the pH and nutrient content of the culture medium were unsuccessful.

Both human asymptomatic and convalescent antisera recognized numerous trophozoite proteins (Fig. 7), thereby excluding the possibility that chronic infection with G. lamblia is a consequence of the immune system's inability to mount an antibody response to the parasite. The blot data are similar for the two antisera, although convalescent serum appeared to recognize more antigens than the asymptomatic antiserum, especially lower molecular weight species. Normal human serum did not react with G. lamblia proteins (data not shown).

Radioimmunoprecipitation of trophozoite surface proteins. Because the variety of trophozoite antigens recognized by Western blotting was so complex, we chose to examine more closely a smaller subset of trophozoite proteins, namely, those surface components radioiodinated by chloramine T and lactoperoxidase-glucose oxidase procedures, which may play a role in pathogenesis and host immunity in giardiasis. Results are illustrated in Fig. 8-10 and summarized in Tables I and II.

Our initial success in radiolabeling surface antigens of G. lamblia with lactoperoxidase (LPO) could not be repeated during subsequent trials. Because both successful and unsuccessful trials were performed in identical fashion (to the best of our knowledge), we feel that the lactoperoxidase or glucose oxidase lost enzymatic activity during

Figure 7.

Antigens recognized by human IgG, detected by incubation with goat second antibody conjugated to horseradish peroxidase. (A), human convalescent antiserum. (B), human asymptomatic antiserum. (1), KSU-1 trophozoites. (2), Portland-1 trophozoites.

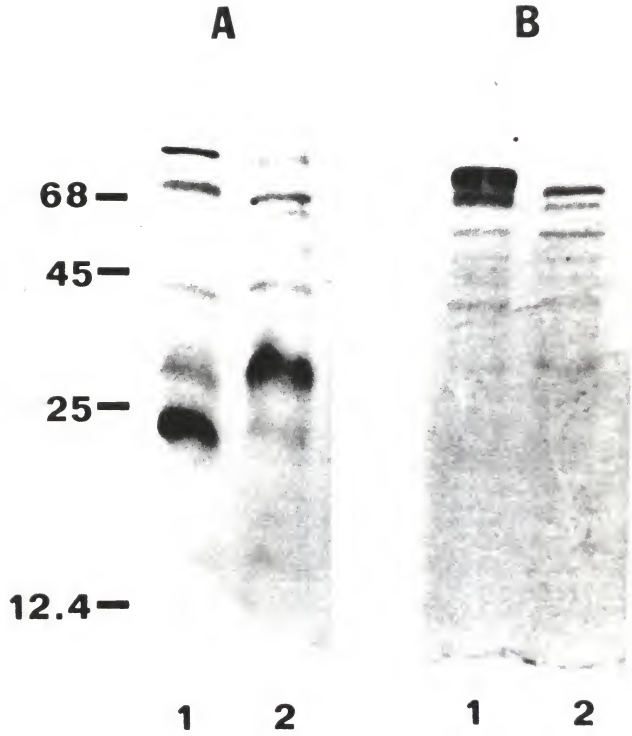

A
B
68
45
25
12.4
1
2
1
2

Figure 8.

Coomassie blue-stained gels (a) and their complementary autoradiograms (b) of trophozoite surface proteins either labeled with LPO (1), or labeled with LPO and then immunoprecipitated with rabbit antiserum to Portland-1 trophozoites (2).

