DISCUSSION

The leishmaniasis (visceral, cutaneous and/or mucocutaneous) is a complex disease syndrome caused by different species of protozoan parasite of the genus *Leishmania* which cause a considerable morbidity and mortality in human especially in tropical and subtropical areas of the world.

Visceral leishmaniasis, is a debilitating often fatal disease in man. The clinical signs may vary from one infected individual to another,depending on the hosts' ability to mount an effective immune defence response. Increasing evidence indicated that both cell mediated immunity (CMI) and humoral immune mechanisms play important role in acquired immunity to visceral leishmaniasis (Preston and Dumonde, 1976; Poulter, 1980 and Read, 1981). These two types of immune responses operate in different stages in varying quantities, and as a result determine the clinical outcome (Nandy *et al,* 1983).

The previous research on visceral leishmaniasis suggested (Anon, 1989) some recommendations particularly to unveil the role of antigens in immunological responses. The antigenic make up of *Leishmania* is very complex, but a few antigens have been found to evoke host immunological responses. (Ghose and Rowe, 1976). However, to improve upon the studies on the immunological responses, more work on leishmanial antigens needed to be carried out so that the specificity of the serological tests for diagnosis can be determined with greater ease, and simple sensitive yet specific diagnostic tests useful at periferal laboratories can be made available.

Leishmania organisms vary in their antigenic composition both qualitatively and quantitavely. Selection of strain for antigenic analysis forms the basis for any comparative meaningful interpretation. In this study, a nonvirulent strain of *L. donovani,* originally isolated from a human patient of kala-azar, provided by Indian Institute of Chemical Biology, Calcutta was selected for its luxurient growth in *in vitro* culture medium and its earlier use in similar studies. The growth of an organisnm, the nature of culture and the bio-chemical behaviour of the antigen and/or antigen components are greately influenced by the mode in which the development and multiplication of the organisms, take place. The *in vitro* grown organisnms (promastigotes) having much antigenic similarity to *in vivo* grown organisms (amastigotes) are preferable for any antigenic or immunological studies for their ease in harvesting and artificially induced conditions for luxurient growth. In this context several media has been tried time to time, by different scientists,

[Chang (1947),Tayler and Baker (1968),Gaugher and Krassner (1971), Berens *et al* (1976), Steiger and Steiger (1977, Ray and Ghosh (1980), Chowdhuri *et al* (1982), Mukherjee and Ghosh (1982), Jalees *et al* (1982)].

Many of these media prepared and tried by different workers were expensive and their ingradients were not indigeneously available in India. Media containing larger number of chemicals yielded variable results because of difficulties in maintaining quality control for varied sources of chemicals not always available in India. However, Ray's media (1932) has been accepted world wide for its sustainable good growth of the promastigote cells.

The present study was conducted on the development of "Modified- Ray's media" using minimum, less expensive, indegenously obtainable ingradients which provided good, sustainable and luxorious growth even after tenth serial passages. Simultaneously, three other liquid media were tested for growth study viz: Medium 199, RPMI-1640, DME-medium. These media although yielded sustainable growth but were not suitable for large scale bulk production of promastigote cells due to difficulties in handling by small laboratories. Morever they required expensive foetal-calf-serum necessitating complicated serum filtration procedure. The "modified Ray's media" developed in this study (Majumder and Sen, 1990) was selected as best suitable medium for bulk culture for large scale antigen preparation. The present study thus provided an avenue for cheaper production of *L. donovani* antigen although thereafter. Gupta and Saran (1991) also reported one *in vitro* cheap, serum free, hemin-based autoclaved culture media for growth of *Leishmania donovani* and maintenance of strains by *in vitro* passage.

It is generally accepted that loss of virulence of a microbe is associated with the change in antigenic structure and determines the change in antigenic value with possible potentiality of possessing immunizing antigens. Further, the pathogeriecity of the cell at times get attenuated or less virulent after a long passage and subppassage in *in vitro* culture medium. Therefore it was felt essential to test the pathogenecity of the study strain to its natural host for infectivity in balb-c-mice, white mice and hamsters. The present study noted the non-pathogenic nature of the promastigotes cells to either of these hosts. The present observations are similar to those made by Winger *et al* (1989) on *Babesia divergens* and other earlier workers on this line.

