Studies on *Giardia lamblia* Trophozoite Antigens

Using Sephacryl S-300 Column Chromatography, Polyacrylamide Gel Electrophoresis and Enzyme-linked Immunosorbent Assay

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Antigen prepared from *Giardia lamblia* trophozoites cultured *in vitro* in Diamond's TYI-S-33 medium was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis, Sephacryl S-300 column chromatography, counter immunoelectrophoresis and enzyme-linked immunosorbent assay. After elution through S-300 column four distinct fractions were obtained. Molecular weights of these fractions were 150,000; 65,000; 50,000 and 10,000 daltons for FI, FII, FIII, and FIV respectively. The SDS-PAGE analysis revealed a minimum of 28 distinct bands with crude antigen and 13, 22, 26 and 30 bands with FI, FII, FIII and FIV fractions respectively. The molecular weight of these bands ranged from 125,000 to 14,000 daltons. Antigenic activity was observed in all four fractions in the CIEP test. However, when assayed by the ELISA test the maximum antigenic activity was linked to the higher molecular weight fraction.

INTRODUCTION

*Giardia lamblia*, a flagellated protozoan parasite that invades the upper intestine of humans and causes a spectrum of diseases, including asymptomatic carriage, acute diarrhoea, and chronic diarrhoea with steatorrhoea. The disease most commonly occurs in children and infants (18), particularly those attending day care centers (8), travelers (5), homosexuals (13), hypoimmunoglobulinaemics and back packers (5) in addition, the organism has been established as the etiological agent of numerous outbreaks of diarrheal disease in various parts of the world (4,7).

Despite the considerable morbidity caused by *G. lamblia* very little is known about the antigenic configuration of this protozoan. Earlier work on this parasite by various workers (11,12,15,16) suggests that the organism is antigenically a complex moiety.

To define the antigenic nature of *G. lamblia* trophozoites (strain Portland-1) further, Sephacryl S-300 column chromatography for fractionation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the comparison of protein constituents in CSA and its fractions, and counter immunoelectrophoresis (CIEP) and enzyme-linked immunosorbent assay (ELISA) for immunological activity were used. The crude soluble antigen (CSA) and its fractions were polydisperse in their molecular weight when analyzed both in Sephacryl S-300 column chromatography and SDS-PAGE with a broad spectrum of immunogenicity.

MATERIALS AND METHODS

Parasite culture. The P-1 strain of *G. lamblia* was subcultured twice per week at 37°C in filter sterilized TYI-S-33 medium (6), supplemented with vitamins and 10% heat inactivated adult bovine serum, penicillin (50 μg/mL) and streptomycin (50 μg/mL) as antibiotics.

Antigen preparation. Actively growing *G. lamblia* trophozoites showing exponential growth (72-96 h) were dislodged from the walls of culture tubes by immersion in an ice bath for 10 minutes followed by centrifugation at 800 x g for 5 minutes. Pooled viable trophozoites were washed 5 times in sterile phosphate buffered saline (PBS pH 7.4, 0.05 M) and finally resuspended in normal saline. This suspension was then sonicated in an ice bath with eight 30 sec bursts (MSE Sonicator, U.K.). The sonicated material was centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatant was collected as crude soluble antigen (CSA) and used for antigenic analysis after the protein contents were estimated by the method of Lowry et al. (10).

Preparation of antisera. Albino rabbits weighing 2-3 kg were immunized with *G. lamblia* CSA. About 2 mg of antigenic protein in 0.5 mL were emulsified with an equal volume of Freund’s complete adjuvant (Difco) and injected subcutaneously into the hind legs of each rabbit. A total of three such injections were given to each rabbit at weekly intervals. This was followed by three intravenous injections with CSA alone (approximately 3-4 mg of antigenic protein per rabbit) at 2 day intervals. Animals were bled a week after the last injection and the precipitating antibody in the immune rabbit serum was detected by the CIEP test against the homologous antigen.

Fractionation of CSA by Sephacryl S-300 (gel-filtration) column chromatography. CSA of *G. lamblia* was subjected to gel filtration through Sephacryl S-300 columns in order to separate its antigenic fractions. About 80 mL of prewollen Sephacryl S-300 (Pharmacia Fine Chemicals, Sweden) wet bed diameter 40-105 μm was poured in a glass column (16 x 40 cm). Pharmacia Fine Chemicals, Sweden). A flow rate of 20 mL per hour was maintained with a peristaltic pump (Pharmacia) throughout the experiment. The void volume of the column was determined by applying Dextran Blue 200-000.