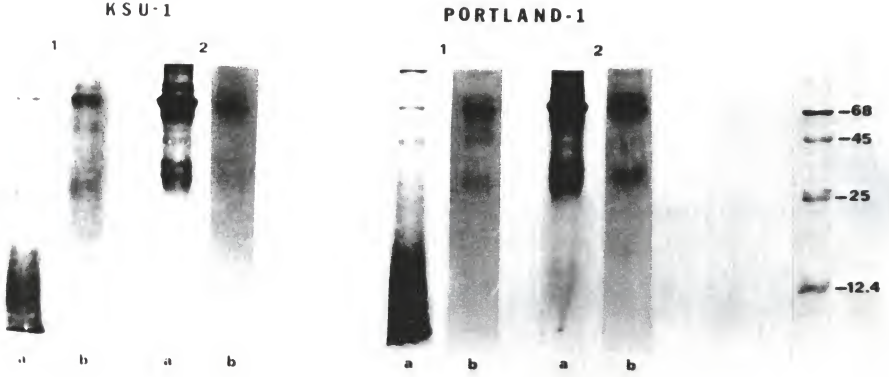
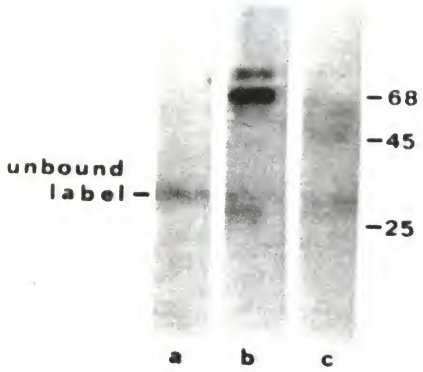

KSU-1
1
2
a
b
a
b
PORTLAND-1
1
2
a
b
a
b
-68
-45
-25
-12.4

Figure 9.

Autoradiograms of Portland-1 trophozoite proteins either surface-labeled by a chloramine T procedure (b), or surface-labeled and then immunoprecipitated by rabbit or human antiserum. Trophozoite proteins were precipitated by (a), preimmune rabbit serum; (c), anti-cyst antiserum; (d), anti-trophozoite antiserum; (e), normal human serum; (f), convalescent human serum; and (g), asymptomatic human serum.


unbound
label
68
45
25
a
b
c

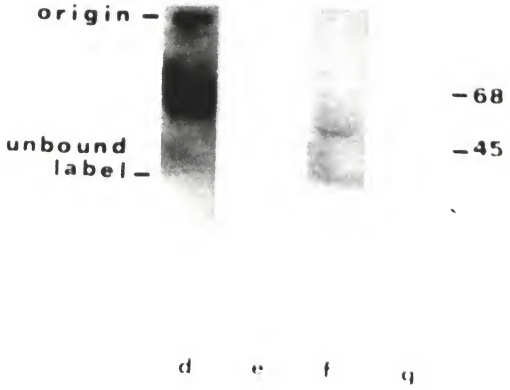

origin
unbound
label
68
45
d
e
f
g

Figure 10.

Autoradiograms of KSU-1 trophozoite proteins either surface-labeled by a chloramine T procedure (a), or surface-labeled and then immunoprecipitated with rabbit or human antiserum. Trophozoite proteins were immunoprecipitated by (b), rabbit anti-cyst antiserum; (c), rabbit anti-trophozoite antiserum; (d), normal rabbit serum; (e), normal human serum; (f), asymptomatic human antiserum; and (g), human convalescent antiserum.

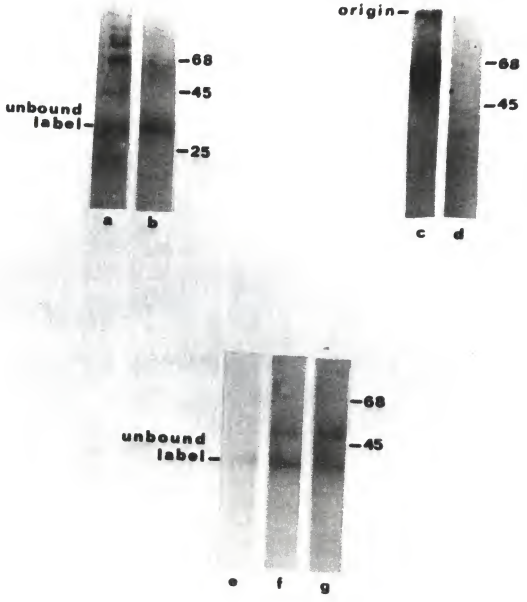

unbound label
-68
-45
-25
a
b
origin
-68
-45
c
d
unbound label
-68
-45
e
f
g

Table I.

