BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON ANTIGENS OF GIARDIA LAMBLIA TROPHOZOITES
3. BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON ANTIGENS OF GIARDIA LAMBLIA TROPHOZOITES

3.1 Materials and Methods

3.1.1 Maintenance of axenic strains of G. lamblia

An axenic strain of G. lamblia Portland-1 (ATCC No. 30888, kindly supplied by Dr. S.R. Das, Central Drug Research Institute, Lucknow) was routinely maintained in TYI-S-33 medium (Diamond et al., 1978).

The composition of TYI-S-33 medium was as follows:

1) Nutrient broth

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase (BBL)</td>
<td>20.0 gm</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.6 gm</td>
</tr>
<tr>
<td>L-cysteine-HCl (Monohydrate)</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.022 gm</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Triple distilled water</td>
<td>875.0 ml</td>
</tr>
</tbody>
</table>

pH was adjusted to 6.8 with 1 N NaOH.

The broth was filter sterilized through a 0.22 μ membrane filter (Millipore, USA) using an autoclaved filter assembly (Millipore, USA).

11) Preparation of vitamin - Tween mixture

Stock solution

Solution A: Vitamin mixture NCTC 107 was prepared as originally described by Evans et al. (1956) for use in tissue culture media.
Solution B: Vitamin B₁₂ (Sigma), 40 mg was dissolved in glass distilled water and the final volume was brought to 100 ml.

Solution C: D-L 6, 8-thioctic acid (Oxidized, Sigma Chem. Co., USA) 100 mg was dissolved in small volume of ethanol and the final volume made to 100 ml by glass distilled water.

Solution D: Tween-80 (Sigma), 50 gm dissolved in ethanol and the final volume was made to 100 ml by glass distilled water.

Stock solution To 1,000 ml of A, 12 ml of B, 4 ml each of C and D and 180 ml of water were added. The working solution was sterilized by passing through 0.22 μ membrane filter (Millipore, USA) and stored at -20°C until use.

111) Preparation of antibiotic solution

Penicillin (10,000,000 IU) and Streptomycin (1.0 gm) were dissolved in 10 ml of sterile distilled water.

1v) Complete TyI-S-33 medium

The constituents of complete TYI-S-33 medium for 100 ml are:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter sterilized TYI-S-33</td>
<td>87 ml</td>
</tr>
<tr>
<td>nutrient broth</td>
<td></td>
</tr>
<tr>
<td>Complete vitamin mixture</td>
<td>3 ml</td>
</tr>
<tr>
<td>Penicillin + streptomycin</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>Sterilized bovine serum</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Aseptic conditions were maintained throughout the preparation.

The complete TYI-S-33 medium was dispensed in 9-10 ml volumes in sterilized screw cap tubes (15x125 mm Borosil, India). The tubes were incubated at 37°C for 24-48 hours to ascertain the sterility of the medium. Subculturing of *Giardia lamblia* strain Portland-1 (P-1) was carried out.
at 72 hrs interval in the above medium.

3.1.2 Studies on crude soluble antigens

1) Preparation of G. lamblia antigen

72- to 96-hour old axenic cultures showing exponential growth were chilled in an ice bath for 5 mins and centrifuged at 500 xg for 5 min in Bechman TJ 6R cold centrifuge. All the cells were pooled and washed once with 0.25 M sucrose solution followed by three successive washes in chilled normal saline (0.85% w/v NaCl). Number of trophozoites were counted in a haemocytometer and the concentration was adjusted to a known value. This suspension was then sonicated with eight 30 sec. bursts at 1.5 mA in MSE sonicator (U.K.). The sonicated material was then centrifuged at 10,000 xg for 20 min at 4°C in a high speed centrifuge (Sorval RC 5C, USA). The supernatant was collected as crude soluble antigen (CSA) and the protein content was estimated by the method of Lowry et al (1951).

II) Estimation of protein

The protein content of the antigen was estimated according to the methods described by Lowry et al (1951), Bradford (1976) and Markwell et al (1978). Bovine serum albumin (Sigma Chemical Co., USA) was used as the standard. The readings were taken at 600-660 nm in spectronic 20 (Bousch and Lomb, USA) using reagent blank as zero. Protein contents of axenic G. lamblia antigen is shown in Table 3.1.

III) Detection of antigenic activity in soluble and particulate fraction (10,000 xg)

After sonication and centrifugation at 10,000 xg, two fractions were obtained. The clear supernatant was termed as crude soluble antigen (CSA) and the pellet as particulate antigen. Using an indirect ELISA,
antigenic activity of both the fractions were tested against the immunized rabbit and symptomatic giardiasis sera. The detailed methodology of the ELISA has been described in section 3.1.2 vi (c).

iv) Production of antisera in rabbits

a) Against G. lamblia trophozoites (IRS-GL)

48-72 hrs old active active trophozoites showing log phase growth were fixed in 1% formal saline for 1 hr at 4°C. After fixation, the cells were washed five times in cold normal saline and resuspended in 0.5 ml of same solution. Initially each rabbit was given an intramuscular injection with 5x10⁶ trophozoites suspended in 0.5 ml of saline and equal volume of adjuvant. This was followed by intravenous injection of Giardia at 3-4 days intervals. The number of Giardia to be injected was increased gradually from 5x10⁶ to 25x10⁶ organisms per rabbit. The first course of immunization consisted of seven such intravenous injections. This was followed by a rest period of two weeks. The second course of immunization also consisted of seven such intravenous injections given at 3-5 days intervals. Animals were bled by intracardial puncture after 10 days of the last immunization. The sera were tested for their reactivity against Giardia crude soluble antigen in gel diffusion precipitin test (GDP). The procedure for performing the GDP is given elsewhere (3.1.2 vi. a).

b) Against crude soluble antigen (IRS-CSA)

Rabbit antiserum to CSA was prepared by injecting rabbit with CSA, emulsified in Freund's complete adjuvant followed by Freund's incomplete adjuvant (Difco Laboratories, Detroit, Michigan, USA) and then by the antigen alone. Half millilitre of CSA (containing about 2 mg of protein) was emulsified with equal volume of Freund's complete adjuvant and injected intra muscually into the hind legs of each rabbit. A total of two
such injections were given to each rabbit at 7 days intervals. This
was followed by two intramuscular injections with 3 mg of CSA in
0.5 ml + 0.5 ml FICA given at 7 days intervals. After 7 days of rest
animals were immunized intravenously with antigen alone. Four such
injections were given and the animals were bled a week after the
last injection and sera were tested for their reactivity against CSA
by GDP test.

c) Against Fraction I (F-I) of CSA (IRS-F-I)

Rabbit antiserum to Fraction-I of CSA was raised by injecting
rabbits intramuscularly with 0.4 ml of F-I (containing 0.4 mg of
protein) emulsified with equal volume of FCA. This was followed by
an identical immunization with FICA at an interval of 15 days. After
10 days of last immunization, animals were given 3 intravenous injec­
tions (each consisting of 0.4 mg protein) at 3-5 days intervals. The
rabbits were bled after 10 days of the last immunization and the sera
were tested for their reactivity against F-I antigen in GDP test.

v) Fractionation of CSA by Sephacryl S-300 (gel filtration)
column chromatography

Crude soluble antigen of _G.lamblia_ trophozoites as prepared
before was subjected to gel filtration through Sephacryl S-300 column
in order to separate its antigenic fractions. About 80 ml of pres­
wollen Sephacryl S-300 (Pharmacia Fine Chemicals, Sweden) wet bead
diameter 40-105 μm was taken in a beaker and washed by repeated
decantation with addition of 0.05 M Tris-HCl buffer pH 7.4. The
slurry of the gel was then poured in a glass column - (1.6 x 40 cm,
Pharmacia LKB United, Sweden) and allowed to pack under gravity upto
the height of 35 cm. The gel column was then equilibrated overnight
by passing the same buffer with a constant flow using peristaltic pump (P-1 - Pharmacia LKB United, Sweden). A constant volume of 20 ml/hr flow rate was adjusted throughout the experiment. The void vol. of the column was determined by applying Dextran blue - 2000.

About 1.5 ml (1-2% of bed volume of column) of CSA which was previously dialyzed (Amicon-Ultrafiltration system, USA, using PM-10 membrane) with eluent buffer containing about 24 mg of protein was applied on the top of the gel column. Before sample application the upper surface of the gel bed was protected with a piece of filter paper. The sample was eluted with 0.05 M Tris-HCl buffer pH 7.4 and about 3 ml eluates were collected in each tube with the help of a fraction collector (Frac-100, Pharmacia LKB United, Sweden). An elution profile was obtained by measuring the optical density (OD) at 280 nm in spectrophotometer (Shimadzu Corporation, Japan) of the eluted material and these values were plotted against the elution volume (ml). According to the OD value, each peak was pooled separately and termed as fractions. These fractions were concentrated by lyophilization and dialysed against 10 mM PBS, centrifuged and stored at 4°C until used for further studies.