About 15 mL (i e 1-2% of bed volume of column) of CSA which was previously dialyzed with PBS buffer containing about 24 mg of protein, was applied and 3.5 mL fractions were collected in each tube with the help of an automatic fraction collector (Fac-100, Pharmacia Fine Chemicals, Sweden). An elution profile was obtained by measuring the optical density (O D)
The protein constituents of CSA as well as the four fractions separated by S-300 column chromatography were compared by SDS-PAGE using 10% separating gels, in 0.5 tris-HCl buffer pH 6.8. The electrode buffer and sample preparation was made after following standard procedures (9).

SDS-PAGE analyses was performed in a vertical slab gel electrophoresis chamber (LKB, Sweden) with a constant temperature of 15°C. A constant 120 V was applied once the GSTA antigen had entered the separating gel. Known molecular weight protein standards (Pharmacia Fine Chemicals, Sweden) were run simultaneously. The gels were fixed, washed and stained for protein with 0.125% Coomassie Brilliant Blue dye.

CIEP test The antigenic activity of CSA and its four fractions were compared in counterimmunoelectrophoresis tests against the different dilutions of rabbit anti-Giardia antibodies. The CIEP test procedure was essentially the same as that described by Sharma et al (14) Briefly, approximately 3 mL of 1% agarose (Sigma Chemicals, U.S.A.) in barbital buffer (pH 8.6, 0.05 M) was layered on micro slides and wells for antigen and antibody were punched according to the standard size. The antibody wells were placed to the anodal side and the antigen wells to the cathodal end. The experiment was run in an electrophoresis chamber (Shandon, U.S.A) at a constant 160 V for 30 minutes. Readings were taken just after the experiment and also after 24 h of incubation at 4°C.

ELISA test The micro ELISA test performed was the same as described earlier in the serodiagnosis of amoebiasis (5), with slight modification. In this experiment a known amount of antibody (i.e. 1 200 dilution) was used to react with variable amounts of antigen. Four pre-immune rabbit sera and four immunized against *Giardia* CSA rabbit sera were pooled in separate batches and used as reference positive and negative sera for the experiment. Before the experiment was conducted a checker board titration was carried out to determine the optimal concentrations of antigen and antibody required. A 20 μg per mL G. *lambia* protein from CSA and a 1 200 dilution of pooled positive and negative sera were found optimal and specific for obtaining a clear cut distinction between positive and negative results.

For comparison of antigenic activity among CSA and its fractions, different concentrations of antigenic protein viz. 20 μg, 10 μg, 5 μg, 2.5 μg and 1.2 μg per mL were used in this test. A 1 1000 dilution of anti-rabbit IgG labelled with horse radish peroxidase (Sigma Chemicals, U.S.A) was used as a conjugate. Results were read photometrically at 490 nm (O.D.) in an automatic ELISA reader (Dynatech Labs, U.S.A).

### RESULTS

The Sephacryl S-300 gel filtration pattern of *Giardia* CSA is presented in Figure 1. Two major light absorbing peaks were observed, one of which appeared in the void volume and the other at the end of total column volume. The last peak mostly consisted of yellowish colouring material (originally seen in the CSA). Eluted materials were appropriately pooled as indicated in Figure 1 to give four different fractions FI to FIV, molecular weights of FI to FIV were 150K, 65K, 50K, and 10K daltons, respectively.

In the CIEP test the FI fraction showed a positive precipitin reaction up to 1 64 titre of immunized rabbit serum, the other fractions as well as CSA itself failed to show reactivity beyond 1 8 titre of immunized rabbit serum, although the same concentration (1 mg/mL) of CSA antigen had entered the separating gel. Known molecular weight protein standards (Pharmacia Fine Chemicals, Sweden) were run simultaneously. The gels were fixed, washed and stained for protein with 0.125% Coomassie Brilliant Blue dye.
TABLE 1  Comparison of optical density values for different concentrations of crude soluble antigen (CSA) and its fractions (FI - FIV)

<table>
<thead>
<tr>
<th>Antigenic protein concentrations (µg/mL)</th>
<th>Mean O.D values*</th>
<th>CSA</th>
<th>FI</th>
<th>FII</th>
<th>FIII</th>
<th>FIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.243</td>
<td>0.556</td>
<td>0.410</td>
<td>0.256</td>
<td>0.156</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.203</td>
<td>0.500</td>
<td>0.318</td>
<td>0.196</td>
<td>0.079</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.138</td>
<td>0.426</td>
<td>0.235</td>
<td>0.110</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0.120</td>
<td>0.250</td>
<td>0.135</td>
<td>0.128</td>
<td>0.023</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>0.039</td>
<td>0.165</td>
<td>0.092</td>
<td>0.059</td>
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</tr>
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</table>