Molecular weights of G. lamblia trophozoite surface proteins radiolabeled by chloramine T (CHL T) and lactoperoxidase-glucose oxidase (LPO) procedures.

| CHL T | | LPO | |
|---|---|---|---|
| KSU-1 | Portland-1 | KSU-1 | Portland-1 |
| 105,000 | 105,000 | 77,000 | 77,000 |
| 92,500 | 77,000 | 67,000 | 67,000 |
| 82,000 | 64-68,000 | 63,000 | 59,000 |
| 77,000 | 30,000 | 45,000 | 51,000 |
| 67,000 | | 20-23,000 | 43,000 |
| 45,000 | | | 25-30,000 |
| 21-25,000 | | | |

Table II.

Molecular weights of G. lamblia trophozoite surface antigens radiolabeled by a chloramine T (CHL T) or lactoperoxidase-glucose oxidase (LPO) procedure, and immunoprecipitated by rabbit or human antisera.

| Rabbit Anti-Trophozoite | | | |
|---|---|---|---|
| CHL T | | LPO | |
| KSU-1 | Portland-1 | KSU-1 | Portland-1 |
| 54-70,000 | 54-70,000 | 67,000 | 71,000 |
| | | 59,000 | 59-63,000 |
| | | 21-30,000 | 21-30,000 |

| Rabbit Anti-Cyst* | |
|---|---|
| KSU-1 | Portland-1 |
| 45-67,000 | 45-67,000 |

| Human Convalescent* | |
|---|---|
| KSU-1 | Portland-1 |
| 50,000 | 50,000 |

| Human Asymptomatic* | |
|---|---|
| KSU-1 | Portland-1 |
| 50,000 | ------ |

*Immunoprecipitated antigens were labeled by the chloramine T method.

storage at -20 C or during thawing and refreezing.

Surface-labeling of trophozoite proteins by the two methods yielded differing results (Table I). Because chloramine T is water-soluble, it is possible that some of the reagent entered the cells and allowed internal proteins to be labeled. The chloramine T method labeled a 105,000 MW component in both strains that the LPO procedure did not. In addition, in the KSU-1 strain, proteins of 92,500 and 82,000 MW were labeled by chloramine T but not by LPO. A 67,000 MW component usually appeared as a single broad band in chloramine T preparations, but could be distinguished as a doublet in LPO-labeled samples. A 77,000 MW protein was common to both strains and labeled by both procedures.

When Nash et al. (48) surface labeled trophozoites of the Portland-1 and WB strains by lactoperoxidase and IODOGEN methods, most of the labeled material was a polydisperse substance with an apparent MW of 94,000 to 224,000. This component was also the major excretory-secretory product released into culture medium. Other iodinated substances were of 45,000; 30,000; and 21,000 MW. The components reacted with homologous, polyspecific goat antiserum, but not with antiserum to the heterologous strain.

In contrast, Einfeld and Stibbs (15) detected a discrete component of 82,000 MW shared by all of four strains tested, including both Portland-1 and WB. Antigens detected at 30,000 and 24,000 MW by Einfeld and Stibbs appeared analogous to antigens detected by Nash et al. at

30,000 and 21,000 MW. Finally, discrete bands of 180,000 and 105,000 MW detected by the Einfeld laboratory fall within the range of Nash's polydisperse antigen.

Our results correlate quite well with these two previously published accounts of surface-labeled proteins in G. lamblia. We have confirmed the presence of the 105,000; 45,000; 30,000; and 21,000 MW components. In most cases, additional proteins were labeled (Table I).

Rabbit antiserum made to trophozoites in our laboratory did not immunoprecipitate an 82,000 MW surface component, in contrast to the results of Einfeld and Stibbs. The major immunoprecipitate we detected with rabbit anti-trophozoite antiserum appeared as a broad band with a molecular weight ranging from 54,000 to 70,000 (Table II). The immunoprecipitate appeared to correlate with a doublet of 67,000 MW found in autoradiograms of labeled, but not immunoprecipitated, surface proteins.