**Molecular weight determination by gel filtration calibration kit.**

Molecular weight of each peak was determined by using gel filtration calibration kit (Pharmacia LKB United, Sweden). A calibration curve was prepared by measuring the Kav values of the standard proteins and these Kav values were plotted against their molecular weights. The molecular weight of unknown substances could be determined from the calibration curve once Kav value was calculated from its measured elution volume.
Kav value was calculated by using the equation:

\[ Kav = \frac{V_e - V_o}{V_t - V_o} \]

Where, \( V_e \) = Elution volume of protein
\( V_o \) = Column void volume
\( V_t \) = Total bed volume

vi) Immunological procedures used in the determination of antigenic activity of crude soluble antigens and its Sepharcryl-S-300 fractions

The antigenic activity of CSA and its fractions were tested against the immunized rabbit sera. The following tests were used during the study:

a) Gel diffusion precipitin (GDP) test

The test was done as detailed by Ouchterlony (1962). About 3 ml of 0.85% agarose (Sigma Chemical Co., USA) in PBS pH 7.4 (containing 0.02% sodium azide) was uniformly layered on a glass slide and kept at 4°C for solidifying. Wells were punched as required; each well was 3 mm in diameter and 5 mm apart from the adjoining wells. Antigens and antisera were introduced in the respective wells twice at hourly intervals, so as to ensure adequate quantity of reactants in the test. The charged slides were incubated in a humid chamber at room temperature for 4-6 hrs for diffusion then at 4°C for overnight for maximum precipitation. Results were noted next morning, a positive reaction usually appeared within 24 hrs.

b) Counterimmunoelectrophoresis

The test was performed according to method described by Sepulveda et al (1971). Three ml of 0.9% agarose in veronal buffer
(pH 8.2, ionic strength 0.05 M) was layered on a clean microslide (2.5 cm x 7.5 cm) in the manner already described for the Ouchterlony test.

**Veronal buffer pH 8.2 (ionic strength 0.05 M)**

Sodium barbital 11.9 g  
Dist. water 1,000 ml

To this 1.17 NHCl was added to adjust the pH to 8.2 and the volume was adjusted to 1066.25 ml with distilled water. For the test, antigen and antiserum wells, each 3 mm in diameter and 5 mm apart were punched and the antigen wells were placed towards the cathode of the power supply. Electrophoresis was carried out in electrophoresis chamber (Shandon, UK) at a constant voltage of 160 volts or 10 milliamps per slide for 20-30 mins. Bromophenol blue stain as an indicator was run simultaneously.

c) **Enzyme-linked immunosorbent assay (ELISA) test**

The method of micro ELISA test was essentially the same as described by Chaudhuri et al (1988).

**Sensitization of micro ELISA plates**

The test was performed in disposable, flat-bottomed, micro-ELISA plates (NUNC, Denmark) having 96 wells. Lyophilized CSA and its fractions prepared from P-1 strain of *G. lamblia* trophozoites were reconstituted with the carbonate-bicarbonate buffer (0.05 M, pH 9.6). The plates were coated with 100 μl of different concentrations of antigenic proteins (viz. 20 μg, 10 μg, 5 μg, 2.5 μg, 1.2 μg per ml) and kept overnight at 4°C in the refrigerator.
Treatment of sera

Next morning plates were removed from the refrigerator and washed thrice with washing solution (PBS, pH 7.4 and 0.05% Tween-20). In this experiment a known dilution (1:200) of antibody was used to react with variable amounts of antigen. Four pre-immune rabbit sera and four immunized rabbit sera (immunized against *Giardia* CSA) were pooled in separate batches and used as reference positive and negative sera. A 100 μl volume of the diluted serum was put into duplicate wells of the plates prepared as above and were incubated at 37°C for 90 minutes in a humid chamber.

Treatment with horse-raddish peroxidase (HRP) conjugated anti-rabbit IgG

After incubation with sera, plates were washed thrice with washing solution and a 100 μl of diluted (1:1000) conjugate solution (anti-rabbit IgG; Sigma Chemical Co., USA) was added to each well except the conjugate and substrate control wells. The contents were incubated as above for 90 minutes.

Detection of enzyme activity

The plates were washed as above and the enzyme activity was estimated using O-phenylene diamine dihydrochloride (OPD) as substrate. The substrate solution was prepared as follows:

- OPD (Sigma Chemical Co., USA) 40 mg
- 30% hydrogen peroxide (BDH) 40 μl
- Phosphate citrate buffer (0.2 M, pH 3.7) 50 ml

To each well 100 μl of freshly prepared substrate solution was added and the reaction was allowed to proceed for exactly 20 mins in the dark. The reaction was terminated by the addition of 25
μl of 5(N) H₂SO₄ to each well. The colour intensity, developed was recorded as optical density (OD) at 490 nm wave length in an automated micro ELISA reader (Dynatech Lab., USA) considering substrate control as blank.

vii) Protein profiles and molecular weight determination of CSA and its fractions by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous SDS-PAGE was performed as described by Laemmli (1970) and Hames & Rickwood (1986) in 10% acrylamide - bis acrylamide separating gel.

During electrophoresis, following major gel recipes were used:

**Stacking gel** - 3% acrylamide-bisacrylamide, 0.187 M Tris-HCl, pH 6.8 and 0.1% sodium dodecylsulphate (SDS).

**Separating gel** - 10% acrylamide-bisacrylamide, 0.375 M Tris-HCl, pH 8.6 and 0.1% SDS.

**Electrophoresis buffer** - Tris-glycine buffer pH 8.3 and 0.1% SDS.

**Standard protein** - Known standard proteins (molecular weight standard kit – from Pharmacia LKB United, Sweden and Sigma Chemical Co., USA) consisting of Myosin B-galactosidase, phosphorylase b, albumin bovine, albumin, egg and carbonic anhydrase were used in this study.

**Preparation of samples and electrophoresis**

Briefly, to 100-200 μg of samples protein in an appropriate volume of sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue in 0.0625 M Tris-HCl, pH 6.8) was added and the suspensions were then boiled for 3 min in boiling water bath and
vortexed immediately. After a brief centrifugation at 1000 × g the samples were introduced into the respective wells of the gels.

Electrophoresis was carried out in rectangular vertical slab gel apparatus (BIORAD Laboratories, Richmond, USA) at a constant temperature (18°C) using 60 volts until the samples touched the top of the separating gel and then increased to 120 volts. Electrophoresis was terminated when the dye front migrated to within 1-2 cm of the bottom of the gel.

Fixation, staining and destaining of gel

Following electrophoresis the gel was immediately removed from the glass plates and transferred to a tray containing 15% (w/v) trichloroacetic acid solution for fixation for 1-2 hrs. Then the gel was stained with 0.1% Coomassie blue in 50% methanol and 10% acetic acid for overnight. Next day, the gel was washed with 50% methanol and 10% acetic acid for one hr and then destained with 5% methanol and 10% acetic acid.

Molecular weight determination

Subunit molecular weights of the test material was determined by comparing their electrophoretic mobilities with the standard proteins (Sigma Chemical Co., USA, Cat. No. SDS-6H and Pharmacia United Ltd., Sweden). The distance of protein migrated and the distance of dye migrated from the origin of the separating gel were measured and relative mobility (Rf) value was calculated using the following formula:

\[
\text{Relative mobility (Rf)} = \frac{\text{Distance of protein migration} \times \text{Gel length before staining}}{\text{Distance of dye migration} \times \text{Gel length after staining}}
\]
A standard graph was plotted using mobility values of standard proteins. The molecular weight of unknown proteins were calculated from the standard graph.

**viii)** Detection of immunoreactive polypeptides of Fraction I (F-I) of CSA by immunoblotting (Western blotting)

Immunoblotting (Western blotting) was employed for the detection of immuno-reactive polypeptide and estimation of their apparent molecular weight after separation in SDS-PAGE. The technique for performing the test was the same as described by Towbin (1979) with slight modification.

The antigens were first separated in 10% SDS-PAGE. They were then transferred to nitrocellulose (NC) strips (Pore Size 0.45 μ, BIORAD, USA) in a transblot apparatus (BIORAD, Richards, USA) keeping gel on cathodal side and NC paper on anodal side, in the presence of transfer buffer (25 mM Tris, 192 mM glycine 20% mechnol) at a constant current of 400 mA at 4°C for 3 hrs.

   a) **Blocking of extra protein binding sites of the nitrocellulose membrane**

   Soon after the transfer was over the nitrocellulose (NC) strips were washed thoroughly in Tris buffer saline (TBS) 10 mM Tris and 150 mM NaCl with few changes and incubated with blocking buffer (10 mM Tris, 150 mM NaCl, 3% BSA pH 7.4) at 4°C for overnight.

   b) **Incubation with first antibody**

   Next morning the NC strips were washed once with TBS and incubated with a 1:200 dilution of IRS-CSA and normal rabbit serum (NRS) prepared in blocking buffer at 37°C for 90 mins with constant shaking on a rocking shaker (Luckham-R-100 TW, England).
c) **Incubation with HRP conjugated second antibody**

After four washes in TBS-Tween (TBS + 0.05% Tween 20) and three washes in TBS only, the NC strips were incubated with 1:4000 dilution of HRP conjugated goat anti-rabbit IgG (Jackson Immuno-research Lab., USA) for 60 mins at 37°C on a rocking shaker.

d) **Development of precipitin colour complex**

The washed nitrocellulose strips were developed in 0.05% solution of 3,3-diaminobenzidine tetra hydrochloride (Sigma Chemical Co., USA) and 0.01% hydrogen peroxide in TBS. Reaction was allowed to proceed for 5-10 min at room temperature with continuous shaking and was stopped after washing in distilled water and blot dried. Intensities of antigen antibody complex was estimated visually.