*O.D values were taken at 490 nm

The antigenic protein was used in each case

In the ELISA test a clear cut distinction in O.D value was observed when 20 µg of crude soluble antigen was used against a 1:200 dilution of positive and negative sera. The four pre-immune negative sera showed an O.D value ranging from 0.05 to 0.09 with a mean O.D of 0.07 at 490 nm. The four immunized rabbit sera with the same dilution showed O.D values ranging between 0.12 and 0.325 with a mean O.D of 0.234. The cut-off value indicating a negative or a positive result was taken as 0.12 (mean O.D of controls). The results of CSA and its fractions when compared using the ELISA test for antigenic activity against the pooled immunized rabbit sera are shown in Table 1. After analysis, fraction I was found to be most antigenic as compared to the parent CSA and the other three fractions in other words, the µg/mL protein of this fraction (FI) showed almost the same O.D value as those obtained with 20 µg/mL of CSA, 5 µg/mL of FII, and 20 µg/mL of FIII, respectively. Fraction IV was found to be the least sensitive in comparison to CSA and other fractions.

The pattern of SDS-PAGE with CSA and its fractions for comparison of their constituent protein subunits are shown in Figure 3. The CSA showed several about 28 discrete protein bands in the molecular weight region of ~12.5 x 10^4 to ~1.4 x 10^4 daltons. Fraction I showed a similar banding pattern with less number of subunits (about 13 bands). Fraction II showed protein bands in the molecular weight range of ~9.4 to ~4.3 x 10^4 daltons. The bands of FIII were mainly confined to the molecular weight region of < 9.4 x 10^4 to 2.1 x 10^4 daltons. However, the FIV contained low molecular weight protein subunits ranging from ~8.2 x 10^4 to ~1.4 X 10^4 daltons.

DISCUSSION

The present investigation demonstrates the complex nature of CSA of *G. lamblia* (strain P-1) trophozoites. They contain about 28 polypeptides according to SDS-PAGE analysis and these findings correspond to the observations of Smith et al. (15). However, the results differ slightly from those of Moore et al. (12) who demonstrated about 20 distinct protein determinants with the same experiment. A similar study (17) using an immunoelectroprecipitation test showed 24 precipitin arcs. These differences in the protein polymers may be due to different strains and methods used by different workers. The results of fractionation in HPLC (12) and Sephacryl S-300 column chromatography of CSA showed almost similar observations. In both cases the maximum antigenic activity was recorded in the high mol. wt. fraction (FI). Fraction I, which showed maximum antigenicity, contained only 13.5% of the total protein present in CSA; this also suggests that only 13.5% of the protein present in the whole *Giardia* extract is serologically active. Fractions II, III and IV, although they had protein contents of 22%, 10% and 2.5% respectively of the total extract, showed significantly lower serological values. Furthermore, the precipitin reactions which have been demonstrated in whole CSA and its various fractions can be explained on the basis that fractions II, III and IV are not pure and are contaminated with the preceding fraction. Fractions III and IV which were found to be relatively less active than the other two fractions by ELISA test could perhaps throw some light on the difference between the ELISA and precipitin test for the detection of clinical *Giardia* cases as described in amoebiasis (5) where, the haemaglutination and IFA test showed negative observations. However, ELISA was found positive with the same sera. It is possible that the two tests detect different subclasses of IgG antibodies.

The demonstration that the immunologic activity was associated with a particular fraction(s) makes it possible to isolate these particular fractions by Sephacryl S-300 gel chromatography and to use these fractions for the production of a more specific anti-*G. lamblia* antiserum. The production of more specific antigens and antibodies will...
allow for more sensitive assays to study and elucidate the role these antigens play during G. lamblia infection

LITERATURE CITED


Identification of Heterogeneity in Human Isolates of *Giardia lamblia* by Isoenzyme Studies

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Running Title: Heterogeneity among human *Giardia* isolates

