MATERIALS
AND
METHODS
3.1 **MATERIALS**

3.1.1 **Mice**

BALB/c By.J. inbred mice were obtained from Jackson Laboratories, Bar Harbor, Maine, U.S.A. These were maintained at the Small Animal Facility of the National Institute of Immunology, New Delhi and were used for the study.

3.1.2 **Entamoeba histolytica strains**

*E. histolytica* strain NIH:200 was obtained from Dr. J C Samantaray, Department of Microbiology, All India Institute of Medical Sciences (AIIMS), New Delhi. *E. histolytica* strains SAW 1734 and HM1 C6 were obtained from Dr. Alok Bhattacharya, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067. All the three strains were maintained during the study. The clinical isolates used in the characterization of monoclonal antibodies were maintained by Dr. Sharon L. Reed at Division of Infectious Disease, UCSD Medical Center, San Diego, CA 92103, U.S.A.

3.1.3 **Myeloma cell line**

Mouse myeloma cell line SP2/O-Ag 14 was available in the Hybridoma lab of National Institute of Immunology, New Delhi.

3.1.4 **Media for *E. histolytica* culture**

Panmede was supplied by Paines and Byrne Limited, Greenford, England and Trypticase was obtained from BBL
Microbiology Systems, Cockeysville, MD 21030, USA. Yeast extract was obtained from Difco Laboratories, Detroit, U.S.A. L-cystein-HCl from Hi media, Bombay 400 086, India; potassium dihydrogen orthophosphate, sucrose, ferric ammonium citrate and dextrose from Sarabhai M Chemicals, Baroda, India. Components for vitamin mixture 107 were bought from Hi media, Bombay and adult bovine serum was purchased from Sera lab, U.K.

3.1.5 Cell culture materials
RPMI-1640 medium, inactivated foetal calf serum (FCS) and fungizone were supplied by Flow Laboratories, Irvine, Scotland, U.K. Hypoxanthine, aminopterin, thymidine, 8-azaguanine and HEPES were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Streptomycin was supplied by Hindustan Antibiotics Limited, Pune, India; penicillin by Alembic Chemical works Limited, Bangalore, India and gentamycin by Eupharma Laboratories Vile-parle, Bombay, India.

3.1.6 Chemicals
Polyethylene glycol-1450 (PEG) was obtained from Feinbiochemica Heidelberg, West Germany. Freund's complete and incomplete adjuvants (CPA,IFA), dimethyl sulfoxide (DMSO), ortho-phenylene diamine (OPD), 4-chloro-1-naphthol, horse radish peroxidase (HRP), bovine serum albumin (BSA; Cohn's fraction V), sodium azide and ascorbic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 2,4,10,14-Tetramethyl pentadecane
(Pristane) was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Protein G-Sepharose was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium periodate and Folin Ciocalteau reagent were obtained from Sisco Research Laboratories Pvt. Ltd., Bombay, India. Sodium chloride, sodium carbonate, sodium bicarbonate, di-sodium orthophosphate, sodium dihydrogen orthophosphate, potassium sodium tartrate, ammonium sulphate, citric acid, sodium acetate, methanol, acetic acid, formaldehyde, sodium hydroxide, acetic anhydride, and ammonium hydroxide were all of analytical grade and were supplied by E. Merck (India) Ltd., Worli, Bombay, India. Trizma base (Tris[hydroxymethyl] aminomethane) was obtained from Sigma Chemical Co. St. Louis MO, USA. Milk protein (Lactogen) was obtained from Food Specialities Ltd., New Delhi, India.

3.1.7 Reagents for electrophoresis
Acrylamide, N,N'-methylene bis-acrylamide, coomassie brilliant blue, Sodium dodecyl sulphate (SDS), N,N,N',N'tetramethylene diamine (TEMED) were obtained from Sigma Chemical Co. St. Louis MO, USA. Amido black and bromophenol blue were obtained from Sisco Research Laboratories, Bombay, India. Ammonium persulphate (APS) and silver nitrate were supplied by E. Merck (India) Ltd., Bombay. Molecular weight marker-proteins and mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, MO, USA. Nitrocellulose membranes were
supplied by Advanced microdevices (Pvt.) Ltd., Ambala Cantt., India.

3.1.8 Plastic ware
Disposable tissue culture flasks, culture plates, petri dishes, culture tubes, pipettes and polyvinyl microtitre ELISA plates were obtained from Flow Laboratories, Irvine, Scotland, U.K.

3.1.9 Miscellaneous reagents
Dipsticks and nitrocellulose paper were bought from Advanced Microdevices Pvt. Ltd., Ambala, India. Activated charcoal was supplied by Glaxo Laboratories (India) Ltd., Bombay. Sheep anti-mouse Ig (H+L) coupled to horse radish peroxidase (HRP) was obtained from Dakopatts, Denmark. Goat anti-mouse μ, τ1, τ2a, τ2b and τ3 chain specific antibodies were obtained from Sigma Chemicals, St. Louis, Mo, USA. Rabbit anti-goat coupled to HRP was obtained from the Reagent Bank Facility of NII.