1x) **Localization of F-1 antigens on the surface of G.lamblia by indirect fluorescent antibody test (IFAT)**

IFAT was carried out to localize the F-I antigens on the surface of G.lamblia. The technique used in IFAT was the same as described by Das et al (1984) with slight modification. In place of fixed cells (Methanol), live trophozoites were used in this study.

Trophozoites of axenic G.lamblia (strain P-I) from 48-72 hrs old cultures were washed thrice with chilled PBS (pH 7.4) in a tube. To this 100 µl of 1:100 dilution (found optimal in earlier standardization study) of immunized rabbit sera to FI (IRS-F-I) was added and incubated at 4°C for 30 min. The cells were then washed three times in PBS (pH 7.4) and allowed to react with 1:50 (optimal in earlier studies) dilution of fluorescein isothiocyanate conjugated antirabbit IgG (Sigma Chemical Co., USA; Cat. No. F-0382, F/P ratio 4.2) diluted in PBS (pH 7.4) for 30 min. at 4°C. The cells
were washed once again to remove the excess conjugate and mounted with buffered glycerol (PBS : Glycerol, 1:9) on a clean glass slide and examined under a fluorescent microscope (Reichert Polyvar-Jung, Austria) using excitor (blue BP-475-495) and barrier filter (B4, BP 520-560).

x) Detection of immunogenic surface components in F-I

The F-I antigen was separated first in 10% SDS-PAGE and transferred to NC paper as described earlier in section 3.1.2. NC strips were then incubated with 1:100 dilution of F-I antibody raised in rabbits. The duplicate NC blot was incubated with same dilution of Giardia trophozoites absorbed (Packed G.lamblia: IRS-F-I, 1:1. Incubated at 4°C overnight) F-I antibody. Further incubations of NC strips with conjugate and substrate solution was essentially the same as described earlier (vide section 3.1.2).

3.1.3 Studies on plasma membrane antigen(s)

1) Isolation and purification of PM

The plasma membrane from axenic G.lamblia trophozoites (strain P-1) was isolated according to the method of Clark and Holberton (1986). Briefly, trophozoites (confluent wall attached cells + suspension cells) from 50 cultures tubes were harvested and washed thrice in cold normal saline and centrifuged at 4,800 g for 15 min at 4°C. The cell pellet was suspended at 0°C in 20 times of the original pellet volume of hypotonic buffer (10 mM Tris-HCl, 30 mM MgCl₂, 0.005% PMSF, pH 7.4) for 10 min. The swollen cells were then homogenised in the shearing field of a polytron tissue homogenizer for 5 min. The swelling step and the subsequent homogenisation were
monitored by phase contrast microscopy. To stabilise nuclei and cytoskeletons, the ionic strength was restored immediately after disruption by adding 0.1 volumes of 70 mM NaCl, 30 mM KCl. The homogenate was then centrifuged at 1000 x g for 1 min at 4°C in a swing out rotor to remove nuclei and any other intact cells remaining after homogenisation. The partly-cleared supernatant (10 ml) was removed and centrifuged again. The second supernatant contained crude plasma membranes and was then made to 25% sucrose solution by adding equal volume of buffered (hypotonic) 50% sucrose. Eight ml aliquot of this sample was layered onto the 4 ml of 50% buffered sucrose and centrifuged in a Beckman SW41T rotor at 100,000 x g for 60 min at 4°C. A fluffy band was seen in the junction of 25%/50% sucrose solution interface. This fluffy band was removed, diluted with buffer (hypotonic), and recentrifuged at 100,000 x g for a further 30 min at 4°C, to pellet the pure plasma membranes.

11) Tests for purity of plasma membrane

a) Morphological examination by electromicroscopy

The sample (plasma membrane) preparation for electronmicroscopic examination was done according to the method of Doane and Anderson (1987). Briefly, one drop of specimen (plasma membrane) was placed on a drop of sterile (filtered) distilled water resting on a waxed surface. A coated grid was touched briefly to the surface of the drop (coated surface down). A drop of 2% phosphotungstic acid (containing 0.01% bovine serum albumin, pH adjusted to 6.5 with 1 N KOH) was then added to the grid. Excess fluid was removed by soaking with a piece of filter paper, and the preparation was air dried. The grids containing the plasma membrane specimens were examined in Phillips 420 transmission electron microscope.
b) **Marker enzyme assay**

Plasma membrane bound different enzymes activity were studied as follows:

**Acid phosphatase**

The activity of acid phosphatase was assayed according to the method of Muller (1973) with slight modifications. The reaction mixture contained 0.2 ml of enzyme source and 1.0 ml of 100 mM acetate buffer (pH 4.0) containing 5 mM p-nitro phenyl phosphate. The tubes were incubated at 37°C water bath shaker for 30 min. To this 0.5 ml of 1.2 M TCA added and the tubes were centrifuged. The supernatants were collected in fresh tubes and 0.2 ml of 4 N NaOH was added to each tube. The colour developed was read at 450 nm in a spectrophotometer (Shimadzu, Japan).

**Alkaline phosphate**

Alkaline phosphatase activity was determined as the Mg^{2+} dependent alkaline p-nitrophenyl phosphatase activity as described by Barden et al (1983). A 200 µl of sample was incubated in 2 ml of a solution containing 10 mM Tris, 5 mM MgCl₂, and 10 mM p-nitrophenyl phosphate at pH 10. After an incubation of 15 min at 37°C, the reaction was stopped by the addition of 0.2 vol of 1 m K₂HPO₄, 0.25 M EDTA, pH 10. Absorbance was measured at 420 nm in a Shimadzu (Japan) Spectrophotometer.

**Na⁺-K⁺-ATPase**

Na⁺-K⁺-ATPase was assayed by the procedure of Hoelzl-Wallach and Kamat (1966). The activity of Na⁺-K⁺-dependent ATPase was estimated by the increase in the rate of ATP hydrolysis which occurs in the presence of both Na⁺ and K⁺ over the rate found when K⁺ lacking.
The reaction mixture for Na\textsuperscript{+}-K\textsuperscript{+}-ATPase consisted of 100 mM NaCl, 100 mM KCl, 5 mM MgCl\textsubscript{2}, 5 mM EDTA, 10 mM Tris-HCl pH 8.6, 5 mM ATP and enzyme source (200-300 μg protein) in a final volume of 1 ml. The reaction was allowed to proceed for 45 mins at 37°C and terminated by adding 0.3 ml of 30% TCA at 4°C.

The samples were centrifuged at 10,000 rpm for 5 min at 4°C; quantitation of the supernatant was made for the inorganic phosphorus generated by the method of Plumer (1976). The μ mole Pi liberated/min/mg protein was considered as specific activity of the enzyme.

5'-Nucleotidase

The 5'-nucleotidase activity was determined by the method of Michell and Hawthorne (1965). The plasma membrane fraction (200-300 μg protein) was incubated at 37°C for 15 min in 2 ml reaction medium containing; 100 mM KCl; 10 mM MgCl\textsubscript{2}; 50 mM Tris-HCl (pH 7.4), 5 mM 5' AMP (disodium salt) and 10 mM sodium potassium tartrate. The reaction was terminated by adding 1 ml of 25% (w/v) TCA and Pi was estimated in 2 ml of the supernatant by the method of Plumer (1976).

