METHODS

A. COLLECTION OF SERA SAMPLES

Human amebiasis sera samples were obtained from patients who were either attending the out patient departments, or were admitted as indoor patients in the J.N. Medical College Hospital, Agra, India. Sera samples were collected from clinically proven cases of invasive amebiasis presenting unambiguous evidence of the presence of amebic liver abscess. A large number of sera samples were also obtained from cases of amebic colitis, and asymptomatic cyst passers. A total of 400 sera samples collected during the course of last five years were included in this study. Microscopic stool examination for the detection of ameba cysts was invariably carried out in all the patients from whom the blood samples were withdrawn. All the sera samples were dispensed in small vials and preserved in 0.2 per cent solution of sodium azide and stored at -20 C.

Hyperimmune sera from experimental animals (rabbits) were also obtained by immunizing the animals.

B. ANIMAL IMMUNIZATION

Anti- _E. histolytica_ rabbit serum was obtained by immunizing the animals with various antigen preparations.
Healthy, male rabbits weighing about 2 kg. to 2.5 kg. were given weekly intraperitoneal inoculations over a period of 5 weeks. No inoculations were made in the 6th week, and the animals were bled from the heart in the 7th week. Antigen preparations mixed with equal volumes of either Freund's (complete) or alumina adjuvant were administered in weekly doses ranging from 0.5 ml to 2 ml volumes. Immune sera were raised against one monoxenic and an axenic strain (NIH 200) of E. histolytica. Both mechanically disrupted, lysed whole cells, and water soluble antigenic extracts were used for immunizing the animals. Essentially, the immunization schedule as described by Ali-Khan and Meerovitch was followed for raising hyperimmune serum.

C. ANTIGEN PREPARATION

a) Ameba Cultures

Antigenic extracts were made by growing the amebeae in the following media:

**Monoxenic Cultivation**

1. Boeck and Drbohlav's Locke-Egg-Serum (LES) medium

2. NIH (National Institute of Health, U.S.A.) medium slants
Axenic Cultivation

1. TP-S-I-monophasic broth supplemented with vitamin mixture number 107 according to the method of Diamond.

Stock culture of *E. histolytica* (Strain NIH : 200) obtained by courtesy of Dr. Louis S. Diamond from the National Institute of Health, Bethesda, Maryland, U.S.A., was used for the mass cultivation of axenic amebae. All ameba cultures were made according to the technic described by Diamond. Amebae grown in 100 ml flasks and incubated for a period of 72 hr were harvested and pooled for making antigenic extracts.

b) Antigen Extraction

1. Water Soluble Antigen

Water soluble antigenic extracts both from axenic and monoxenastic amebae were made according to the technic described by Kessel et al. Amebae were chilled in an ice water-bath to make them fall off from the glass walls of the container. The cells were given several washings and resuspended in physiological saline.
Total cell counts in a Neubauer hemacytometer counting chamber were made from ameba pools which were subsequently used for antigenic extraction. Harvesting of the amebae was accomplished after a final run of centrifugation at 300 X G. Ameba cell packed volumes were determined in cold distilled water at pH 10.0. The suspensions were kept in an ice water-bath while being vigorously passed several times through a 20 - gauge needle attached to a 20 ml syringe. Disrupting of the cells by the application of shear force, along with alternate freezing and thawing was continued till the cells were completely lysed. The extracting fluid was left undisturbed for several hours, after which the cells were restored to isotonicity by the addition of an equal volume of 1.7 per cent saline. Soluble components were separated by centrifugation at 16000 X G for 30 min in a refrigerated centrifuge. Extracted antigens in supernatant fluids were dispensed in 10 ml volumes in vials, merthiolated (1:10,000) and stored at -20 C. Antigen preparations, based on the ratio of ameba cell packed volumes and the extracting fluids, were designated as 1:40 extracts.