3.1.10 Clinical specimens
Serum samples of suspected ALA patients were obtained from the Deptt. of Microbiology, AIIMS, New Delhi. Finger prick blood and serum samples of ALA patients and other controls e.g. non-ALA patients suffering from liver ailments other than ALA were collected from G B Pant Hospital, New Delhi. Serum samples for follow-up studies were also collected from patients visiting G B Pant Hospital at different time intervals. Stool samples
from patients visiting outdoor patient department of AIIMS with a variety of bowel syndromes, were collected from Deptt. of Microbiology, AIIMS, where microscopic examination and culture of the samples were done.

3.1.11 Preparation of buffers and other solutions

3.1.11.1 Phosphate buffered saline (PBS), pH 7.4

Na$_2$HPO$_4$ 40.5mM  
NaH$_2$PO$_4$.2H$_2$O 9.49mM  
NaCl 150mM

3.1.11.2 PBS-Tween

0.5ml of Tween-20 was added per liter of PBS.

3.1.11.3 Carbonate buffer, pH 9.5

Na$_2$CO$_3$ 16mM  
NaHCO$_3$ 36.9mM

3.1.11.4 Citrate phosphate buffer, pH 5.6

Citric acid 22.1mM  
Na$_2$HPO$_4$ 51.4mM

3.1.12 Medium for cultivation of E. histolytica

3.1.12.1 Preparation of vitamin mixture 107

(I) Preparation of water soluble vitamin B

Solution A: Niacin 62.5 mg  
      p-aminobenzoic acid 125.0 mg  
Dissolved in 150 ml boiling double distilled water
Solution B: Niacinamide 62.5 mg  
Pyridoxine hydrochloride 62.5 mg  
Pyridomal hydrochloride 62.5 mg  
Calcium pantothenate 25.0 mg  
i-inositol 25.0 mg  
choline chloride 1250.0 mg  
Dissolved in 150 ml of double distilled water

Solution C: Riboflavin 25.0 mg  
Dissolved in 75 ml of double distilled water with the aid of 0.1 N NaOH added drop by drop. Total volume was made up to 100ml.

Solutions A, B and C were mixed and the total volume made up to 500 ml with double distilled water.

(II) Preparation of biotin solution
30 mg D-biotin was dissolved in 200 ml of double distilled water with the aid of 0.1 N NaOH and total volume was made upto 300 ml with double distilled water.

(III) Preparation of folic acid solution
30 mg of folic acid was dissolved in 200 ml of double distilled water with the aid of 0.1 N NaOH and total volume was made upto 300 ml with double distilled water.

(IV) Preparation of lipid soluble vitamins A, D and K.
Solution a: 300 mg vitamin D\textsubscript{2} calciferol was dissolved in 63 ml of 95\% (v\textsubscript{v}) ethyl alcohol. To this 300 mg of vitamin A was added.
Solution b: 60 mg of vitamin K (sodium metabisulfite) was dissolved in 300 ml of 5\% v/v solution of Tween 80. Solution b was added to solution a and final volume made to 3000 ml with distilled water.

(V) Vitamin E: 25 mg of Vitamin E (alpha tocopherol acetone) in 250 ml of double distilled water.
The working mixture of vitamins was prepared by combining the five primary stock solutions in the following proportions:

(I) Water soluble vitamin B solution 500 ml
(II) Biotin solution 250 ml
(III) Folic acid solution 250 ml
(IV) Lipid soluble vitamin A, D and K 2500 ml
(V) Vitamin E solution 250 ml

Sterilization was done by filtering the solution through sterile membrane filter (pore size 0.22um). It was aliquoted and stored at -20°C till use.

3.1.12.2 Composition of TPS-1 medium (for 100 ml)

Panmede 2.00 gm
Trypticase 1.00 gm
Dextrose 0.50 gm
NaCl 0.50 gm
L-cystein-HCl 0.10 gm
Disodium hydrogen phosphate 0.10 gm
Potassium dihydrogen phosphate 0.06 gm
Ascorbic acid 0.02 gm

Preparation: The above ingredients were dissolved in about 70 ml of glass distilled water and pH adjusted to 6.8 with 1 N NaOH and made the volume made to 87.5 ml. Medium was filter sterilized and 2.5 ml of sterile vitamin mixture 107 and 10 ml of sterile, heat inactivated adult bovine serum was added. Twelve ml of
complete medium was poured aseptically in sterile screw cap glass tubes and incubated for 24 hours at 37°C to check the sterility of the medium.

3.1.12.3 Composition of TYI-S-33 medium (for 100 ml)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>2 gm</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1 gm</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>L-Cystein-HCl</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.02 gm</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>22.8 mg</td>
</tr>
</tbody>
</table>

The above ingredients were dissolved in about 70 ml of glass distilled water, pH adjusted to 6.8 with 1 N NaOH and the volume made to 87.5 ml. This solution was filter sterilized and 2.5 ml of sterile vitamin mixture 107 and 10 ml of sterile, heat-inactivated adult bovine serum was added to it. Twelve ml of medium was poured aseptically in sterile screw cap glass tubes and kept for 24 hours at 37°C to check the sterility of the medium.