Some antigenic constituents from the non-virulent or attenuated culture of promastigote cells of *Leishmania donovani* might posses the potentiality for use as vaccine for the protection against visceral symptoms— of course which does not form the parameter in this study.

The growth of microbes in a given environment is measurable either by cell count or by optical density measurement or by estimating their protein content of the suspension. Vattuone and Yanovky*et al* (1973) studied the antigen concentration with the change of optical density of the cell suspension of *Leishmania donovani.* In the present study, a standard optical density calibration to denote the number of *L. donovani* cells per millilitre of suspension has been established. The optical density of cell suspension when 1.205, it represented 7×10^7 cells/ml and when optical density was 0.681, the number of cells were 3.5×10^6 cells/ml (Majumder and Sen, 1990). The development of optical density calibration for expression of number of cells per millilitre is of unique advantage in fixing the concentration of cells for antigenic extraction. The variation from smooth to rough form of cells are sometimes associated with loss of the heat stable somatic antigen that characterized the surface of normal virulent microbial cells. In this study the nonvirulent *L. donovani* strain having been used, tests for smoothness were felt to be a prerequisite. Thermo-agglutination test, acriflavin test and tri-phenyl-tetrazolium chloride test were applied for assessing the variation to rough form and the results indicated the smoothness of the growth for their use as antigen and extraction of components. However, uncovering of some somatic antigens dominating the antigenic behaviour of non-virulent strain could not be overruled.

Elicitation of parasitaemias in rabbits tested by injecting promastigote cell suspension, with change in the body temperature was not alarming, i.e. no change in the body temperature even after 48 hours of inoculation. The differential leucocytic count however showed gradual increase in polymorphs and decrease in lymphocyte counts from 4 hours after inoculation, and these reached highest and lowest count respectively at 48 hours. Thereafter the polymorph count started declining and lymphocyte count started increasing reaching preinoculation count after 96 hours. This could be expected as host initial response to sudden exposure to foreign cells— here the promastigotes.

The present study concerning characterisation of antigens from *L.donovani* embraces the studies with whole cell promastigote antigen,particulate antigens of promastigote and antigen fractions derived from the promastigotes as well as extra celular antigen excreated/elaborated by growing promastigotes. Since the log-phase of growth contains the full complements of antigenic components, the cells at this stage were harvested for particulate antigen and isolation of antigen fractions. The soluble antigehn and as well as particulate antigens were also isolated earlier from promastigote cells by Dwyer (1976) for studies of surface membrane antigen antibody reactions.

The promastigotes of *L. donovani* have well defined flagella which led to their motility. Formalin treatment of motile Gram-negative bacteria for obtaining flagellar 'H' antigen is well accepted. Same principle has been utilised in this study for obtaining the flagellar antigen: by treating the cell suspension with 0.1% formalin. In order to evaluate the effects of absolute alcohol and high temperature on antigen mosaic of surface membranes of promastigotes, the somatic antigen or 'O' antigen was prepared from the promastigote cells as in many Gram-negative bacteria, by treating with 20 volumes of absolute alcohol. The heat-killed somatic antigen was prepared by heating the cell suspension at 100°c temperature. Another heat-killed particulate antigen was prepared by killing at 65°c water by bath for one hour followed by resuspension of particulate antigen in normal saline containing 0.5% carbolic acid as used in standard tube agglutination test for Brucellosis diagnosis (Alton *et al* 1975). The last type of particulate antigen made was 2, 3, 5, triphenyl-tetrazolium chloride stained antigen which would allow for better visible coloured reaction. Vital staining of the antigen was carried out on the line of production methodology of milk ring test antigen for diagnosis of bovine Brucellosis described by Alton *et al* (1975) so as to ensure non participation of chemical dye in the test which could possibly result to non specific agglutination.

These particulate antigens isolated represented the native state as per their location (flagellar, or membrane or whole cell) in the parasite body, and when exposed to either anti-H or anti-O or anti-whole cell sera, they make the parasite agglutinable.