2. Whole Antigen Preparation

The harvested amebae were washed several times in cold physiological saline. Cell packed amebae were diluted to two and a half times of their volume by the addition of
2.5 ml/ml (V/V) of normal saline. The cell suspensions were left undisturbed in a refrigerator for several hours before being subjected to mechanical disruption and lysis. The cells were also subjected to alternate freezing and thawing till all the cells were completely lysed and disrupted as confirmed by microscopic examination. Antigenic suspensions were later merthiolated (1 : 10,000), dispensed in small vials and stored at -20 C.

D. ANTIGEN AND ANTIBODY CHARACTERIZATION

a) Antigen Characterization

Antigenic extracts were subjected to chromatographic separations for studying the antigenicity of the various isolated fractions.

1. Chromatographic Studies

Sephadex G-200 was packed in a glass column measuring 45 x 2.5 cm. The gel was allowed to swell for several hours on a boiling water bath, and subsequently deaerated under vacuum for about half an hour. The supernatant fluid was sucked off till its volume was reduced to approximately half of the sedimented gel. The gel slurry of the required consistency was then poured into the column. Packing of the column was continued till a bed of desired compactness
was obtained. The column was equilibrated with the eluant buffer. The packing of the gel bed into the column was further checked by determining the void volume with the application of blue dextran - 2000.

Antigen samples were applied to the column with the help of a pipette. Chromatographic separations were allowed to continue by collecting the effluent fractions at an adjusted flow rate of 12 ml/hr. Protein estimations on the eluted fractions were carried out by the method of Lowry et al.\textsuperscript{150}, as also by optical density measurements.

b) Antibody Characterization

Column chromatographic separations for isolating antiamebic antibodies were also carried out on immunized human and rabbit sera samples.

Gamma globulins from human and immunized rabbit sera samples were precipitated by the addition of a saturated (40 per cent) solution of ammonium sulfate. Precipitating sera samples were then left overnight in the refrigerator. The obtained precipitate from each tube was washed and dissolved in 0.15 M sodium chloride. The precipitated gamma globulins were subsequently dialysed in 0.15 M sodium chloride till such time that no further precipitate was obtainable on subsequent additions of barium chloride.
Later, the rabbit and human gamma globulin samples were also dialysed against 0.017 M phosphate buffer of pH 6.3.

Salt fractionated gamma globulin samples were subsequently used for the isolation and purification of antimitic antibodies on (Diethylaminoethyl) DEAE-cellulose and Sephadex G-200 columns.

1. DEAE - Cellulose Column Chromatography

About 10 g cellulose was suspended in 200 ml of 0.1N hydrochloric acid and stirred at room temperature for 30 min. After filtering the suspension through Buchner funnel under partial pressure, the cellulose cake was resuspended in 200 ml of 0.1N NaOH and filtered again. After filtration, the final suspension was made in 200 ml of 0.1N NaOH. The cellulose was finally washed with 200 ml of 0.0175 M phosphate buffer (pH 6.3) till an ionic equilibrium between the cellulose and the effluent buffer was achieved. Finally, the cellulose was resuspended in 200 ml of the above buffer. The gel slurry before being pressure packed in a column measuring 15 X 32 cm was completely deacrated with the help of a vacuum pump. The column was also equilibrated with the starting buffer.

Precipitated gamma globulin samples were concentrated by dialysis, and also equilibrated against starting buffer before being applied for chromatographic runs. Samples
containing 12 mg gamma globulins were applied to the column. The column was connected to a buffer reservoir - a device which maintained an ionic gradient of 0.0175 M and 0.5175 M in the initial and final sodium phosphate buffer solutions, respectively. The two buffer solutions were adjusted at a pH of 6.3. Two ml fraction samples were collected at a flow rate of 15 ml/hr. Protein concentrations were determined by the same method as described above. A graph showing fraction number versus optical density was plotted for indicating the results.