Production of immunized rabbit sera against plasma membrane antigens (IR5-PM)

Two young male albino rabbits weighing 1.5 - 2 kg, were immunized with 0.5 ml of purified plasma membrane fractions containing 300-400 μg of antigenic protein, emulsified with equal volume of Freund's complete adjuvant (Difco, USA) and injected subcutaneously at different sites. A total of three such injections were given at 7 days intervals. This was followed by the three Intramuscular injections with plasma membrane antigens alone at an interval of seven days in between. Animals were bled a week after the last immunization and sera were tested for its reactivity against homologous antigens.
iv) Physico-chemical characterization of plasma membrane antigen(s)

a) Heat Treatment

About 1 mg of purified plasma membrane was rehydrated in 6 ml of PBS (pH 7.4) and distributed in six screw capped tubes. Each tube was heated at different temperature (viz. 60-100°C) in a thermo-regulated water bath for 10-20 mins. Antigenic activity either lost or retained after the heat treatment was tested against immunized rabbit sera (IRS-PM).

b) Proteolytic digestion

Hundred microliter of different concentration of trypsin and pronase (starting from 10 μg – 1000 μg) were added in duplicate wells of antigen coated ELISA plate. The plates were incubated at 37°C for 2 hrs and washed three times with PBS (pH 7.4). The antigenic activity of the plasma membrane antigen was tested against immunized rabbit sera (IRS-PM) in ELISA.

c) Periodate oxidation

Periodate oxidation was carried out by incubating the antigen (plasma membrane) coated ELISA plate with 0.05 M sodium meta-periodate in 10 mM sodium acetate buffer pH 4.5 for overnight at 4°C in dark. Appropriate control (i.e. incubation of antigen with 10 mM sodium acetate buffer) was also employed with same plate. The plates were washed thrice with PBS (pH 7.4). The antigenic activity after periodate oxidation was tested against immunized rabbit sera (IRS-PM) in ELISA.

v) Agglutination studies

A micro agglutination assay was carried out as described by Hill et al (1981) to assess the binding of lectins with the Giardia
trophozoites as well as to demonstrate the presence of glycoproteins on the surface of the trophozoites. The lectins used were wheat germ agglutinin (WGA), phytohaemagglutinin (PHA), Concanavalin A (Con A), peanut agglutinin (PNA), and soy bean agglutinin (SBA). All the lectins were procured from Sigma Chemical Co., USA. A $5 \times 10^5$ trophozoites of Giardia were incubated at room temperature with different concentration of lectins viz., 0, 1, 10, 25, 50, 100, 250 and 500 $\mu$g/ml prepared in PBS (pH 7.4). The lectin-trophozoite suspension was then centrifuged at 250xg for 5 min. Approximately 0.7 ml of supernatant was discarded, and the pellet was gently dispersed in the remaining 0.3 ml volume.

The specific monosaccharides used to inhibit these lectins and their inhibitory doses are as below:

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Sugar specificity</th>
<th>Inhibitory dose of the specific sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Wheat germ agglutinin (WGA)</td>
<td>N-acetyl-D-glucosamine (GLC NAc) and N-acetyl-D-neuraminic acid (NANA)</td>
<td>5 mM</td>
</tr>
<tr>
<td>2. Phytohaemagglutinin (PHA)</td>
<td>N-acetyl-D-galactosamine (Gal NAc)</td>
<td>&quot;</td>
</tr>
<tr>
<td>3. Soybean agglutinin (SBA)</td>
<td>- do -</td>
<td>&quot;</td>
</tr>
<tr>
<td>4. Concanavalin (Con A)</td>
<td>D-glucose</td>
<td>&quot;</td>
</tr>
<tr>
<td>5. Peanut agglutinin</td>
<td>D-galactose</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
A drop of this suspension was placed on a haemocytometer and examined microscopically. Agglutination was assessed by counting the number of trophozoites that were agglutinated in the groups of >3 with flagella-body, body-body, flagella-flagella and the agglutination was determined as suggested by Hill et al (1981).

vi) SDS-PAGE analysis of plasma membrane antigens

The protein constituents of plasma membrane antigens were analysed by SDS-PAGE according to the method of Laemmli (1970). Before the plasma membrane antigens were loaded onto the gel, the protein contents were estimated by modified Lowry method (Markwell et al, 1978). The detailed methodology of SDS-PAGE has been described in section (3.1.2 vii).

vii) Lectin blot assay

The major glycoproteins of the plasma membrane antigens were detected by Lectin blot analysis using WGA and Con A lectins as probe (Hawkes, 1982; Ward et al, 1988).

The plasma membrane antigens were first separated in 10% SDS-PAGE and transferred to NC paper (Towbin et al, 1979). It was then incubated with 10% solution of fetal calf serum (FCS) in TBS (pH 7.4) for 1 hr to block the extra protein binding sites. After incubation, blocking solution was aspirated out and freshly prepared Con A-HRP and WGA-HRP (Sigma Chem. Co., USA) solution (50 μg/ml in blocking solution was found optimal) was added. After an hour of incubation at 37°C, the nitrocellulose strips were washed thoroughly for 15 min with three changes of TBS. The bound peroxidase was detected by the addition of a freshly prepared substrate solution (described in Section 3.1.2 viii). The blots were then washed in distilled water air dried and stored in the dark. The glycopeptide bands appeared dark brown.
viii) Detection of antigenic components of plasma membrane by immunoblot assay against IRS-PM

The method for immunoblotting was essentially the same as described previously (vide section 3.1.2 viii). Briefly, purified membrane was run in 10% SDS-PAGE and transferred to NC paper. After blocking, NC strips were incubated with IRS-PM. Subsequent incubation in second antibody and addition of substrate solution was same as described earlier.

ix) Immunoblot analysis of plasma membrane antigen against acute giardiasis patients sera

The method for immunoblotting was essentially the same as described previously (vide section viii). Precisely, after SDS-PAGE the plasma membrane proteins were transferred to nitrocellulose paper. The nitrocellulose strips were incubated at 4°C overnight in blocking buffer. Next morning, the strips were washed three times in TBS and incubated with pooled acute giardiasis patients sera (five patients) at 37°C for 90 min with constant shaking (Luckham, England). A 1:50 dilution of pooled acute giardiasis patients' sera was found optimal in this study. After incubation the nitrocellulose strips were washed (three times with TBS + 0.05% Tween 20 and five times with TBS only) as and before incubated again with anti-human immunoglobulins conjugated with HRP (Jackson Immuno Research Lab., Inc., USA) for 90 min at 37°C with constant shaking. A 1:4000 dilution of conjugate was found optimal in this study. Strips were once again washed and developed in substrate solution (0.05% 3'-3' diamino benzidine tetra hydrochloride, Sigma Chemical Co., USA; 0.01% hydrogen peroxide in TBS) for 15 min. The reaction was stopped by washing the strips in distilled water. Strips were then blot dried and results were read visually.
Production of immunized rabbit sera against highly immunoreactive polypeptide (≈118 kDa) of plasma membrane

The method for immunization was essentially the same as described previously (vide section 3.1.2 iv).

Briefly, the plasma membrane protein was separated in 10% SDS-PAGE and 118 kDa polypeptide was marked and isolated by simply slicing off the proper band. This was achieved by staining the gel with 0.2% coomassie blue which also contained the known marker protein along with the sample. The 118 kDa protein band was located very near to the known protein β-galactosidase band, having the mol wt. of 116 kDa (Sigma Chemical Co., USA). About 0.25 cm wide sliced gel strip containing 118 kDa protein was taken and washed twice in distilled water. Washed gel was then homogenized in a glass homogenizer (Pisco, India) in 1.0 ml normal saline. The homogenized gel was emulsified with equal volume of FCA and injected subcutaneously at four different sites on the back of albino rabbits. The further immunization schedule was the same as described earlier (section 3.1.2 iv). The production of immune sera was checked in GDP as well as in immunoblot assay.

Effect of different antisera on the immobilization of Giardia trophozoites

The in vitro immobilization effect of different antisera viz., IRS-CSA, IRS-G1, IRS-PM, IRS-118kDa and NRS was tested on live trophozoites of Giardia. The immobilization study was performed according to method of Lehmann and Wallis (1988). Briefly, 0.1 ml of Giardia suspension (1x10^6 cells/ml) was mixed with an equal volume of serum on a glass slide and covered with glass cover slip. Normal serum was also diluted 1:1 so that the concentrations of all serum factors should be same. The periphery of the coverslip and slide interfaces were sealed with wax
and incubated in a humid chamber for different time periods viz., 30, 60, 90, 120 and 240 hrs at 37°C. Reactions were examined under a phase contrast microscope at 400 x (Leitz West Germany). Immobilization was determined by movement or lack of movement of the flagellae. Fifty such fields were examined and results were expressed at the mean immobilization of the antibody.

3.2 Results

3.2.1 Antigenic reactivity of soluble and particulate fraction of Giardia

The antigenic activity of the crude soluble antigen (supernatant) and its sediment (pellet) after 10,000 x g was compared against pooled IRS-G1 and acute giardiasis cases in ELISA test (Table 3.2). Crude soluble antigen always showed better O.D. values than the sediment fraction, when used against same concentrations of antigenic protein and equal dilution of antibody in ELISA test. The antigenic activity of the soluble fraction was found more than two fold in both the groups when compared with sediment fraction.

3.2.2 Reproducibility of antigenic activity of axenic G. lamblia (P-1) trophozoites

A standard lyophilized Giardia antigen was prepared from axenic G. lamblia trophozoites (strain Portland-1) and stored at -20°C for several months. The reproducibility and specific reactivity of the antigen was tested by ELISA (Table 3.3). Almost similar results (in OD values) were observed when the same sera were tested against two different batches of G. lamblia antigens.
**Figure 3.1**

Immunodiffusion pattern of different antisera against CSA preparation (Centre well A). Outer wells contained, IRS-CSA (1), IRS-G1 (2), IRS-F-I (3) and preimmune rabbit serum (4). About 100 µg of antigenic (CSA) protein was charged in the Centre well.