3.1.12.4 Robinsons culture method (BRS) (for clinical isolates of E. histolytica)

1. Bactopeptone: 20% solution, Difco, autoclaved and stored at 4°C
2. Robinsons medium 10x 1 Litre

| NaCl | 50 gm |
Citric Acid 20 gm
KH₂PO₄ 50 gm
Ammonium sulphate 10 gm
Magnesium sulphate (7H₂O) 0.5 gm
Lactic acid 85% solution 40 ml

Preparation: The above ingredients were dissolved in one liter of double distilled water and autoclaved. The stock was diluted (1:10) and pH adjusted to 7.0 and autoclaved. The above medium was inoculated with E. coli:0111 followed by incubation at 37°C waterbath till it got cloudy (Overnight to 48 hours) and stored at RT. Heat inactivated bovine serum (1:1) was added and incubated overnight at 37°C.

3. Phalate
Potassium hydrogen phalate 204 gm
40% NaOH 100 ml

4. Erythromycin
Erythromycin 50 mg/ml

5. Complete medium
Bactopeptone 20 ml
Erythromycin 5% 2 ml
BRS 150 ml
Phalate 1x 850 ml

6. Starch suspension
During subculture 100ul of 12.5% of starch suspension made in phalate was added to each flask.
3.1.13 Preparation of cell culture media

RPMI-1640 medium supplied as dry powder by Flow Laboratories was made up according to the instructions given by the suppliers. One liter of medium was supplemented by 2g NaHCO₃ and 2.38g HEPES. It was sterilized by filtration through 0.22 µm millipore membranes, aliquoted and stored at 4°C. Complete medium was made by adding 10% or 20% FCS (v/v) to RPMI-1640. When contamination was suspected, penicillin was used at a concentration of 100 IU/ml, streptomycin at 100 µg/ml and fungizone at 2.5 µg/ml concentration.

100X HT stock solution: 10mM hypoxanthine,
1.6mM thymidine.

100-fold concentrated HT stock solution was prepared by dissolving 136mg hypoxanthine and 39mg thymidine in 100ml double distilled water warmed to 70-80°C. It was sterilized by filtration and stored in aliquots at -20°C.

100X HAT stock solution: 10mM hypoxanthine,
4 x 10⁻⁵M aminopterin,
1.6mM thymidine.

100X HAT solution was prepared by adding 1.8mg aminopterin to 100ml of 100X HT stock solution. A few drops of 1M NaOH were added to dissolve aminopterin. It was filter sterilized and stored in aliquots at -20°C.
3.1.14 Polyethylene glycol (PEG) solution
1 gm of PEG was sterilized by autoclaving at 15 lb/sq.in. for 15 min. 1ml of RPMI was added to it to make 50% PEG. The solution was kept at 37°C before use.

3.1.15 Substrate for plate-ELISA
0.5 mg of ortho-phenylene diamine (OPD) was dissolved in 1ml of citrate phosphate buffer (section 3.1.11.4) and 1 ul of 30% hydrogen peroxide (H₂O₂) was added to it. The substrate was prepared just before use.

3.1.16 Substrate for dot-ELISA
3 mg of 4-chloro-1-naphthol was dissolved in 1 ml of methanol and then 5 ml of PBS was mixed and finally 6 ul of H₂O₂ was added. The substrate was prepared fresh each time.

3.2 METHODS
3.2.1 Maintenance of axenic E. histolytica cultures
E. histolytica NIH 200 was grown axenically in TPS-1 medium supplemented with vitamin mixture 107 (2.5%) and 10% heat inactivated adult bovine serum. E. histolytica HM1 C6 and SAW 1734 were also grown axenically in TYI-S-33 medium supplemented with 2.5% of vitamin mixture 107 and 10% of adult bovine serum. E. histolytica cultures were examined under the inverted microscope daily. Trophozoites can be seen adhering to the glass surface with pseudopodia extending in different directions with or without locomotion. Tubes to be subcultured were
chilled in ice water for 5-10 minutes then inverted a number of times to evenly suspend the trophozoites. The inoculum of approximately 2000-5000 trophozoites were transferred to the tubes containing fresh medium. The tubes were kept at 35-36°C tilted at an angle of 45° so that the trophozoites could settle on the wall of the tube. After 48-72 hours of incubation, the tubes were examined and harvested if the growth was confluent or further subcultured. To harvest the amoebae, tubes were incubated in ice cold water for 5-10 minutes and then inverted several times to suspend the trophozoites and centrifuged at 500 x g at room temperature. The trophozoites were washed thrice with chilled normal saline (0.9%) or phosphate buffered saline (50mM phosphate and 0.9% saline; pH 7.4).

3.2.2 Preparation of antigens

The harvested trophozoites were counted using haemocytometer (Neubauer, Fein-optik Blankenburg, GDR). Approximately 5x10^6 trophozoites suspended in normal saline containing PMSF (2 mM) were exposed to ultrasonic waves in a Sonifier (Model B-30, Branson Sonic Power Co., Danbury Conn. USA) at 20% duty cycle for 3 minutes. The sonicate was centrifuged at 10,000 x g and the supernatants used as antigen.