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Abstract

Electrophoretic mobility patterns of six enzymes viz. Alkaline phosphatase E.C. 3.1.3.1., Acid phosphatase E.C 3.1 3.2, Malic enzyme E.C. 1.1.1 40., Isocitrate dehydrogenase E.C 1 1.1.42, Glucose-6-phosphate dehydrogenase E.C. 1 1.1.49 of two axenically cultured human Giardia lamblia isolated from India (PD-1 and PD-2) and one from Portland (P-1) were compared using polyacrylamide gel electrophoresis (PAGE). Based on the difference in mobility patterns of enzymes PGM, ICDH and ME, the PD-1 and PD-2 appeared to be quite different from P-1. The ICDH and ALP enzymes were used for the first time in the present study for differentiation of Giardia isolates. In case of PD-1 two ALP bands could be seen whereas only one band was observed in PD-2 and P-1. Thus, the three strains could be grouped into three different zymodemes. These findings reveal the significant heterogeneity in G. lamblia isolates both from widely separated areas and within a single region. Heterogeneity among G. lamblia strains may explain the variable clinical manifestations, host response and treatment efficacy characteristic of human giardiasis.
Introduction

Giardia lamblia, the causative organism for giardiasis, is a flagellated, protozoan parasite found in the intestine of man and animals. Infection with Giardia may be asymptomatic or symptomatic characterized by diarrhoea, flatulence, abdominal pain, and anorexia etc. In some cases chronic infections develop resulting in severe malabsorption syndromes. Recent evidences demonstrate that at least some species of Giardia are capable of infecting more than one animal species. Controversies are still existing whether human Giardia species is homogeneous or consists of multiple strains exhibiting differences in behaviourl characteristics such as virulence, infectivity, antigenicity and susceptibility to drugs. As such a study of genetic variation is needed in this regard to differentiate the isolates of Giardia from the same and different hosts as also from the same and different geographical areas. Methodology is now available in the form of DNA and isoenzyme analysis for characterization of Giardia isolates. The isoenzymes are different molecular forms of enzymes with the same enzymatic activity. They are frequently used to differentiate morphologically similar strains of other human parasitic protozoa e.g Entamoeba histolytica, Trypanosoma and Leishmania. The isoenzyme analysis has become popular as it is economical, simple to perform and more or equally sensitive in comparison to DNA analysis.
In this study we have compared the two Indian human isolates of *G. lamblia* (which produced different symptomatology in patients) with Portland-1 (U.S.A), a reference strain, by isoenzyme electrophoresis using six enzyme systems in polyacrylamide gel.

**Materials and Methods**

**Source of Parasite and Cultivation**

Stools from patients admitted to the Infectious Diseases Hospital, Calcutta, India, with various gastrointestinal disorders and diarrhoea were routinely examined for the presence of diarrhoeagenic pathogens. Ten such patients, aged between 10-18 yrs. had abdominal pain, flatulence, diarrhoea, fever, and whose stool samples were repeatedly (3 times) positive for *Giardia* cysts and trophozoites only were selected for duodenal intubation. About 0.1 ml of the aspirated duodenal fluid containing $2 \times 10^5$ *Giardia* trophozoites were injected immediately into the culture tubes in filter sterilized TPS-I medium, supplemented with 10% fetal calf serum, 10% NCTC 135 and different combinations of antibiotics (8).

Attempts were made to axenize all the isolates. However, only two strains (PD-1 and PD-2) could be axenized. These two Indian isolates and Portland-1 (P-1, ATCC No. 30888) isolate were routinely maintained in filter sterilized TYI-S-33 medium (7) containing vitamin mixture and 10% heat-inactivated adult bovine serum. These isolates were subcultured twice-a-week.
Isoenzyme analysis

Active trophozoites from late log phase of growth were harvested and centrifuged at 600 x g for 10 min. The pellet was washed three times with normal saline supplemented with 250 mM sucrose. Lysates of cells were made by freezing at -70°C and then thawing at 4°C several times, till no intact trophozoites could be seen under microscope. The lysates were centrifuged at 20,000 x g for 20 min and the cell free supernatant of soluble antigen was obtained. The antigen was stored at -70°C with equal volume of stabilizing reagent (250 mM sucrose, 1 mM DTT, 1 mM aminocaproic acid and bromophenol blue). The protein contents were estimated by the method of Bradford (4) using bovine serum albumin as standard. Electrophoretic isoenzyme analysis was carried out using polyacrylamide gel electrophoresis (PAGE) as described by Siciliano and Shaw (19). Gels were run in a horizontal LKB Multiphor II electrophoresis apparatus (Pharmacia LKB Biotechnology, Upsala, Sweden) for 5 hrs. at 300 v in cold condition. Six enzymes, Alkaline phosphatase (ALP - E.C.3.1.3.1), Acid phosphatase (ACP - E.C.3.1.3.2) Glucose-6-phosphate dehydrogenase (G6PDH- E.C.1.1.1.49), Isocitrate dehydrogenase (ICDH - E.C.1.1.1.42), Malic enzyme (ME - E.C.1.1.1.40) and Phosphoglucomutase (PGM- E.C.2.7.5.1) were studied following electrophoresis and staining for various enzyme systems. The staining process was same as described by Siciliano and Shaw (19) for ACP, G6PDH, ICDH, ME and PGM and that for ALP was done according to Harris & Hopkinson (9). Complete axenic medium (7) or bovine serum and standard enzymes were used as controls.
Results