As a possible explanation for the discrepancy between our data and that of Einfeld et al., the major immunoprecipitated component of our autoradiograms may have undergone slight proteolysis during extraction into PBS-Triton X-100 or during elution from Staph A cells into SDS sample buffer. The presence of an 82,000 MW component infrequently seen in autoradiograms (Table I) supports this possibility, perhaps representing occasions when proteolysis did not occur in all copies of the protein. Alternatively, the 54-70,000 MW protein may indeed be a separate protein

species resulting from differences in the culturing methods employed by our two laboratories. Einfeld et al. proposed that differences in their results, compared to those of Nash et al., may have been due to the fact that the latter group supplemented their TYI-S-33 medium with bile, while the former group did not. Einfeld further suggested that components of bile, such as bile salts, might influence the molecular architecture of the parasite's surface; bile salts have been reported to stimulate the uptake of phospholipid from culture medium by Giardia sp. Our culture technique differs even more radically in that we use TPS-1 as the growth medium rather than TYI-S-33.

A diffuse band of 45,000 to 67,000 MW labeled by chloramine T was immunoprecipitated by rabbit anti-cyst antiserum in both trophozoite strains (Table II). Human convalescent antiserum recognized a 50,000 MW component in both trophozoite strains, while human asymptomatic antiserum recognized a protein of similar size in the KSU-1 strain but not in the Portland-1 strain. In no instance did preimmune rabbit serum or normal human serum precipitate any G. lamblia proteins.

## DISCUSSION

Giardia lamblia is responsible for more cases of waterborne epidemic diarrhea in the United States than any other known parasite. The resistance of the cystic stage to conventional water purification procedures and the existence of significant animal reserviors appear to to be responsible for the protozoan's ubiquity. Clinical manifestations of giardiasis range from short-lived, self-limiting diarrhea to acute disease with symptoms of abdominal cramps, flatulence and malabsorption. Asymptomatic carriers who pass cysts contribute to the spread of the disease.

In spite of the high incidence of giardiasis, little is known about the immune response the parasite elicits in the human host. Animal models of giardiasis in the mouse and mongolian gerbil have recently implicated the importance of macrophage activity, T lymphocyte function, and IgG and IgA production to the eradication of the parasite and resistance to reinfection. In humans, the role of IgA in immunity to G. lamblia remains debatable. Circulating IgG molecules appear to provide protection against reinfection in 86% of cases, but the epitopes to which antibodies in human serum are directed have not been characterized. The possibility exists that immunogens may vary with the infecting strain, since antigenic differences among strains have been demonstrated.

The present study was initiated to: 1)examine the

differences between two axenic strains of G. lamblia, one of which was isolated from an asymptomatic carrier; 2)investigate the components shared among, or unique to, the cyst and trophozoite stages; and 3)examine the reactivity of convalescent versus asymptomatic human sera to G. lamblia antigens, in order to determine whether chronicity of infection is dependent upon differences in antibody specificities.

During the course of our investigation, differences between the KSU-1 strain, axenized in our laboratory from an asymptomatic carrier, and the Portland-1 strain, obtained from other laboratories, became apparent. Growth curve data demonstrated that generation times for the two trophozoite strains differ significantly when grown in TPS-1 medium. In Toxoplasma gondii, virulence appears to correlate with generation time, those strains with shorter generation times being more pathogenic (27). A similar correlation may be drawn in the Giardia system. The KSU-1 strain, which has a relatively long generation time, was obviously much less virulent in its host than the Portland-1 strain, axenized from a symptomatic patient, which replicates more rapidly.

Gel immunodiffusion assays suggested that homologous reactions between trophozoites and rabbit antiserum are stronger than heterologous reactions. Anti-Portland-1 antiserum recognized more antigenic determinants within Portland-1 trophozoites than within KSU-1 trophozoites. In

addition, antiserum made to KSU-1 cysts reacted slightly with the KSU-1 trophozoite strain in the gel tests, but not at all with Portland-1. Strain differences were also apparent by SDS-PAGE and Western blotting techniques.