**Figure 3.2**

Immunodiffusion pattern of different antisera against F-I antigen (Centre well A). Outer well contained IRS-CSA (1), IRS-F-I (2) and preimmune (3 and 4) rabbit sera.
3.2.3 Reactivity of CSA against immunized rabbit sera

The CSA preparation was tested for its reactivity against antisera (IRS-CSA, IRS-F-I and IRS-G1) raised in rabbits. Distinct precipitin bands were observed both in IRS-CSA and IRS-G1 in agar gel diffusion. However, multiple and intensified precipitin lines were noticed in IRS-CSA (Fig. 3.1).

3.2.4 Reactivity of fraction I (F-I) antigen against immunized rabbit sera

Similarly, as above the F-I antigen was tested against its homologous antibody (IRS-F-I) and IRS-CSA (Fig. 3.2). A pattern of cross reactivity was observed with F-I antibody to IRS-CSA.

3.2.5 Studies on crude soluble antigen

1) Fractionation of CSA by Sephacryl S-300 (gel filtration) column chromatography

The Sephacryl S-300 gel filtration pattern is presented in Figure 3.3. Blue dextran - 2000 and marker proteins were used for determination of void volume and standard calibration curve, respectively. Two major light absorbing peaks were observed, one of which appeared in the void volume and another 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 3.3, to give four different fractions FI to FIV. These fractions were concentrated; dialysed against PBS (pH 7.4) and stored at 4°C.

The molecular weight of each peak was determined by obtaining Kav value for each peak. Kav value for standard proteins were also measured and standard curve was drawn against Log of molecular weights. The
Figure 3.3

Elution profile of crude soluble antigen of axenic G. lamblia trophozoites chromatographed on Sephacryl S-300 gel. Column dimension 1.6 x 40 cm; sample size 24 mg/1.5 ml; 0.05 M Tris-HCl buffer pH 7.6.
Figure 3.4

Immunodiffusion patterns of IRS-CSA (Centre well) against CSA (well no. 5), and different fractions viz. F-I (well no. 4), F-II (well no. 1), F-III (well no. 3), F-IV (well no. 2), obtained after Sephacryl S-300 column chromatography.

Figure 3.5

Bar diagrammatic representation of antigenic activity in Counter immunoelectrophoresis test using constant amount of antigenic proteins of CSA and its fractions (F-I to F-IV) against different dilutions of pooled IRS-CSA.
molecular weights of F-I to F-IV were 150 K, 65 K, 50 K and 10 K daltons, respectively.

11) **Immunological reactivity of different fractions (F-I to F-IV) of CSA**

The presence of antigenic activity in different fractions of CSA were determined against IRS-CSA by GDP, CIEF and ELISA tests.

a) **GDP**

The fractions I to III as well as the parent CSA were found reactive against IRS-CSA in GDP test but F-IV failed to do so. Although the amount of antigenic proteins were taken constant (2 mg/ml) in each cases. It was also noticed the precipitin bands appearing with these fractions showed fusion with each other (Fig. 3.4). The precipitin band of F-I was found to be most prominent as compared to other fractions and CSA.

b) **CIEP**

In this experiment the protein concentrations of all the fractions (F-I to F-IV) and parent CSA were made constant (2 mg/ml) and their reactivity was tested with different dilutions of IRS-CSA. This was done to assess the activity of each antigen in their ability to produce precipitin bands at different antibody dilutions. In CIEP test, F-I fraction showed a positive precipitin reaction upto 1:64 titre of IRS-CSA; the other fractions as well as CSA failed to show reactivity beyond 1:8 titre of IRS-CSA (Fig. 3.5).

c) **ELISA**

The ELISA was employed to quantitate the antigenicity of all the fractions (F-I to F-IV) and CSA against a particular dilution of IRS-CSA. In the ELISA test a clear cut distinction in OD value was observed when
Figure 3.6

Coomassie blue stained SDS-PAGE profiles of *G. lamblia* crude soluble antigen (A) and its Sephacryl S-300 column chromatographed fractions (F-I to F-IV for lane B to E, respectively). In lane M low molecular weight marker proteins were used.
20 µg of CSA was used against a 1:200 dilution of positive and negative serum. The four pre-immune negative sera at 1:200 dilution showed an OD value ranging from 0.05 to 0.09 with a mean OD of 0.07 at 490 nm. However, the four immunized rabbit sera at the same dilution showed OD values ranging between 0.12 to 0.325 with a mean OD of 0.234. Various concentration of CSA and its fractions were compared in the ELISA test for antigenic activity against 1:200 dilution of pooled immunized rabbit sera (Table 3.4). As observed earlier, fraction-I was found to be most antigenic as compared to the parent (CSA) and the other three fractions, in the sense that, 2.5 µg/ml protein of this fraction (F-I) showed almost the same OD value as obtained with 20 µg/ml of CSA, 5 µg/ml of F-II and 20 µg/ml of F-III, respectively. Fraction IV was found to be the least immunogenic in comparison to CSA and other fractions.

iii) SDS-PAGE analysis of CSA and its fractions (F-I to F-IV)

The SDS-PAGE pattern of F-I to F-IV along with the parent material i.e., CSA is shown in Figure 3.6. CSA showed several (about 28) discrete protein bands in the molecular weight region of >125 kDa to ≤ 14 kDa 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 ≈ 94 kDa to 43 kDa. The bands of F-III were mainly confined to the molecular weight region of <94 kDa to 21 kDa daltons. However, F-IV contained low molecular weight protein subunits ranging from <82 kDa to 10 kDa.

iv) Detection of immunoreactive polypeptides of Fraction-I of CSA by immunoblot assay

The immuno-reactive polypeptides of fraction I (F-I) was identified by incubating the NC paper with optimal dilution of IRS-CSA and
Figure 3.7

Showing immunoreactive polypeptides of fraction-I (F-I) antigens against IRS-CSA. Antigens transferred to N.C. strips were incubated with IRS-CSA (A) and NRS (B). Known molecular weight proteins (M) were stained with Ponceau S for comparison.
anti-rabbit IgG conjugate and substrate solution. Immunogenic polypeptides were detected throughout the lane. Highly immunoreactive bands were observed at apparent molecular weight regions of 170, 118, 55, 32, 30 and 23 kDa. However, 118 kDa polypeptide was found less prominent in protein staining (coomassie blue) experiment. Less immunoreactive polypeptide was observed in mol. wt. range of 30 kDa to 45 kDa (Figure 3.7). Same experiment with normal pre-immune rabbit sera, did not show any immunoreactive polypeptide.

v) Localization of F-I antigens on the surface of G. lamblia by IFAT

Rabbit antiserum raised against the fraction-I was used in IFAT to visualize the F-I antigen on the parasite. The antibody to the F-I protein reacted primarily to the surface of the trophozoites and to a lesser extent on the flagella. However, fluorescence of the ventral disc cytoskeleton region was also noticed. The fluorescence in the rims (outer periphery) of Giardia trophozoites were found remarkably brighter than the internal organelles (Fig. 3.8).

vi) Detection of immunogenic surface components in F-I

The identification of immunoreactive surface components in fraction I antigen was performed by immunoblot assay using G. lamblia trophozoites absorbed and unabsorbed F-I antibody (IRS-F-I).

The prominent immunoreactive surface proteins were observed in apparent molecular weight regions of 170 kDa, 118 kDa, 55 kDa, 38 kDa and 30 kDa respectively. These findings were confirmed by using the absorbed IRS-F-I antisera (previously absorbed with live Giardia trophozoites). The absorbed versus unabsorbed data revealed that the immunoreactive prominent protein bands of F-I, mentioned above were either abolished or
Figure 3.8

Localization of F-I antigens on the surface of the trophozoites of _G. lamblia_ as done by IFAT against IRS-F-I.
Figure 3.9

Immunoreactive banding pattern of F-I antigens, against the F-I antisera absorbed with live G. lamblia trophozoites (lane B), unabsorbed serum (lane A) and normal rabbit serum (lane C). Known marker proteins (lane M) were also included.
Fig. 3-9
reduced in intensity when the same F-I blot was incubated with absorbed IRS-F-I (Fig. 3.9).

3.2.6 Studies on the plasma membrane antigens

1) Purity of the plasma membrane

The purity of the plasma membrane was confirmed morphologically by electron microscopy and biochemically by marker enzyme study.

a) Morphological examination of plasma membrane by electron microscope

After the plasma membrane was isolated it was subjected to morphological examination by electron microscope. Under electron microscope the membrane fixed with 1% phosphotungstic acid, appeared as vesicles. No recognizable intracellular organelles or nuclei could be seen in this preparation (Fig. 3.10).

b) Marker enzyme study

Before attempting to isolate the plasma membrane, the cell homogenate was assayed for various enzyme markers generally used in plasma membrane studies with other organisms. The presence of Na\(^+\)-K\(^+\) ATPase, alkaline phosphatase, acid phosphatase and 5'-nucleotidase was tested and found that cell homogenate contained all these enzymes.