3.2.2.1 Protein estimation by the method of Lowry et al. (1951)

Solutions A: 0.5 ml of 1% CuSO_4·5H_2O was mixed with 0.5
ml of 1% potassium sodium tartarate and added to 50 ml of 2% sodium carbonate made in 0.1N NaOH. This solution was prepared just before use.

**B:** Folin's reagent diluted 1:1 with distilled water.

**Standard:** Bovine serum albumin 1 mg/ml in distilled water.

**Procedure:** 100 ul of standard solution containing 10 to 50 ug BSA and 100 ul of the test sample were dispensed in glass tubes in duplicates. 1ml of solution A was added and the mixture incubated for 10 to 15 minutes at room temperature. 100 µl of solution B was then added, mixed well and incubated for another 30 minutes at room temperature. Optical density was measured at 750 nm. The protein concentration of the unknown sample was obtained from the graph plotted for the standard.

3.2.3 **Harvesting clinical isolates of* E. histolytica**

Flasks with confluent growth of the trophozoites were chilled for 10 minutes over ice and centrifuged at 500xg for 10 minutes. Pellet was resuspended in 25 ml of PBS-Cystein and again centrifuged 500 x g for 10 minutes. Finally the Pellet was resuspended in 4 ml of PBS-Cystein and overlaid carefully over 7 ml of diluted Percoll (9 parts Percoll: 1 part 10 x PBS) and centrifuged at 750 x g for 10 minutes. Cell interface was carefully removed and resuspended in PBS-Cystein and washed thrice. Finally, trophozoites were counted using a haemocytometer.
3.2.4 Polyacrylamide gel electrophoresis

Solutions

Acrylamide 30%
Bis-acrylamide 1%
Tris-HCl buffer 1.5 M; pH 8.7
Tris-HCl buffer 0.5 M; pH 6.8
Sodium dodecyl sulphate (SDS) 10%
Ammonium persulphate (APS) 10%
(prepared just before use)
Electrode buffer 0.025 M Tris base, 0.192 M glycine and 0.1% SDS;
pH 8.3
Sample buffer 0.0625 M Tris base, 2.3% SDS, 10% glycerol,
5% mercaptoethanol and
0.1% bromophenol blue;
pH 6.8

Coomassie blue stain (0.25%) was prepared in 40% methanol and 10% acetic acid, 50% water. The destaining solution consisted of 40% methanol and 10% acetic acid in water.

Procedure: Recipes for the preparation of separating gels of two different porosities are given in the following table.
Solutions

<table>
<thead>
<tr>
<th></th>
<th>10% gel</th>
<th>12.5% gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide 30% and</td>
<td>3.3 ml</td>
<td>4.2 ml</td>
</tr>
<tr>
<td>.25% Bis-acrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris-HCl (1.5M)</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>pH 8.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>4.0 ml</td>
<td>3.17 ml</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>APS 10%</td>
<td>33.3 μl</td>
<td>33.3 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10.0 μl</td>
<td>10.0 μl</td>
</tr>
</tbody>
</table>

The stacking gel was prepared according to the following formula.

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide 30% and</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>0.25% Bis-acrylamide</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl (0.5M) pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3.2 ml</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>50.0 μl</td>
</tr>
<tr>
<td>APS 10%</td>
<td>25.0 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5.0 μl</td>
</tr>
</tbody>
</table>

The separating gel was polymerized in a Hoeffer mini gel apparatus for 30 minutes and the stacking gel for 20 minutes. The thickness of the gel was either 1.5 mm or 1.0 mm. Samples to be electrophoresed were mixed with an equal volume of 2x sample buffer and incubated in a boiling water bath for 5 min. The samples were applied in the wells and electrophoresis carried out at a constant current of 20 mA per gel.
Staining and destaining of the gels
Gels were stained with coomassie blue for one hour and destained overnight using the destaining solution on a rocker with a few changes.

3.2.5 Generation of monoclonal antibodies

3.2.5.1 Immunization: BALB/c mice were immunized subcutaneously with 40 μg of soluble *E. histolytica* antigen with CFA and after 3 weeks a booster containing similar dose was given. Four months later, mice were given live trophozoites (1x10^5) intraperitoneally and 11 days thereafter one final booster of similar dose was given and on 14th day the mouse was killed and spleen was removed aseptically and used for fusion.

3.2.5.2 Fusion: Fusions were done essentially following Kohler & Milstein (1975) method with minor modifications. The spleen from immunized mouse was placed in a sterile glass petri dish containing 10 ml of RPMI was crushed using sterile forceps to release cells. In order to obtain single cell suspension, the spleen cells were passed through a 10 ml syringe with a 21 gauge needle 2-3 times. This process was repeated 2-3 times with a 23 gauge needle. The cell suspension was centrifuged at 1200 xg for 5 minutes. The cell pellet was resuspended in 10 ml of RPMI. 50 μl of this suspension was mixed with 450 μl of 0.93% ammonium chloride to lyse RBCs and lymphocytes were counted using
haemocytometer. The cell suspension was centrifuged again at 1000 x g for 5 minutes and the spleen cell pellet was resuspended again in 10 ml RPMI.