When antisera to cysts and trophozoites were made in rabbits to probe dissociated trophozoites for components unique to each of the two developmental forms, striking results were obtained. Anticyst antiserum appeared to identify higher molecular weight Giardia antigens. Anti-trophozoite antiserum recognized lower molecular weight components. Studies employing other protozoa, such as Naegleria gruberi (21) and Acanthamoeba castellanii (31) have shown that differentiation from one developmental form to another requires alterations in both transcription and translation. In Acanthamoeba, the synthesis of some proteins, namely of actin and of ribosomal proteins, is reduced more than 20-fold in the cyst. In Naegleria, the ameboid form has actin as its major contractile protein. During differentiation to the flagellate form, actin ceases to be synthesized, but the rate of tubulin synthesis increases. Tubulin is the major protein of the flagellate developmental form.

If the assumption is made that similar changes in the relative concentrations of contractile proteins (and other proteins as well) occur during the differentiation of G. lamblia, Western blots employing anti-cyst and anti-trophozoite antisera should detect such changes. Actin and tubulin have been shown by immunostaining to be

major components of the adhesive disc in the trophozoite (16). However, immediately after emergence of the trophozoite from the cyst, no sign of an adhesive disc can be detected (10), suggesting that actin and tubulin are not present in large quantities within the cyst, and the bulk of these proteins must be synthesized by the trophozoite during or after excystation. Actin has a molecular weight of 42,000 daltons; that of tubulin is 55,000. It is interesting to note that proteins of these particular sizes were avidly recognized by anti-trophozoite antiserum, but not by anti-cyst antiserum, in Western blots of dissociated trophozoites. Additional blots employing antiserum to purified trophozoite actin and tubulin could be used to identify the 42,000 and 55,000 MW components. Thus, antiserum made to a particular stage of G. lamblia may prove to be a useful tool in investigating the differentiation process of the organism.

Antiserum from a convalescent giardiasis patient and from an asymptomatic carrier yielded remarkably different Western blot patterns when reacted with dissociated trophozoites. As expected, many fewer antigens were recognized by antisera from naturally-infected humans than by antisera from hyperimmunized animals, demonstrating that not all antigens within Giardia trophozoites are recognized by the natural host. Human asymptomatic antiserum appeared to react with predominantly higher molecular weight antigens, weights, a pattern reminiscent of anti-cyst and anti-trop-

hozoite serum specificities. It is intriguing to speculate that these specificities have a direct bearing on chronicity of infection. For example, if a chronically-infected carrier primarily recognizes cyst antigens, the antibodies formed will be of little utility in combating trophozoite infection. In contrast, humans who are able to spontaneously clear the parasite must form antibodies directed at relevant trophozoite antigens. Trophozoite-directed antibodies could act to prevent attachment of the organisms to the intestinal wall, to induce the complement cascade, or to act as opsonizing agents.

To determine whether the important immunogens in G. lamblia are located on the surface of the organism, the parasites were labeled by chloramine T and lactoperoxidase techniques, followed by immunoprecipitation with various antisera. The lactoperoxidase method has previously been used with success to label G. lamblia antigens, but has the disadvantage of introducing extraneous protein into the system, which can itself become labeled (44). In addition, loss of enzymatic activity can prevent labeling of antigens. Another commonly used iodinating reagent, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (IODOGEN) is gentle and nonenzymatic, but has been shown to label fewer surface antigens than the lactoperoxidase technique in Leishmania tropica (22).

A standard chloramine T labeling method has been successfully applied to Trichomonas vaginalis (1). The

primary criticisms of this procedure are that the reagent is a powerful oxidant which requires a strong reducing agent to terminate the reaction (44), and that some internal proteins may become labeled, since chloramine T is water soluble and may enter into the cells. To avoid the addition of a strong reductant, excess tyrosine was used in place of sodium metabisulfite to terminate the reaction. The oxidizing activity of chloramine T appeared to have no detrimental effect on the trophozoites, since most of the organisms were actively motile after iodination and washing.