The specific enzyme activity of 5'-nucleotidase of the crude homogenate of *G. lamblia* trophozoites (P-1) exhibited a mean specific activity value of 0.408 (μmol of product/mg protein/hr) in three experiments and the corresponding mean value for plasma membrane preparation was 7.25 (Table 3.5). The Na\(^+\)-K\(^+\) ATPase activity of the crude homogenate had a mean specific activity value of 0.408; the corresponding mean value for the purified plasma membrane preparation was 5.3. The mean specific activity of alkaline phosphatase in homogenate and membrane preparation
Figure 3.10

Showing electron microscopic photograph of purified *G. lamblia* plasma membrane (20,000x), isolated after sucrose gradient centrifugation.
was 0.533 and 1.75, respectively. The acid phosphatase had lowest mean specific activity value in plasma membrane fraction i.e., 0.46, whereas a higher mean specific activity value was discernible in the homogenate. The membrane bound enzyme activity in the plasma membrane preparation and crude homogenate indicated an enrichment of 17 folds of 5'-nucleotidase, 7 fold of Na⁺, K⁺ ATPase and 2 to 3 fold of alkaline phosphatase, respectively (Table 3.5).

11) Physico chemical characterization of plasma membrane antigens

Further studies were carried out to establish the physico chemical nature of the membrane when treated separately with heat, proteolytic enzymes (trypsin and pronase) and sodium metaperiodate.

After heat treatment at 100°C maximum reduction (Fig. 3.11) in antigenic activity of plasma membrane preparation was observed (O.D. values 0.30 in ELISA against PM antibody). An almost equal reduction in O.D. value was observed in comparison to control when the same antigen (plasma membrane) was subjected to periodate oxidation. The maximum effect was achieved at 150 mM concentration of sodium metaperiodate (Fig. 3.12). However, a varied degree of reduction in antigenic activity was noticed when the plasma membrane antigen was treated with pronase and trypsin separately. 100 µg/ml of pronase could reduce the O.D. values from 0.65 to 0.23. However, the same degree of reduction in O.D. values was produced by trypsin at 400 µg/ml (Fig. 3.13). Overall, all the three treatments reduced the antigenicity of the plasma membrane to some extent.

111) Agglutination study

The agglutination of Giardia trophozoites by lectins of various saccharide specificities are shown in Figure. 3.14
EFFECT OF HEAT TREATMENT ON PLASMA MEMBRANE ANTIGENS
EFFECT OF PERIODATE OXIDATION ON PLASMA MEMBRANE ANTIGENS

Figure 3.12
Sodium Meta Periode Conc.(mM)
EFFECT OF PROTEOLYTIC ENZYMES TREATMENT ON PLASMA MEMBRANE ANTIGENS

Figure 3.13
Agglutination of *G. lamblia* trophozoites by lectins
Maximum agglutination was observed by WGA at 250 μg/ml, at which 23% of Giardia trophozoite was agglutinated. More specifically Glc-NAc reduced WGA agglutination by 56% and NANA reduced by 38%. No inhibition was observed with Gal-NAc, D-galactose, D-mannose or D-fucose, confirming the specificity of WGA-Giardia binding. Prior treatment of neuraminidase to Giardia trophozoite did not significantly alter agglutination by WGA, indicating, that the agglutination was mainly due to the N-acetyl D-glucosamine residue (Fig. 3.14). In the absence of lectins 1.7% of the Giardia spontaneously agglutinated.

iv) Sodium dodecyl sulphate polyacrylamide gel electrophoretic analysis of plasma membrane

The polypeptide composition of the plasma membrane was investigated by SDS-PAGE (Fig. 3.15). The protein profile of cell homogenates of Giardia was also compared. Enrichment of some protein bands in the plasma membrane was noticed, although these were only minor components of the total proteins. Similarly, the major total cell proteins were undetectable or present as minor components in membrane. As expected, the pattern from total giardial protein was found more complex than plasma membrane. About 10 major and 15 minor bands were visible in the plasma membrane profile in the molecular weight range of about 225 kDa to 20 kDa. The most prominent bands had an apparent molecular weights of 118 kDa, 84 kDa, 55 kDa and 38 kDa to 32 kDa, respectively.

v) Lectin blot assay

Lectins are now being used as a common tool in analytical biochemistry for the detection of glycoprotein containing structures. To ascertain the nature of the surface glycoproteins, the plasma membrane of G.lamblia was first separated in 10% SDS-PAGE, transferred to nitrocellulose. These blots were then incubated with peroxidase labelled WGA and
SDS-PAGE patterns of purified plasma membrane protein (lane A), crude plasma membrane protein (lane C) and homogenates (lane B) of *G. lamblia* in 10% polyacrylamide gel. Molecular weight marker was also included (lane M).
Lectin blot analysis of major glycoproteins of *G. lamblia* plasma membrane. NC strips were incubated with HRP-conjugated WGA (lane A) and HRP-conjugated Con A (lane B).
Fig. 3.15
Fig. 3.16
Con A, used as a probe. The D-Glc NAc residues recognized by WGA were present on trophozoite surface membrane glycoproteins in the apparent molecular weight regions of 118 kDa, 84 kDa, 55 kDa and three bands in between 30 kDa to 20 kDa, respectively (Fig. 3.16).

The D-glucose and D-mannose containing glycoproteins (specific for Con A lectin) were identified in the molecular region of 60-67 kDa and 43 kDa region respectively (Fig. 3.16). The surface sugar specificity of these WGA/Con A were further confirmed by incubating the blots further, with WGA/Con A-HRP which was previously absorbed with live G. lamblia trophozoites. This experiment resulted in the appearance of a few faint bands; however, most of the major bands disappeared.

vi) Detection of antigenic components of plasma membrane by immunoblotting with homologous antibody

Figure 3.17 shows the gel diffusion precipitin band of plasma membrane antigen against the homologous antisera raised in rabbits.

The same antisera when used in immunoblotting experiment against plasma membrane antigen, showed immuno-reactive polypeptides throughout the lane, ranging from an apparent mol. wt. of >200 kDa to 25 kDa. However, prominent conserved antigens were detected at 160 kDa, 118 kDa, 55 kDa and 30 kDa regions, respectively. The antigenic polypeptide of an apparent mol. wt. of 118 kDa was found to be highly immunoreactive, in the sense that the intensity of this band was found to be more than the other immuno-reactive polypeptides (Fig. 3.18).

vii) Immunoblot analysis of plasma membrane antigens against acute giardiasis patients' sera

The 10% SDS-PAGE separated plasma membrane antigens were transferred to NC paper and allowed to react with pooled acute giardiasis
patients' sera having no history of other immune defect. Several polypeptides were found antigenic when reacted with the patients' sera, viz., 118 kDa, 84 kDa, 55 kDa and in between 30 kDa to 1 kDa (Figure 3.19). However, after comparing the intensity of the bands, the polypeptide with an apparent mol. wt. of 118 kDa was found to be immunodominant. Presence of 118 kDa mol. wt. polypeptide was observed as the major antigen in all the acute giardiasis patients' sera tested.

viii) Reactivity of 118 kDa polypeptide against immunized rabbit sera

Rise of antibody (IRS-118 kDa) against this polypeptide was checked in GDP test (Fig. 3.20). The cross reactivity between the plasma membrane antibody (IRS-PM) and IRS-118 kDa was evident. However, the antibody titre of IRS-118 kDa was not so high as was observed with IRS-PM. The monospecificity of the antiserum was detected in immunoblot assay (Fig. 3.21).

ix) Immobilization studies

The in vitro immobilization effect of different G. lamblia antisera (i.e., IRS-CSA, IRS-G1, IRS-PM, IRS-118 kDa and NRS) on live G. lamblia trophozoites was assessed. Sera were used in two different conditions viz., inactivated at 56°C and non-inactivated. The percentage of immobilization was much higher in non-inactivated sera (Table 3.6). Maximum percentage of immobilization was observed with non-inactivated IRS-PM serum (84%) followed by IRS-G1 (70%) and IRS-118 kDa (65%). However, normal rabbit sera could also immobilize 25% of the Giardia trophozoites.
Figure 3.17

Immunodiffusion pattern of different antisera against plasma membrane antigens (Centre well). Outer wells contain IRS-G1 (1 and 3), IRS-PM (2) and preimmune serum (4).

Figure 3.18

Showing immunoreactive polypeptides of plasma membrane antigens against homologous antibody raised in rabbit (lane A), and normal rabbit serum (lane B) in immunoblot assay. Major immunoreactive polypeptides were found as dark intense brown bands.
Figure 3.19

Immunoblot analysis of plasma membrane antigens of *G. lamblia* against pooled acute giardiasis patients sera (lane A). Arrow represents a prominent immunoreactive band at 118 kDa region. Prestained marker proteins (lane B).
Fig. 3.19.
Figure 3.20

Immunodiffusion pattern of different antisera against 118 kDa antigen (Centre well). Outer wells contained 118 kDa antisera (1), IRS-G1 (2), IRS-PM (3) and pre-immune serum (4).