2. Sephadex G-200 Column Chromatography

Sephadex G-200 gel suspended in 0.0175 sodium phosphate buffer (pH 6.3) was allowed to swell for 5 hr in a boiling water bath. The deaerated gel slurry was poured into a column measuring 2.2 X 38 cm. After complete packing of the column, the flow rate was adjusted at 15 ml/hr. The void volume of the column was determined by using blue dextran 2000. Two ml gamma globulin samples containing 50 mg of protein were applied to the column. Samples were eluted and collected in 2 ml fractions at an adjusted flow rate of 15 ml/hr. The determination of protein concentrations on the eluted samples were carried out according to the method described earlier.

The purity of the isolated fractions was further checked in polyacrylamide gel (PAG) electrophoresis carried
out in 7 per cent gel (pH 8.3) according to the method of Davis. Large pore solution containing about 200 mcg of protein was layered on the top of the gel. The photopolymerisation was carried out for 30 min. A current of 5 mA/tube was maintained for 4 hr. After the electrophoresis, the gels were stained with 1 per cent amidoschwarz dissolved in 7 per cent acetic acid.

E. DETECTION OF ANTI-AMEBIC ANTIBODY

The detection of antibody activity in human and hyperimmune animal sera and as well as from various fractions was carried out by making use of several antigen-antibody reactions. The following antigen-antibody reactions were used for the detection of antibody activity:

a) Precipitin Reactions

Swift - Wilson - Lancefield capillary tube test, which is a modification of the interfacial precipitin ring test, was used for the detection of antigen-antibody reactions. The two reactants separately placed on a clean glass slide were taken up in a disposable capillary tube (1.0. 75 mm X 1.3 mm). Antiserum was the first to be taken up, followed by the antigen. The two reactants rose up to a certain height, after which about 6 mm column of air was introduced by tilting the capillary tube. The tip of the
tube was plunged into a wax platform and the precipitin reactions at the interface were read at room temperature after about 90 min. The tests were run in parallel with appropriate control tubes.

Preliminary antigen titrations, for determining the optimum antigen dilutions to be used in subsequent tests, were also carried out with the help of a quantitative precipitin reaction. Optimum antigen–antibody ratio was obtained by allowing highest antigen dilution, capable of giving a precipitate, to react with decreasing amounts of serum antibody. The test set up also included antiserum, normal serum, antigen and saline control tubes. The tubes were thoroughly shaken and incubated at 38°C for 4 hr. The test set up was left overnight in the refrigerator after which the contents from each tube were centrifuged at 1500 rpm for 15 min. Discarding the supernatant, the precipitate in each tube was washed three times with normal saline and redissolved in 0.5 ml of 0.1N NaOH. Protein contents from each tube were determined by the Lowry's method. The optical densities obtained at 700 nm were plotted against antigen dilutions.

b) Immunodiffusion (ID) Tests

Agar-gel diffusion reactions were carried out according to the technics initially described by Ouchterlony.
and subsequently modified by Mancini et al. A thin film of 1 per cent ion agar (containing 0.1 per cent merthiolate) was made on a glass slide. The central and peripheral wells were respectively charged with appropriate dilutions of antigen and antiserum. Similarly, the immunodiffusion tests were also carried out on the chromatographically isolated human immunoglobulin fractions IgG, IgM and IgA. Only axenic antigen preparation containing 1 mg/ml antigen protein was used in these tests.

Chromatographic eluants representing IgG, IgA and IgM classes of immunoglobulins from human immune sera samples were also assayed by radial immunodiffusion technique of Pereira et al. Sequentially obtained sera samples from immunised rabbits were also monitored by means of radial immunodiffusion tests for the presence of specific antibody activity. Sera samples obtained at weekly intervals from rabbits immunized with axenic antigen preparation were employed for the detection of antibody activity in various immunoglobulin fractions from the primary and secondary response sera. Appropriate amounts of monospecific antiserum to human and rabbit IgG and IgM immunoglobulins were incorporated in agar. Halos surrounding each well containing test serum were measured for estimating the immunoglobulin levels against a reference serum.
c) Immunoelectrophoresis