The suitability of applying the chloramine T procedure to G. lamblia was supported by the similarity of our results to those previously published (15, 48). A total of seven separate components were surface-labeled in each strain by either the lactoperoxidase or chloramine T method, but the sets of proteins labeled were not identical with respect to labeling technique or trophozoite strain. Common to both strains were proteins of 105,000; 77,000; 67,000; 63,000 or 64,000; and 43,000 or 45,000. Proteins previously described in the literature and confirmed by our data include the 105,000 and 45,000 MW components, plus 21,000 and 82,000 MW components in KSU-1, and a 30,000 MW structure in Portland-1.

Both surface proteins and glycoproteins may be radioiodinated by chloramine T and lactoperoxidase. Carbohydrate moieties, if present on a cell surface, can play a role in adherence and colonization, microbial susce-

ptibility to complement and phagocytosis, or can actually impair the host's immune response. As an example of the last point, blood-form trypomastigotes of Trypanosoma cruzi have been shown to possess a major 90,000 MW surface membrane glycoprotein which appears to be responsible for a marked antiphagocytic effect (49).

In a study conducted by Hill et al., G. lamblia surface components were tested against a panel of plant lectins including wheat germ agglutinin, concavalin A, peanut agglutinin, soybean agglutinin, and fucose binding protein by microagglutination and fluorescein-labeled lectin assays. Wheat germ agglutinin was the only lectin tested that significantly agglutinated or labeled the trophozoites. Because WGA binds both N-acetyl-D-glucosamine and sialic acid, the trophozoites were treated with neuraminidase to cleave any sialic residues that might be present on the membrane glycoproteins. This treatment resulted in no significant decrease in WGA binding. Therefore, GlcNAc appears to be the major carbohydrate present of those tested. These results are in contrast with other protozoa, most of which strongly agglutinate in the presence of various lectins.

Not all surface proteins or glycoproteins are immunogenic. We therefore compared the surface structures which were immunoprecipitated by various antisera, in the hope of identifying candidate vaccine molecules which mimic a desirable component of the natural host response to

infection. All previous work in this area has been done with antisera made in hyperimmunized animals. Our study represents the first effort using human sera, and the results demonstrate that while animal models are extremely beneficial, they do not always mimic the human condition. For example, Einfeld et al. found an 82,000 MW component recognized by rabbit antiserum in all four of the strains of G. lamblia they tested. The major component we immunoprecipitated with anti-trophozoite antiserum was a 67,000 MW antigen. Human sera weakly recognized a 50,000 MW structure. Because the 50,000 MW antigen was recognized by the asymptomatic carrier, the antibodies it elicits are probably not protective. We must therefore conclude that the antibodies to Giardia antigens in human sera detected by Western blot analysis are directed toward internal, rather than surface, immunogens.

The question remains as to why surface antigens of trophozoites are not recognized by human sera. Perhaps humans make antibodies to some large tertiary membrane structure which is not immunoreactive unless it remains intact. Dissociation by SDS and reduction by β-mercaptoethanol may disrupt the component to the degree that it can no longer bind antibody. Previous work done in our laboratory by G. L. Marchin suggests that this might be the case. When Marchin applied dissociated trophozoites to nitrocellulose and then blotted with human antisera followed by $^{125}$I-Staph A, he found that the dissociated trophozoites did not bind antibody. However, when the experiment was

repeated using whole, undissociated trophozoites, reactivity was noted on the resulting autoradiograms (personal communication).

A second possibility is that the surface antigens recognized by rabbit antisera but not by human sera may be those that mimic the blood group A antigen. Additionally, both the chloramine T and lactoperoxidase procedures primarily label tyrosine residues. An antigen which does not contain this amino acid would thus go undetected.