Figure 3.21

Immunoblot assay of plasma membrane antigens against antiserum to 118 kDa polypeptide (lane A) and normal rabbit serum (lane B). Known molecular weight marker proteins (lane M) were also included in the experiment.
Fig. 3.20

Fig. 3.21
Table 3.1. Protein contents of axenic *G. lamblia* antigen

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>No. of <em>Giardia</em> trophozoites</th>
<th>Protein contents per ml (mg)</th>
<th>Protein concentration per million <em>Giardia</em> (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>$10 \times 10^6$</td>
<td>5.0</td>
<td>0.5</td>
</tr>
<tr>
<td>2.</td>
<td>$4 \times 10^6$</td>
<td>2.2</td>
<td>0.55</td>
</tr>
<tr>
<td>3.</td>
<td>$15 \times 10^6$</td>
<td>6.9</td>
<td>0.46</td>
</tr>
<tr>
<td>4.</td>
<td>$22 \times 10^6$</td>
<td>12.4</td>
<td>0.56</td>
</tr>
<tr>
<td>5.</td>
<td>$14 \times 10^6$</td>
<td>6.8</td>
<td>0.48</td>
</tr>
<tr>
<td>Average</td>
<td>$13 \times 10^6$</td>
<td>6.66</td>
<td>0.51</td>
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</table>
Table 3.2. ELISA activity of CSA and its sediment against IRS-G1 and human acute giardiasis sera

<table>
<thead>
<tr>
<th>Serum Code</th>
<th>Antigen</th>
<th>Protein µg conc. (µg/ml)</th>
<th>Mean ELISA OD values</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS-G1</td>
<td>10,000 xg supernatant (CSA)</td>
<td>20</td>
<td>0.898</td>
</tr>
<tr>
<td>Acute giardiasis (Pool of five)</td>
<td>- Do -</td>
<td>20</td>
<td>0.476</td>
</tr>
<tr>
<td>IRS-G1</td>
<td>10,000 xg pellet</td>
<td>20</td>
<td>0.496</td>
</tr>
<tr>
<td>Acute giardiasis (Pool of five)</td>
<td>- Do -</td>
<td>20</td>
<td>0.273</td>
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</table>
**Table 3.3. Reproducibility of axenic *G.lamblia* antigen in ELISA against giardiasis patients sera**

<table>
<thead>
<tr>
<th>Protein concentration</th>
<th>Sera code</th>
<th>No. of sera</th>
<th>Mean ELISA O.D. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µg/ml (1st lot)</td>
<td>Acute giardiasis</td>
<td>15</td>
<td>0.437</td>
</tr>
<tr>
<td></td>
<td>patients sera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µg/ml (2nd lot)</td>
<td>Acute giardiasis</td>
<td>15</td>
<td>0.425</td>
</tr>
<tr>
<td></td>
<td>patients sera</td>
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Table 3.4. 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$^a$</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>
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<tr>
<td>10</td>
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<tr>
<td>2.5</td>
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<td>0.250</td>
<td>0.135</td>
<td>0.128</td>
<td>0.023</td>
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</tr>
<tr>
<td>1.2</td>
<td>0.039</td>
<td>0.165</td>
<td>0.092</td>
<td>0.059</td>
<td>-</td>
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</table>

$^a$ O D values were taken at 490 nm.
Table 3.5. Distribution of marker enzymes in the membrane fraction of *Giardia lamblia*

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Specific activity (a) (μmol of product/mg protein/hr)</th>
<th>Fold of enrichment in membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma membrane</td>
<td>Cell homogenate</td>
</tr>
<tr>
<td>1. 5'-nucleotidase</td>
<td>7.25±0.4</td>
<td>0.408±0.08</td>
</tr>
<tr>
<td>2. Na(^+)K(^+)ATPase</td>
<td>5.3 ± 0.641</td>
<td>0.768±0.031</td>
</tr>
<tr>
<td>3. Alkaline phosphatase</td>
<td>1.75±0.25</td>
<td>0.533±0.07</td>
</tr>
<tr>
<td>4. Acid phosphatase</td>
<td>0.46±0.10</td>
<td>1.8±0.65</td>
</tr>
</tbody>
</table>

(a) All values are mean ± SD of three experiments.
Table 3.6. Percentage immobilization of *G. lamblia* trophozoites at 37°C after one hour incubation with different sera

<table>
<thead>
<tr>
<th>Condition of antibody used</th>
<th>Antibody code</th>
<th>% trophozoites immobilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Heat inactivated</td>
<td>IRS-CSA</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>IRS-G1</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>IRS-PM</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>IRS-118 kDa</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>5</td>
</tr>
<tr>
<td>II Non-inactivated</td>
<td>IRS-CSA</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>IRS-G1</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>IRS-PM</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>IRS-118 kDa</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>25</td>
</tr>
</tbody>
</table>
3.3 Discussion

The antigenic analysis of *Giardia lamblia* trophozoites reveals that the antigenic configuration of this organism is highly complex and presents a battery of antigenic determinants. The major antigenic reactivity was restricted to the soluble fraction (Supernatant after 10,000 xg) as compared to the particulate fraction (pellet after 10,000 xg). It is quite likely that the soluble antigen(s) probably possess a higher proportion of the total amount of antigen present in the intact parasite. This may indeed be suggested by the fact that the residual debris also possessed some antigenic activity. Several reports are available which suggest that surface membrane of *E.histolytica* contains antigen(s) common to both the developmental form of the parasite that help in antibody induction in the infected host (Ravdin *et al*, 1980).

The complex nature of soluble antigens of *G.lamblia* (strain P-1) trophozoites was once again seen in SDS-PAGE, where 28 polypeptides were demonstrated. These observations accord well with the findings of Smith *et al* (1982) in almost similar type of experiments. However, the results differ slightly from those of Moore *et al* (1982), who demonstrated about 20 distinct protein determinants. A similar study (Visvesvara, 1981) using an immunoelectro-precipitation test showed 24 precipitin arcs in agarose gel. These differences in the protein polymers may be due to the different strains and methods used by different workers. The results of fractionation in HPLC by Moore *et al* (1982) and the Sephacryl S-300 column chromatography of CSA showed almost similar observations. In both instances, four peaks were noticed and the maximum antigenic activity was recorded in the high mol.wt. peak (Fraction I). 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 of F-I is serologically more active than F-II, F-III and F-IV. Fraction-I was found eight times more potent than the parent antigen (CSA) in the sense that 2.5 μg/ml of this protein gave almost same OD values with 20 μg/ml of CSA in ELISA (Table 3.4). The other three fractions i.e., F-II, F-III and F-IV contained 22%, 10% and 2.5%, respectively of the total protein contents. Furthermore, the precipitin reactions which have been demonstrated in whole CSA and its various fractions can be explained on the basis that fraction II, III and IV are not pure and are contaminated with the preceding fraction. Fraction III and IV which were found to be relatively less active than the other two fractions (F-I and F-II) in ELISA can be explained on the basis that lower mol.wt. proteins were not as potent as high mol. wt. proteins (Sawhney et al, 1980).

Analysis of SDS-PAGE of different fractions of CSA after reduction showed distinct polypeptide pattern with mol. wt. ranging from >125 kDa to 14 kDa (Fig. 3.6).

The major immunoreactive polypeptides of the F-I were detected at apparent mol.wt. regions of 170 kDa, 118 kDa, 55 kDa and 30 kDa. The 118 kDa polypeptide which was found highly immunoreactive in immunoblotting was not prominently stained in the coomassie blue staining of the gel. The possible reason may be that the polypeptide had some constituents of non-proteineous substances probably carbohydrate which interfered in clear visualization by protein staining. However, the same was found to be highly immunogenic in immunoblot assay. The 55 kDa and 30 kDa polypeptides observed in the present study are probably the tubulin and giardin proteins, respectively, as described by Crossley and Holberton (1983). A similar 30 kDa mol. wt. membrane protein has also been identified from
both *G. lamblia* and *G. muris* (Crossley et al., 1986, Erlich et al., 1983; Taylor and Wenman, 1986). Presence of 55 kDa, polypeptide in F-I corresponds well with the membrane associated tubulin of *G. lamblia* (Clark and Holberton, 1986). An excretory secretory component of 170 kDa has been reported by Nash and Aggarwal (1986). However, specific report on 118 kDa immunoreactive protein has not yet been reported by any group.

The expression/association of F-I antigen with the membrane was demonstrated by indirect fluorescent antibody test. In this study, live *Giardia* trophozoites showed bright fluorescence against IRS-F-I on the surface. Further characterization of these antigens by immunoblot assay against live *Giardia* trophozoite absorbed versus unabsorbed IRS-F-I antisera revealed that some immunogenic polypeptides of F-I are common with the surface antigens of *Giardia* trophozoites. The major immunoreactive polypeptides of F-I antigens were detected in the molecular weight regions of 170 kDa. This result matches well with the protein analysis of cytoplasm and membrane/cytoskeleton fractions of *Giardia* as demonstrated by Butscher and Faubert (1988), where they detected some common antigens in cytosol and membrane fraction. They suggested that the precursors of some membrane proteins are usually found in the cytoplasm. So it is quite apparent that some of the membrane proteins which were present in very small amount in the F-I of CSA were of immense importance as far as their antigenicity was concerned.

From the antigenic point of view these studies suggest that membrane proteins are of much importance for studying the immuneresponse to the parasite and their role in host parasite interaction.