Antigen - antibody reactions were also studied by means of immunoelectrophoretic tests run according to the technic of Scheidegger and Tanner and Gregory. A 2 mm layer of 1 per cent ion agar in 0.1 M sodium barbital (Hcl buffer of pH 8.6) was prepared on 75 X 25 mm slide. Appropriate channels and wells for receiving the antiserum and antigen respectively were cut in the agar layer. Electrophoresis was carried out at 4°C by providing a current of 8 milliamperes at 150 volts for about 120 min. After the initial run with antigen loaded in the well, the antiserum was delivered to the two parallel channels cut 15 mm apart. The slides were incubated for a period of 72 hr and subsequently read and photographed.

d) Indirect Hemagglutination Test

The test was performed according to the procedures described by Krupp. Preparation of Agents

1. Alsever's Solution

Alsever's solution was prepared by dissolving 2.05 gm glucose; 0.8 gm sodium citrate and 0.42 gm sodium chloride in
100 ml distilled water. The pH of the solution was adjusted at 6.1 with 10 per cent citric acid solution prepared by dissolving 1 g citric acid in 10 ml of distilled water. The solution was sterilized by autoclaving at 12 lbs pressure for 15 min.

2. Glutaraldehyde Solution

A 1 per cent solution was prepared by adding 4 ml of 25 per cent glutaraldehyde to a flask containing 57.6 ml 0.15 M NaCl, 6.5 ml 0.15 M Na2HPO4 and 32 ml double distilled water.

3. Agammaglobulin Normal Rabbit Serum

Gamma globulin fraction of the normal rabbit serum was isolated by salt fractionation with 40 per cent ammonium sulfate. The gamma globulin fraction in the supernatant was removed by centrifugation at 2000 rpm for 15 min. Excess of ammonium sulfate was removed by dialysis.

4. Sensitization of Sheep Erythrocyte

Sheep blood collected in Alsever's solution was centrifuged and the erythrocytes were separated and washed several times with 0.15 M sodium chloride. Washed red cells were first chilled at 4°C in an ice water bath and then
brought into a 2 per cent suspension in 1 per cent glutaraldehyde salt solution. The erythrocytes were washed several times and finally brought to 2.5 per cent suspension in 0.15 % buffered saline of pH 7.2.

Equal volumes of 1:60,000 solution of tannic acid and 2.5 per cent precooled suspension of glutaraldehyde fixed erythrocytes, in parallel with untreated red cells, were mixed by gently rotating the mixture in an ice water bath. Tannic acid treated cells were washed twice with phosphate buffered saline of pH 7.2 and re-suspended (2.5 per cent) in physiological saline. Fixed and tanned erythrocytes were sensitized by mixing an equal volume of such cells with the antigen samples. The treated erythrocytes were washed twice in one per cent agammaglobulin normal rabbit serum, prepared in 7.2 per cent phosphate buffer solution (1 per cent ARS - PBS). All serum dilutions for the test were prepared in one per cent ARS - PBS solution. Sheep erythrocytes were brought into a 0.5 per cent suspension in one per cent ARS - PBS solution. All antisera and rabbit serum gamma globulin samples were earlier heated at 56°C for 30 min to inactivate the complement.

**Test Procedure**

All IHA tests were performed in Flexiglass microtiter plates. Two-fold serum dilutions were prepared to obtain a
dilution range of 1 : 16 through 1 : 512. Each well received 0.05 ml serum dilution along with 0.025 ml of a 0.5 per cent suspension of antigen sensitized erythrocytes. The plates were sealed and gently rotated at room temperature. The plates were left overnight in the refrigerator. The hemagglutination patterns were read and recorded next morning. The patterns in various serum dilutions were recorded as (++) through (+) reactions. Appropriate controls were included in all the tests.

Isolated human gamma globulin fractions were also similarly employed in these tests.

e) Bentonite Slide Floculation Test

The technic as described by Tupasi and Healy was followed for carrying out the above tests.