As a final possibility for the failure of human antisera to recognize surface proteins or glycoproteins of G. lamblia trophozoites, some evidence already exists suggesting that the parasites may lose antigenicity during prolonged axenic culture. The results of crossed immunoelectrophoresis showed that the Portland-1 strain, which has been in culture for many years, lacks an antigen present in more recently axenized strains (71). Therefore, antigens to which humans mount antibodies may no longer exist in the KSU-1 and Portland-1 strains. The radical difference in generation times for trophozoites *in vivo* (5 hours) and *in vitro* (13-16 hours) suggests that axenic culture conditions are inferior to the natural habitat of the trophozoite for the growth of the parasite. As a result, the trophozoite may become more selective in the protein species it must synthesize. Since receptor proteins or receptor glycoproteins are of little use to a trophozoite attached to the side of glass tube, these

proteins may become expendable and their synthesis abandoned.

It is of interest that anti-cyst antiserum recognized a diffuse surface component of the trophozoite. The molecular weight range of the immunoprecipitated antigen (45-67,000) included the 50,000 MW entity recognized by human sera. Perhaps this component represents an antigen common to all developmental forms of all strains of G. lamblia. As mentioned before, antibodies elicited by this antigen are probably not protective, since serum from a chronic carrier also recognized the component, but the presence of the antigen remains interesting from a developmental point of view.

In summary, we have shown that strain differences exist between KSU-1 and Portland-1 trophozoites, both in generation time, and in antigenicity. There appear to be components which are differentially enriched in the cyst and trophozoite stages, and these differences may be detected using antiserum made separately to trophozoites or cysts. Human convalescent antiserum recognizes predominantly lower molecular weight internal trophozoite antigens, which may induce the formation of protective antibodies. Human asymptomatic antiserum selectively binds to higher molecular weight antigens. These may be primarily cyst antigens and thus antibodies directed toward them would be of little value in clearing a trophozoite infection. Finally, the serum specificities of antisera made by hyperimmunization of animals toward surface antigens of

Giardia lamblia may not accurately represent the serum specificities of humans.

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ACKNOWLEDGEMENTS

I would like to thank Dr. George Marchin for the patience and guidance he has provided during the course of this work, and for providing me with the opportunity to learn in his laboratory.

I am grateful to my committee members, Dr. Bill Fortner and Dr. John Iandolo, for their contributions and helpful suggestions during the preparation of this manuscript.

I would also like to thank Mr. Steve Gill and Mr. John Ludlow for technical advice, and John Ludlow for the photography appearing in the manuscript.

# A COMPARATIVE STUDY OF SERUM ANTIBODY SPECIFICITIES AND ANTIGENIC DIFFERENCES AMONG STRAINS AS CONTRIBUTING FACTORS TO CHRONIC INFECTION WITH *GIARDIA LAMBLIA* IN HUMANS

by

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B. S., Missouri Western State College, 1983

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Division of Biology
Microbiology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1985

Giardia lamblia, a single-celled protozoan which parasitizes the small intestine of man, is the most frequently-documented cause of waterborne epidemic diarrhea in the United States. In spite of the high incidence of giardiasis, little is known about the immune response elicited by the parasite in the human host.

We have demonstrated by double immunodiffusion, SDS-PAGE, immunoblotting, and immunoprecipitation techniques that antigenic differences exist between two axenic strains of G. lamblia. Antisera made separately to the cyst and trophozoite stages of the parasite can be used to detect components shared among, or unique to, the two developmental forms of the protozoan.

The antibody specificities of convalescent human serum differ from those of serum collected from a chronically-infected, asymptomatic Giardia carrier. Antigens recognized by convalescent serum, but not by asymptomatic serum, may induce the formation of protective antibodies. Protective antibodies appear to be directed toward lower molecular weight, internally disposed trophozoite antigens. In contrast, antibodies in asymptomatic human serum recognize higher molecular weight antigens reminiscent of those detected by rabbit antiserum made to cysts. These data suggest that chronicity of infection with Giardia lamblia may be dependent upon the formation of irrelevant antibodies directed toward the cyst, which offer the host no protection against infection by the trophozoite.