Diagramatic representation of six enzymes revealed three different zymodemes among the three (Fig. 1) isolates of Giardia. A distinct electrophoretic heterogeneity was observed in two enzymes namely PGM and ICDH. PD-1 and PD-2 appeared to be quite different from P-1 since the PGM profile showed three bands in PD-1 and PD-2 whereas only one band appeared in P-1 isolate. The migration band of enzyme ICDH in P-1 isolate was found to be slower than PD-1 and PD-2. Significant heterogeneity was also observed in the zymographs of the other two enzymes namely ALP and ME. Two ALP bands could be seen in PD-1 whereas only one band appeared in PD-2 and P-1. The band of ME was found to migrate fast, moderate and slow in PD-2, PD-1 and P-1 isolates, respectively. No difference in the mobility of the two enzymes namely ACP and G6PDH was discernible in the three isolates.

No enzymatic activity was observed in the axenic medium or in bovine serum, a major constituent of the axenic medium, which served as control.
Discussion

Electrophoretic isoenzyme patterns have been employed to identify genetic heterogeneity among the isolates of parasitic protozoa (1, 2, 14, 16, 18, 19).

The isoenzyme profiles of the three isolates of Giardia, PD-1, PD-2 & P-1, revealed three different zymodemes and each strain differed from the other in two or more enzymes out of six enzymes studied. The significant differences were observed among PD-1 and PD-2, (the two Indian isolates) in ICDH, ALP and ME enzymes (may be indicative of different pathogenic forms of G.lamblia).

Korman et al (10) have analysed four human Giardia isolates from Jerusalem and one isolate from Bethesda by five isoenzymes and observed four enzyme profiles. The two Jerusalem strains were indistinguishable but the rest differed from each other in one or many enzymes. Bertram et al (2) have compared isoenzyme patterns of Giardia isolates from different mammalian sources including three human isolates from Portland, Bethesda, and England. The latter two were indistinguishable but differed significantly from the Portland isolates. These findings demonstrated considerable heterogeneity among Giardia species isolated from same geographic area as well as from different geographic origins.

Beveja et al (1) studied the isoenzymes of three G.lamblia strains isolated from Indian patients. The zymodeme patterns of these isolates differed significantly from the
enzyme profiles of our Indian isolates (PD-1 and PD-2). These observations strongly suggest the existence of multiple genetic forms of G. lamblia in this subcontinent.

The differences in the enzyme profiles of Portland-1 strain in our study with those obtained by Baveja et al (1) may be explained by the fact that more than one P-1 strain exists in the Scientific Community (17).

The consistent reproducible profiles of the enzymes over a period of ten months were observed among all the isolates indicating the stable nature of these enzymes. Multiple and double banded profiles of PGM and ALP were observed respectively among the Indian isolates. The genetic interpretation of multiple banded pattern of PGM and ALP in two Indian isolates raise the possibility of genetically different clones or diploid nature of the isolates with isoenzymes specified by different alleles at the same locus (14). Since we did not clone the Giardia, these questions are yet to be answered. Meloni et al (15) have used clonal culture of Giardia and found that the original multiple banded profiles in PGM were retained in the clones, thus ruling out the probability of genetically different clones in one isolate. The double banded pattern of ALP enzyme observed in PD-1 isolate could explain it as a heterozygous organism in which the enzyme might be coded by different alleles at the same locus.

This is probably the first report on ICDH and ALP isoenzymes studies in Giardia and considerable differences were observed in these enzyme profiles of the three isolates.
Although only a few strains could be processed as difficulties exist in axenization, however, the heterogeneity among the strains has been well established by the differences in zymodeme pattern, a well known marker of genetic variation. On the other hand these can be related to the behavioural characteristics of the parasites (15), such as pathogenicity, virulence and susceptibility to drugs. The study may thus be helpful in predicting the control and dissemination of the parasitic infection.
References


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