Stock suspension was prepared by suspending 0.5 g bentonite particles in 100 ml distilled water. Care was taken to obtain a suspension of medium size particles by means of differential centrifugation. Sensitization of bentonite particles with various antigen dilutions was accomplished by mixing bentonite suspension with antigen samples in the ratio of 2 : 1. The opalescent suspension was constantly agitated while being left for several hours at room temperature. The excess antigen was washed off
by the addition of 10 ml of 0.85 per cent saline, followed by centrifugation. The supernatant fluid was discarded and the sensitized particles were resuspended in saline, bringing the volume to initial suspension. Non-specific false positive flocculation was checked and, subsequently removed by the addition of 0.4 per cent suspension of Tween 80.

The actual tests were then performed with human and animal sera samples. Isolated human immunoglobulin fractions were also similarly employed. The test was routinely used for the initial detection of antibodies in various sera samples. Negative and positive serum controls were always included in the tests. Serum complement was always inactivated before using the samples in the test procedure. One drop each, of the antigen-sensitized suspension and the test serum was placed side by side on the glass slide. The two drops were then thoroughly mixed and the slide gently rocked before reading the results. Qualitative observations were recorded as (−) and (+) through (+++) reactions depending on the degree of flocculation.

f) Complement Fixation Test

The complement fixation tests were carried out by modifying the technic of Lennette and Schmidt. The complement for the tests obtained from fresh guinea pig
blood was preserved by mixing with equal volumes of 12 per cent sodium acetate and 4 per cent boric acid solution in distilled water. The sheep erythrocytes were obtained by collecting sheep's blood in an equal volume of Useny's solution. The erythrocytes were washed, centrifuged and resuspended in a similar manner as described earlier in IHA tests. A commercially available hemolytic antibody system (amboceptor - 1:5000) was used for sensitizing the sheep red blood cells. Appropriate dilutions of the various reactants to be used in the actual tests were first determined by carrying out several preliminary titrations of antigen, complement and hemolysin. Quantitative complement fixation tests were performed by using serial dilutions of the test sera (whole and isolated fractions) samples. Proper controls were included in all the tests.

Results were recorded on the basis of hemolysis or lack of it, in the serially diluted sera samples of the test series.

g) Immunofluorescent Tests

All fluorescent antibody tests (FAT) on whole sera samples were performed according to the procedure described by Boonpucknavig and Narm97. Fluorescein isothiocyanate (FITC) - conjugated monospecific anti - human immunoglobulin sera were also used on teflon coated slides for detecting
anti-amebic antibody activity in isolated gamma globulin fractions.

Antigen preparations were dropped by micro pipettes onto a clean glass slide. The slides were dried at room temperature and then fixed with absolute methyl alcohol for 1 min. The slides were then washed with phosphate buffer saline of pH 7.2.

Whole immune serum and fractionated immunoglobulins were applied to antigen spots for about 30 min in a damp chamber at room temperature. The slides were later washed in phosphate buffer for 30 min. Subsequently, FITC-conjugated immunoglobulin antisera samples were applied to the antigen-antibody complex in the ratio of 1:5 and left for 30 min. These slides were later washed for 1 hr in phosphate buffer, rinsed and counter stained with 0.1 per cent Evans Blue. After destaining and washing the excess stain in buffer, the slides were briefly air dried at room temperature. Finally, the slides were mounted with coverslips in buffered glycerol (9 parts glycerol and 1 part phosphate buffer) for fluorescent microscopy. Negative and positive controls were always included in the tests. Readings were recorded on the basis of qualitative visual observations.
ASSOCIATION OF IMMUNE RESPONSES

a) Role of Antibody in Protective Immunity

The assessment and evaluation of the role of cellular and humoral immune responses, with special reference to the production of immunity, was carried out in experimental animal models. Fifty rabbits were used for setting up five animal models for studying the protective role of ameba-specific antibodies. Immunization of animals was carried out by using whole-ameba antigen (axenic) preparations alone and in combination with 
E.C. and tetanus toxoid vaccines. Simultaneously, the unprotected control animals were also given parallel inoculations of Freund’s complete adjuvant and normal saline.