Myeloma cells growing in log phase were harvested and centrifuged at 1200 x g for 5 minutes and suspended in 10 ml of RPMI. Myeloma cells were counted using haemocytometer and mixed with spleen cell suspension in a 1:2 ratio in a 50 ml centrifuge tube. The mixture was centrifuged at 500 x g for 5 min and the supernatant was discarded and the pellet was loosened. One ml of PEG solution was added drop by drop to the cell pellet over a period of 30 seconds and the cell suspension was mixed gently with the pipette for another 30 seconds. The tube was left undisturbed for 30 seconds. 5 ml of RPMI was added slowly into the tube over a period of 90 seconds while gently rocking the tube and finally 5 ml of RPMI was added all at once. The cell mixture was incubated for 5 minutes at room temperature and then centrifuged at 600 x g for 5 minutes and the pellet resuspended in HAT medium at a spleen cell concentration of $10^7$/ml. 1 ml of HAT medium was dispensed into each well of a 24 well plate. Finally 50 ul of fused cell suspension was added into the wells of the plate, leaving two wells for control. A drop of spleen cell suspension in one of the control wells and a drop of myeloma cells into the other control well were added. The plates were kept in a humidified 5% CO$_2$ incubator at 37°C. After four to five
days of fusion 0.5 ml of medium was removed from each well and the same amount of fresh HAT medium was added gently. After about 10 days of fusion macroscopic colonies of hybrids were visible and the spleen and myeloma control cells were dead. On the 11th day, the supernatant from each well was removed and screened by plate-ELISA as described in Section 3.2.11. Anti-mouse HRP conjugate was used instead of protein A-HRP. Second screening of hybrids was done after 48 hours. Consistently positive wells were cloned and subcloned thrice to obtain stable hybrids. Cells were frozen after every cloning.

3.2.5.3 Cloning of hybrids: Cloning was done by limiting dilution. The principle of this method is diluting cells up to a stage where there is, statistically, only one viable cell per well in the culture plate. Dispensing single cells into wells is clearly a random process. Cells suspended in RPMI containing 20% FCS were dispensed at a density of 0.5 cell/well and 1 cell/well in 96-well culture plates. Spleen cells from an unimmunized mouse were used as feeder cells at a concentration of $10^5$ cells/well. Positive clones from the first cloning were recloned twice to get stable antibody secreting hybridomas.

3.2.5.4 Maintenance of hybridomas
After third subcloning, the clones were transferred to culture flasks and maintained in RPMI containing 10% FCS
at 37°C in a humidified incubator with 5% CO₂. Cells were routinely screened for antibody production and were also frozen. Bulk amounts of antibodies were produced by propagating the cells as ascitic fluid (section 3.2.5.6).

3.2.5.5 Freezing and thawing of cells

Cells were suspended at a density of 2-5x10⁶/ml in a medium containing 90% FCS and 10% DMSO and 1 ml aliquots were dispensed in 2 ml cryostat ampoules. The ampoules were frozen at -70°C overnight and then transferred to liquid nitrogen.

For thawing, the ampoules were removed from the liquid nitrogen and RPMI medium prewarmed to 37°C was added. Cells were washed twice with RPMI, suspended in the medium containing 10% FCS and cultured at 37°C in a CO₂ incubator.

3.2.5.6 Production of monoclonal antibodies in ascites

Antibodies were produced in bulk by growing the cells in the peritoneal cavity of pristane-primed mice. BALB/c inbred mice were injected intraperitoneally with 0.5 ml of pristane. After one week, 3-4x10⁶ hybrid cells were injected i.p into these mice. Ascitic fluid obtained after about ten days was made cell-free by centrifugation at 1000 xg. The supernatant was further centrifuged at 15,000 xg to remove the debris.
3.2.6 Characterization of monoclonal antibodies

3.2.6.1 Determination of specificity of the antibodies

Nine of MAbs were reacted with three different strains of *E. histolytica* namely, NIH 200, HM 1 and SAW 1734 in ELISA. The plates were coated with 100 μl of 25 μg/ml protein concentration of the sonicate of these strains. Rest of the protocol was same as described in section 3.2.11 except that the culture supernatant from growing hybridomas was taken instead of serum. Using the same plate-ELISA protocol the specificity of the MAbs was determined by their reactivity with *Giardia lamblia*, *E. coli* and *S. typhi*. Plates were coated with $10^5$ cells of *Giardia lamblia*, 20 μg of protein/ml of *E. coli* and *S. typhi* sonicate.