In the present study, attempts were made to isolate and purify the plasma membrane from trophozoites of axenic *G. lamblia* (strain P-l).
and its characterization by biochemical and immunological parameters.

Purified plasma membrane was prepared successfully from axenic G. lamblia trophozoites (strain P-1) and its purity was checked by electron microscopy and membrane bound enzyme studies. True membrane vesicles with no intracellular organelles were seen in electron micrograph. However, a significant enrichment of 5′-nucleotidase marker enzyme in the membrane fraction was observed. In particular, 5′-nucleotidase has received considerable attention, because of its association with the surface of many cell types. 5′-nucleotidase activity is often regarded as an enzyme marker for plasma membranes especially from mammalian sources (DePierre and Karnovsky, 1973). Gottlieb and Dwyer (1983) have demonstrated the association of 5′-nucleotidase with the parasite surface membrane of Leishmania. Alley et al (1980) have demonstrated the significant enrichment of Na\(^+\)-K\(^+\) ATPase, a well known marker enzyme in the membrane fraction of E. histolytica. However, in this study such significant enrichment of Na\(^+\)-K\(^+\) ATPase in membrane preparation was not observed.

Experiments aimed to characterize the plasma membrane antigen by physico-chemical treatments revealed that pronase, trypsin, periodate and heat could alter the antigenic activity. The results of these experiments suggest that the antigens are mostly glycoprotein in nature. Further biochemical characterization study revealed that the parasites are mainly agglutinable to WGA treatment rather than to Con A. The sugar residues were identified as N-acetyl D-glucosamine and N-acetyl D-neuraminic acid. These sugars were linked with the surface proteins to form the surface glycoproteins. The subunits of these protein were identified in the apparent mol. wt. regions of 118 kDa, 84 kDa, 55 kDa and in between 30 kDa to 20 kDa. However, D-glucose and D-mannose residues (meant for Con A lectin)
were identified at 60-70 kDa and 43 kDa regions. Einfeld and Stibbs (1984) have characterized a 84 kDa surface glycoprotein of *G. lamblia*. The present study also confirms the earlier findings of Hill et al (1981), who demonstrated that WGA was one of the six lectins studied that agglutinated *G. lamblia* trophozoites more vigorously than the other lectins. Recent study of Ward et al (1988) has also suggested that β-linked N-acetyl-D-glucosamine is the only detectable saccharide moiety present on the plasma membrane of *G. lamblia*. They further revealed that the number of surface proteins were predominantly or exclusively linked with this sugar moiety which constitutes the surface glycoproteins of this parasite. However, in the present study, 3-4 glycopeptides were also detected in Con A blotting experiment, thereby suggesting the presence of a few D-glucose and D-mannose residues on the surface of *G. lamblia* trophozoites. This could be due to differences in system and materials used by different workers.

SDS-PAGE analysis of *Giardia lamblia* plasma membrane revealed 10 major and 15 minor polypeptides in the mol. wt. ranging 225 kDa to 20 kDa. However, the most prominent bands were found in the apparent mol. wt. of 118 kDa, 84 kDa, 55 kDa and in between 38 kDa and 32 kDa. Some of these bands resembled the banding pattern obtained by Einfeld and Stibbs (1984) after radiiodination of surface proteins (using IODOGEN). In all the four strains they studied, the labelled components showed the major polypeptide at 82 kDa and less heavily labelled bands at 180 kDa, 105 kDa, 63 kDa, 55 kDa, 37 kDa, 30 kDa and 24 kDa. The radiiodination pattern of the plasma membrane polypeptides obtained by Clark and Holberton (1986) revealed that the major polypeptides exposed on the cell surface were in approximate mol. wt. of 75 kDa, 58/54 kDa and 22 kDa. However, the minor labelled components were detected at 19 kDa, 160 kDa, 120 kDa and 28 kDa.
Edson et al (1986) have observed only one major iodinated trophozoite surface protein (88 kDa) which was recognized by human sera, by either lactoperoxidase or IODOGEN procedures as demonstrated by earlier workers (Einfield and Stibbs, 1984; Clark and Holberton, 1986). On the basis of this study and earlier studies using radiiodination of *Giardia* surface antigens, detection of a major polypeptide or polypeptides probably do not represent the whole spectrum of proteins which are only antigenic in nature or present on the surface of the parasite, as both (lactoperoxidase and IODOGEN) the reagent iodinate only the tyrosine residue of the protein. Therefore, if the protein has a low content of tyrosine, histidine, tryptophan, and sulphydryl groups, it may not be iodinated by surface labeling techniques but may still be an abundant surface antigen (Upcroft et al, 1988). Farthing et al (1986) have demonstrated the presence of at least 20 *G. lamblia* surface proteins by using Bolton-Hunter reagent. Probably for this reason only some of the SDS-PAGE bands of plasma membrane proteins in this study were in common with the protein profiles of surface membranes after radiolabeling with IODOGEN or lactoperoxidase techniques.

This study also revealed the existence of immunologically active polypeptides in plasma membrane, recognized by giardiasis patients' sera. The major antigen was evident at an apparent mol. wt. of 118 kDa which seems to be a glycoprotein, containing N-acetyl D-glucosamine as the major sugar content, as reflected by its ability to bind with WGA lectin. This antigen produced an immune reaction, usually strong in almost all the cases of acute infection. Other important antigens were found at the mol. wt. of 84 kDa, 55 kDa and in between 30 and 20 kDa. Although these results are extrapolated using a single human strain (P-1), studies on
other strains of *G. lamblia* isolated from humans either from one or different geographic area are also antigenically very similar (for details please see Chapter 5). It is worth mentioning that all of these strains of *G. lamblia* possess the major common antigenic epitope with an apparent mol. wt. of 118 kDa.

A major 170 kDa excretory secretory surface antigen shown by n- and Aggarwal (1986) could not be detected in the plasma membrane fraction. The possible reason could be that this polypeptide was lost during the purification process or could relate to the differences in the strain used by the above workers. Kumkum et al (1988) also failed to detect the 170 kDa polypeptide in their plasma membrane study of *G. lamblia* trophozoites. However, in the antigenic analysis of F-I, a 170 kDa antigen was observed in minor quantity in the immunoblot assay.

Major immunoreactive polypeptides have been demonstrated by different groups of workers. Edson et al (1986) have demonstrated an 88 kDa major immunoreactive surface antigen. Taylor and Werman (1987) have reported a major 31 kDa *G. lamblia* antigen during human infection. However, in the present study, major reactivity of the patients' sera against the aforementioned polypeptides could not be detected.

The major antigenic activity recorded in this study against the acute giardiasis patients' sera and immunized rabbit sera was associated with a 118 kDa polypeptide. Similar type of antigenic activity was described by Ortega Pierres et al (1988) with giardiasis patients' sera at 121 kDa and by Clark and Holberton (1986) 120 kDa. From these observations, it can be concluded that 118 kDa polypeptide is the same polypeptide as described by the above workers, or it is an entirely new entity which interacts with the host of this community during active infection. The
later may be true as 118 kDa polypeptide was found immunodominant in all the strains isolated from the two different geographic areas or from the same locality when allowed to react with the antibodies of the acute giardiasis patients of this community (vide Chapter 5 for details). Furthermore, the differences in the immune responses against the different antigenic components among the individuals, reported by various groups of workers may be explained by the fact that humans are outbred populations, which possibly cause a more widely varying response to the complex array of antigenic determinants presented during the course of parasitic infection. On the other hand, the observed pattern of recognition of _G._lamblia antigens might also be influenced by the balance of different Ig isotypes as well as by the titre of antibodies present at the time of sample collection.

A significant percentage of _Giardia_ immobilization was observed by different antisera. Maximum immobilization was observed against IRS-PM followed by IRS-118 kDa. The higher percentage of immobilization by IRS-PM may be explained as the cumulative effect of this antisera raised against several inner and outer plasma membrane associated antigens. The significant increase in immobilization of _Giardia_ trophozoites against non-inactivated (fresh) sera as compared to host inactivated sera is probably indicative of a complement mediated immune response against this organism. However, the role of complement-mediated killing in _G._lamblia is still unclear. Killing of _G._lamblia trophozoites by fresh (non-inactivated) sera from humans with and without a history of giardiasis has been demonstrated (Hill et al., 1984). The abrogation of killing by heat-inactivated sera and the role of complement in the lethal effect have also been documented by the same workers (Hill et al., 1984). This capacity of serum to
kill or immobilize trophozoites may limit the invasion of the parasite into the submucosa.

It is generally assumed that antigen accessible to the protective immune response and located at the parasite surface will be more vulnerable to immune effector mechanisms. These antigens may be naturally immunogenic and therefore targets of anti-parasite immune response. The present study has allowed the identification and characterization of such immunogenic components which might potentially be useful for diagnosis of the disease. Thus, the isolation and identification of *G. lamblia* specific components which are immunogenic in human would hopefully provide the basis for a rational improvement of a universally used diagnostic test for giardiasis. Finally, it would be of great interest to know whether the major immunogenic proteins reported in this study plays any role in the pathogenesis of the parasite or in the adhesion to intestinal epithelium, thereby initiating the disease.