The details of the experimental protocol followed for checking the protective role of antibodies in animal models is shown in Table I.

A total blood picture of all the animals before and during the course of immunization was obtained by counting the red blood cells and white blood cells in Neubauer hemacytometer counting chamber. In every animal, the blood counts were made before the start of immunization schedule and then at weekly intervals. A final blood count after the administration of the challenging dose was made just before sacrificing the animals.
Table I

Immunization Schedule in Animal Models*

<table>
<thead>
<tr>
<th>Serial Number</th>
<th>Inoculations</th>
<th>Number of doses at weekly intervals</th>
<th>Total amount inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Protected</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>BCG : 1.0 ml</td>
<td>6</td>
<td>BCG : 0.6 ml</td>
</tr>
<tr>
<td></td>
<td>Saline : 0.9 ml</td>
<td></td>
<td>Saline : 5.4 ml</td>
</tr>
<tr>
<td>2.</td>
<td>BCG : 0.1 ml</td>
<td>6</td>
<td>BCG : 0.6 ml</td>
</tr>
<tr>
<td></td>
<td>Ameba antigen : 0.9 ml</td>
<td></td>
<td>Ameba antigen : 5.4 ml</td>
</tr>
<tr>
<td>3.</td>
<td>Tetanus toxoid : 0.1 ml</td>
<td>6</td>
<td>Tetanus toxoid : 0.6 ml</td>
</tr>
<tr>
<td></td>
<td>Saline : 0.9 ml</td>
<td></td>
<td>Saline : 5.4 ml</td>
</tr>
<tr>
<td>4.</td>
<td>Tetanus toxoid : 0.1 ml</td>
<td>6</td>
<td>Tetanus toxoid : 0.6 ml</td>
</tr>
<tr>
<td></td>
<td>Ameba antigen : 0.9 ml</td>
<td></td>
<td>Ameba antigen : 5.4 ml</td>
</tr>
<tr>
<td>5.</td>
<td>Ameba antigen : 0.9 ml</td>
<td>6</td>
<td>Ameba antigen : 5.4 ml</td>
</tr>
<tr>
<td></td>
<td>Saline : 0.1 ml</td>
<td></td>
<td>Saline : 0.6 ml</td>
</tr>
<tr>
<td><strong>Untreated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Adjuvant : 0.1 ml</td>
<td>6</td>
<td>Adjuvant : 0.6 ml</td>
</tr>
<tr>
<td></td>
<td>Saline : 0.9 ml</td>
<td></td>
<td>Saline : 5.4 ml</td>
</tr>
<tr>
<td>7.</td>
<td>Saline : 1.0 ml</td>
<td>6</td>
<td>Saline : 6.0 ml</td>
</tr>
</tbody>
</table>

* All antigen preparations along with an equal volume of Freund’s complete adjuvant were inoculated intramuscularly.
The protected and unprotected animals were subsequently challenged by inoculating lethal doses of a strain of ameba of known pathogenicity. Challenging of animals in various groups was carried out by inoculating 125,000 ameba cells/ml of a pathogenic strain directly in the liver (intrahepatic), or in the caecum (intracecal). After giving the challenging doses, the animals were observed over a period of 3 days for the appearance of any signs and symptoms of the disease. The animals were subsequently sacrificed for obtaining the infected tissue specimens.

1. Histopathological Studies

The infected tissues were observed for the appearance of gross and microscopic lesions. The presence of motile amebae in the lesions was also checked in microscopic examinations. Portions of infected tissues were then subjected to histopathological investigations for establishing the extent of tissue damage, or protection.