3.2.6.2 Immunoagglutination

Axenically grown *E. histolytica* NIH 200 trophozoites were harvested and washed thrice with PBS. $10^5$ cells, suspended in 200 ul of PBS, were taken in each well of the 96 well plate and mixed with an equal volume of culture supernatant or diluted ascites. The plates were observed under the microscope at different time intervals for agglutination of trophozoites. Serum from a patient with invasive amoebiasis and culture supernatant from Sp2/0 were taken as positive and negative control respectively.
3.2.6.3 Immunofluorescence

Trophozoites of *E. histolytica* NIH 200 were harvested and washed thrice with PBS. $10^5$ cells were dispensed in microfuge tubes and suspended in 500ul of culture supernatant or diluted ascites and incubated at 4°C for 30 minutes. A control set with 500 µl of Sp2/O culture supernatant and another with some irrelevant monoclonal antibody was also included. The cells were washed at least four times with PBS and incubated with 200 µl of goat anti-mouse-FITC for 30 minutes at 4°C. Finally, the cells were washed four times using PBS and mounted on glass slides in 50% glycerol and PBS. The slides were observed under UV using a fluorescent microscope (Nikon, Microphot).

3.2.6.4 Competitive ELISA

One of the MAbs (AC55) was coupled to HRP and was used in competitive ELISA along with other monoclonal antibodies. Plates were coated with sonicate of *E. histolytica* and non-reactive sites were blocked with milk protein (1%) in PBS. The plates were incubated with a mixture of equal volumes of AC55-HRP (1:1000) and other monoclonal antibodies (doubling dilutions ranging from 1:100 to 1:1600) and incubated at room temperature for one hour. After washing thrice with PBS-T, the enzyme reactivity was revealed using OPD (Section 3.1.15).
3.2.6.5 Western blot

The reactivity of MAbs with *E. histolytica* (10⁵ cells/lane) was studied by Western blot. Live trophozoites after harvesting and washing at least three times with PBS, were counted and incubated in boiling water for three minutes with protease inhibitors (EDTA, TLCK, MEM, PMSF, E64). An equal amount of 2x sample buffer was added and again incubated in boiling water for five minutes. The sample was centrifuged and the supernatant was subjected to 10% or 12.5% SDS-PAGE electrophoresis (section 3.2.4). Polypeptides separated on gels were transferred onto nitrocellulose paper by immunoblot technique (Towbin *et al.*, 1979) at constant current of 30 mA for overnight followed by one hour at 300 mA. The nitrocellulose strips were saturated with 1% milk protein in PBS for one hour at RT on a shaker. The strips were incubated with appropriate dilution of ascitic fluids containing MAbs for one hour at RT. After washing the strips with PBS-T, these were incubated for one hour at RT with sheep anti-mouse Ig-HRP diluted in PBS-Milk protein (1%)-Tween (0.5%). The strips were subsequently washed with PBS-Tween and developed with 4-chloro-1-naphthol (Section 3.1.16). In order to characterize the chemical nature of antigen, the blots were reacted with proteinase k (5 μg/ml) for one hour at 37°C. Another similar blot was treated with periodic acid (100 mM) in sodium acetate buffer 50 mM, pH 4.46, followed by 1 M glycine in PBS for 30 minutes.
in the dark. Third blot was untreated and used as positive control. All these blots were then reacted with MAbs as described above.

3.2.6.6 Determination of class and subclass of antibodies

Heavy chain subclass of MAbs was determined by indirect ELISA using goat anti-mouse $\mu$, $\gamma_1$, $\gamma_2a$, $\gamma_2b$ and $\gamma_3$ specific antibodies and rabbit anti-goat antibodies coupled to HRP. The procedure was same as in section 3.2.11.

3.2.7 Purification of MAbs by affinity chromatography

Equal volumes of ascites and phosphate buffer (0.5 M, pH 8) were mixed and loaded on Protein G-sepharose column. Equilibration was done for 30-45 minutes. The column was washed with 0.1 M PB, pH 8.0 containing 0.15 M NaCl till O.D. was less 0.05 at 280 nm. Elution was done with 0.1 M acetic acid containing 0.15 M. The eluted antibody solution was neutralized with carbonate bicarbonate buffer pH 9.5. Finally, the fractions containing antibodies were pooled and dialyzed against PBS.

3.2.8 Coupling of antibodies to horse radish peroxidase

MAbs were coupled to HRP by the method described by Nakane and Kawaoi (1974). Two mg of HRP was dissolved in 0.9 ml of fresh glass distilled water. 0.1 ml of
freshly prepared sodium periodate solution (0.2 M) was added and the mixture was stirred at room temperature for 20 minutes. The solution was dialyzed overnight at 4°C against sodium acetate buffer 1 mM, pH 4.5. The pH of the solution was adjusted to 9-9.5 with 0.2 M carbonate buffer. Four mg of purified antibody dialyzed against carbonate buffer (10 mM, pH 9.5) was immediately added to the reaction mixture and stirred for two hours at room temperature. 0.05 ml of freshly prepared ascorbic acid solution (4 mg/ml) was added to give a final concentration of 0.18 mg/ml and allowed to stand for 24-48 hours at 4°C. The antibody-HRP conjugate was dialyzed against PBS and stored with 50% glycerol at -20°C. The conjugate was titrated in direct ELISA. Briefly, polyvinyl ELISA plates were coated with *E. histolytica* antigen (25µg/ml of protein concentrations) and unreacted sites were blocked with 1% milk protein and then different dilutions of conjugate (diluted in PBS-Tween 20 0.05% with 1% milk protein) were dispensed. After incubation for one hour at 37°C plates were washed and colour was developed using OPD (section 3.1.15). The results are shown in figure 3.1.