The excised portions of colon and liver were immediately preserved in 10 per cent formalin solution. Tissues showing apparent damage and the ones without any damage were separately processed for histological studies. Preserved tissues were subsequently washed to remove the formalin after about 10 days. The tissues were then dehydrated in 30 through 100 per cent grades of alcohol and
embedded in paraffin blocks. Tissue blocks were serially sectioned and fixed on microscopic glass slides. The sections were cut at 4 μ in a microtome. The slides with fixed sections were again processed through grades of alcohol, and stained with iron haematoxyline and eosin. The serial sections were mounted in Canada balsam with cover glasses.

b) Skin Hypersensitivity Reactions

A group of 12 guinea pigs were used for the detection of skin hypersensitivity reactions. The experimental protocol employed for sensitization of animals and subsequent administration of challenging doses were initially those as described earlier by Lunde et al.\textsuperscript{134} and Miller and Scott.\textsuperscript{136} Animals were immunized with 1.0 ml to 2.0 soluble axenic antigen preparations mixed with an equal volume of Freund's complete adjuvant over a period of six weeks. A total of 10 inoculations containing about 3 mg antigen protein were administered intramuscularly and intraperitoneally at 3 day intervals. The immunized animals were found to have a high circulating antibody levels as monitored by periodic serum antibody titrations.

The test animals were given the challenging doses in the seventh week. The challenging doses consisting of antigen protein in the range of 1 - 20 micrograms were given in 0.1 ml volumes on the shaved areas at the back of the animal.
Parallel doses of saline controls were also similarly administered. Skin hypersensitivity reactions were read immediately after the administration of challenging doses and subsequently after every 5 hr up to a period of 72 hr. The results were recorded by computing the mean of the diameter of the reaction site, measuring in two perpendicular directions. The tests were routinely recorded on the basis of the appearance of a wheal and flare reaction along with the red indurated area.

c) **In Vitro Interaction**

**In vitro** interaction between *E. histolytica* and the humoral antibodies was also studied for assessing the protective role of circulating antibodies. Human sera samples from patients with clinically proven cases of liver abscesses were obtained for studying the inhibitory effect of humoral antibodies. Control sera samples from healthy individuals and having no demonstrable antibody activity were also included in the test runs. The inhibitory effect of circulating antibodies confined to the isolated gamma globulin fractions was also checked from the obtained sera samples. The gamma globulin was isolated by the addition of 40 per cent ammonium sulfate to the sera samples. The isolation of gamma globulin was accomplished in the same manner as described earlier.
The complement from whole sera samples was inactivated by heating the serum at 56°C in a water bath for 30 min. Fresh guinea pig serum was used as a source of complement in the studies on in vitro growth inhibition. Antibody activity in all the sera samples, prior to their use in the in vitro tests, was determined by titrating the precipitinogens. The precipitin titrations of the serum antibody activity were carried out against the standardized axenic antigen. The antibody titres in the test sera were generally found in the range of 1:1024 to 1:2048.

1. Growth Inhibition Studies

Studies on in vitro growth characteristics of ameba in the presence of humoral antibodies were carried out on *E. histolytica* (NIH Strain: 200) axenically grown in TP-S-1-monophasic medium. The medium was dispensed in 15 ml screw-capped tubes. Each tube was inoculated with 0.2 million amebae and incubated over a period of 120 hr at 37°C. All ameba counts were made in a hemocytometer counting chamber at 12 hr intervals. The final count was determined by taking an average of three counts per culture tube. The experimental protocol followed for setting up the culture tubes was as follows:

1. Ameba grown with different concentrations of immune serum.
2. Ameba grown with different concentrations of normal serum.

3. Ameba grown with equivalent concentrations of immune gamma globulin.

4. Ameba grown with inactivated immune serum.

5. Ameba grown with inactivated immune serum reconstituted with fresh guinea pig complement.

6. Ameba grown with normal gamma globulin.

The growth curves were plotted by counting the number of amebae versus incubation period.