3.2.9 Standardization of sandwich enzyme immunoassay for detection of *E. histolytica* antigen(s) 
MAb (AC55) was used in the sandwich EIA both for capturing and revealing the antigen. AC55 purified from ascitic fluids were diluted in PBS, pH 7.4 and
Figure 3.1: Titration of AC55-HRP in direct ELISA using 100 μl/well of *E. histolytica* sonicate (protein concentration 25μg/ml) coated on ELISA plate. O.D. values shown are averages of duplicate. The procedure is described in section 3.2.8.
carbonate buffer, pH 9.5 and coated at different protein concentrations on polyvinyl microtitre plates (100 µl/well) and incubated at (i) 37°C overnight, (ii) 37°C one hour, (iii) drying, (iv) 37°C one hour followed by overnight at 4°C. Plates were washed with PBS-T and non-specific sites were saturated with 1% milk protein in PBS for one hour at room temperature. After washing, different concentrations of the soluble antigens or trophozoites were put into the wells (100 µl/well) and incubated for one hour at room temperature. Control wells had no antigen or trophozoites. Subsequent washing with PBS-T, the plates were incubated with an appropriate dilution of AC55-HRP, diluted in PBS containing 1% milk protein and 0.05% Tween, for one hour at room temperature. Plates were washed thrice with PBS-T and the enzyme activity was revealed by putting 100 µl/well of freshly prepared OPD solution (section 3.1.15). The reaction was stopped with 5N H₂SO₄ and the absorbance measured at 490 nm in a Biotek ELISA Reader. The sensitivity of the assay was determined by taking different concentrations of sonicated E. histolytica NIH 200 antigens and predetermined number of trophozoites of E. histolytica HM 1 and other clinical isolates.

3.2.10 Detection of antigen(s) in stool samples
Stool samples from patients with abdominal disorders were obtained from the Deptt. of Microbiology, AIIMS,
New Delhi. Microscopic examination and culture of the stool samples were carried out at AIIMS. Sandwich ELISA was conducted on coded samples. The stool sample was homogenized in PBS and centrifuged at 2500 xg for 10 minutes and supernatant was assayed in ELISA. The protocol of sandwich ELISA is described in Fig 3.2. 107 stool samples and 6 culture supernatants were tested. Control samples were taken from apparently healthy subjects.

3.2.11 Standardization of plate-ELISA for detection of anti-\( E.\ histolytica \) antibodies in ALA patients

Standardization of antigen concentration and serum dilutions were done by checkard board analysis. Wells of ELISA plate (Titertek) were coated with 100 µl of antigen (protein concentration varying from 12 µg to 0.3125 µg/well). Incubation was carried out at 37°C for one hour and then the plates were washed twice with PBS Tween 20 (0.05%). 200 µl of PBS-MP 1% was added to each well and incubated at 37°C for one hour. Plates were washed twice and incubated with different dilutions (1:100-1:40,000) of pooled serum of five confirmed ALA patients and also with pooled serum of five non-ALA healthy subjects. Plates were incubated at 37°C for one hour. Plates were washed with PBS Tween three times and incubated with Protein A-HRP (1:1000 dilution in PBS tween containing 1% milk protein) for one hour at 37°C.
Figure 3.2: PROCEDURE OF SANDWICH EIA FOR DETECTION OF *E. HISTOLYTICA* ANTIGEN

Purified antibody AC 55 (12.5ug/ml) coated on ELISA plate
incubated at 37°C for 1 hour
↓
 washed twice with PBS-T

Unreacted sites blocked with 1% MP in PBS
incubated at 37°C for 1 hour
↓
 washed twice

Antigen dispensed (stool suspension in PBS)
incubated at 37°C for 1 hour
↓
 washed twice

Diluted AC 55-HRP dispensed
incubated at 37°C for 1 hour
↓
 washed thrice

Colour developed with OPD
incubated at RT for 5 minutes
↓
 Read at 490 nm
Plates were washed thrice with PBS Tween and incubated with 100 μl of OPD solution (section 3.1.15) for five minutes at room temperature. The reaction was stopped by adding 50 μl of 5N H₂SO₄ in each well. 50 ul of reaction mixture was removed from each well before reading plates at 490 nm in Biotek ELISA reader. For further assays 2.5 μg/100μl/well of antigen and 1:200 dilution of patients sera were used (fig 3.3, 3.4). The above ELISA was repeated at 37°C and room temperature and no significant difference was observed hence in future experiments all the incubations were carried out at room temperature (RT).

In order to establish a cut off optical density (O.D.) for discrimination of positive and negative serum samples for diagnosis, sera from 25 apparently normal healthy subjects, were taken and ELISA was done at 1:200 dilution of serum. Mean of the O.D. values + 3 standard deviation was taken as cut off value. Using this standardized protocol, serum samples of suspected ALA patients from AIIMS, New Delhi, were tested and compared with IHA test (done at AIIMS) and clinical diagnosis.

3.2.12 Standardization of dipstick dot-ELISA
Standardization of dipstick dot-ELISA was carried out on nitrocellulose strips. On each strip different protein concentration of antigen was spotted in the form
Figure 3.3: Titration of different protein concentration of coating antigen (soluble sonicate of *E. histolytica*) in plate ELISA using pooled serum of five confirmed amoebic liver abscess (ALA) patients and five normal healthy controls (NHS). The O.D. values are average of duplicates. Procedure is described in section 3.2.11.
Figure 3.4: Titration of different dilutions of pooled serum of five confirmed amoebic liver abscess (ALA) patients and five normal healthy controls (NHS) in plate ELISA using 2.5μg antigen protein concentration (soluble sonicate of *E. histolytica*). The O.D. values are average of duplicates. The procedure is described in section 3.2.11.
of a dot (figure 3.5a) ranging from 15 µg to 0.312 µg/dot. Strips were air dried and unreacted sites were blocked using 1% milk protein in PBS for one hour at RT. Strips were washed with PBS Tween twice and incubated with different dilutions (1:50-1:3200) of pooled patients’ sera (n=5) and pooled normal sera (n=5) for one hour at 37°C. Strips were washed with PBS-T twice and incubated with protein A-HRP (1:1000 dilution in PBS Tween 20 containing 1% milk protein) for one hour at 37°C. Finally strips were washed with PBS-T and incubated with 4-chloro-1-naphthol solution (see section 3.1.16) for five minutes at room temperature and then washed with distilled water to stop the reaction. The intensity of the dot was read visually and graded as 0, 1+ up to 4+.

The assay was then performed on dipsticks (figure 3.5b). It is a 7.6 x 0.7 cm plastic strip to which two 0.7 cm sq. nitrocellulose pads are fixed. 2.5 µg of antigen (1 µl) is spotted on the lower pad and allowed to dry. The upper pad without antigen serves as reagent control. Unreacted sites are blocked with 1% milk protein. Dipsticks, thus prepared, can be stored refrigerated and used up to six months without loss of activity. Precoated dipsticks can also be stored in dry state, sealed in polyethylene aluminium foils. These dipsticks can withstand temperatures up to 45°C for many weeks. Different incubation timings for serum and Protein A-HRP were tried (e.g. 10, 15, 30, 60 minutes). After
Patient Serum
Ag.conc. µg/spot
15
10
5
2.5
1.25
0.625
0.312

(b)

Normal Serum
Ag.conc. µg/spot
15
10
5
2.5
1.25
0.625
0.312

(A) (B)
repeating the experiments on a large number of serum samples, 15 minutes incubation with serum and 15 minutes with conjugate was found to be optimal when compared with 60 minutes incubation time.

In order to simplify the procedure and reduce the number of components in the assay, different washing buffers were used e.g. PBS-T, PBS, distilled water and tap water.

Final form of the method for dipstick dot-ELISA is shown in flow chart (fig 3.6).

3.2.13 *Standardization of method for use of finger prick blood in dipstick dot-ELISA*

Selection of filter paper: Discs of 6 mm diameter were cut from Whatman filter paper no.1 and 3. Different volumes of blood were spotted to saturate the disc. Whatman no. 1 absorbed 4 ul of blood and Whatman no. 3 absorbed 8 ul of blood for complete saturation.

Standardization of blood elution conditions such as time and temperature: Elution time of 10 and 30 minutes at RT and at 37°C were tried. Blood eluted at RT for 30 minutes was selected for further experiments because the results under such conditions were comparable with serum control.

Reproducibility of dot-ELISA was checked using blood eluted from different discs of the same patient's
Figure 3.6: Method of Dipstick Dot-ELISA

Immobilization of E. histolytica antigens on lower pad of NC of dipstick.

Blocked with PBS-Milk protein (M.P.) 1%

Stored at 4°C until use

Coated dipstick incubated with patients serum (1:200) in PBS-MP (1%)

15 mins at RT
washed with tap water

Incubated with Protein A-HRPO

15 min. at RT
washed with tap water

Colour development with 4-chloro-1-naphthol

Washed with tap water

Read visually
sample. Finger prick blood from five confirmed ALA patients was used for the study.

Stability of discs were studied for a period of four months at RT. There was no significant loss of antibody activity for upto three months.

3.2.14 Follow-up of ALA patients for the assessment of clinical improvement and for the study of anti-

*E. histolytica* class-specific antibodies

Patients: Patients suffering from amoebic liver abscess, admitted to GB Pant Hospital, New Delhi, were selected for the present study. The criteria for ALA diagnosis was (a) ultrasound scan of liver abscess, (b) pain in the right hypochondrium, (c) fever, (d) response to tinidazole, (e) needle aspirate (if needed), (f) dot-ELISA for anti-*E. histolytica* antibodies in serum. A total of 21 patients were inducted in the study and finally six patients could be followed up till one year of post treatment.

Follow-up of patients: Patients were followed up at different time intervals for routine clinical examination including ultrasound scan of liver and plate-ELISA for titration of serum antibodies (IgG, IgM, IgA).

Plate-ELISA: This was carried out, as described in section 3.2.11, with a variation that instead of Protein A-HRP as conjugate, goat anti-human IgG, IgM and IgA coupled to HRP were used.