Thus, the particulate form of antigen distributed within the structural framework of promastigote cells; as in *Salmonella, Shigella, E. coli* and others, might be responsible for antigenic specificity, and the stability of antigens at high temperature, on acid and alcohol treatment, is expected to be useful in evaluating the humoral immunity mechanism.

The soluble antigens were extracted mainly by following two methods:- Autoclaving and Sonication although freezing and thawing was also attempted. The crude antigen following autoclaving or sonication was separated into supernatant having solubilised antigen and the residue. one aliquote portion of residues was further fractioned by 80% phenol treatment of Middlebrook and Dubos (1948) as in *Mycobacterium tuberculosis* for extraction of tubercular protein. Another aliquote of the residue was treated with 2.5% NaCl following the method of Baker *et al* (1952) used for isolation of proteins from *Pasturella pestis*. Thereafter, the supernatant was precipitated with 68% ethanol and the residue was further fractioned by 45% phenol on the lines of Westphal *et al* (1952) for liporotein polysaccharides. Thus, the extraction procedure followed

in this study allowed not only the isolation of surface antigens but also antigen complex from cytoplasm of the cell and kinetplast. The freezing and thawing of the cell suspension yielded soluble protein but the yield in this process was very poor, and required much more time and homogenization. Therefore, the freeze-thawing method of antigen preparation was not further continued for rest of the tests done.

The exometabolites or Excretory Secretory Antigen (ESA) was isolated from the liquid growth media where the promastigote cells were grown for 7 days and the exometabolities accumulated. It is possible that during prolonged (7 days) incubation in liquid medium, some of the promastigotes might have got autolysed or disrupted. Thus, the ESA might contain,beside the excretory and secretory material of growth process of promastigotes, some of the cytoplasmic aand cell membrane fragment antigens which could not be overruled. This ESA was also further fractioned by adding trichloroacetic acid treatment and ammonium sulphate treatement. The excess salts were removed by dialysis against distilled water.

It is pertinent to mention in this context that cell wall was partitioned between aqueous and phenol phases. Phenol phase contained most of the protein, as in bacteria, and also contain cell debris and DNA. The aqueous phase contained mainly the lipolysaccharide and RNA. They were dialysed to remove contaminating phenol. Fraction IIA, IIC and IID were prepared from AP or Fraction II and Fraction IVA, IVC and IVD were made from SP or Fraction IV. The cell wall debris was also fractioned by only phenolic (80%) treatment of the debris, and finally suspended in a solution containing 0.55% NaCl, 0.5% Na_HPO_4 and 20% methanol in 0.5:65 ratio and the fraction named as Fraction IIB from AP of Fraction II, and Fraction IVB fron SP or Fraction IV. It may be claimed at this syage that none of the published work contained reports of isolation of so many varied type of antigens although particulate unstained antigen (Senekjier and Liewis 1944, Nandy *et al,* 1983) and stained antigen (Allain and Kagan, 1975; Harith *et al* 1986) as well as soluble antigen (Dwyer 1976, Rezai 1977; Ray and Ghosh 1984; 1985), Sonicated antigen (Bray and Lainson, 1967, Datta *et al* 1987 and Mittal *et al* 1991), excretory Secretary antigen (El-on *et al* 1979, Ray and Ghose 1985) were time to time worked upon by various workers.

The knowledge on the chemical nature of the antigens isolated is of importance for correlating their immunological behaviour. In this study the soluble antigens underwent qualitative and quantitative bio-chemical tests for proteins and carbohydrate moities. The qualitative test for the protein content revealed the "albumin type" nature of protein in the SAS or Fraction I, SSS or Fraction III, Excretory Secretory antigen or ESA or Fraction V, and

ammonium-sulphate precipitate of ESA, known as Fraction VB; and the fractions of II, IV and VA of the antigen might contain lower molecular weight protein of gelatin or peptone type, Carbohydrate moities were detected only in very few fractions like Fraction IIB, IVB, IVC from autoclaved residue and sonicated residue. Fraction IVA from sonicated residue and fraction VB from ESA evinced positive reactions in half saturation to reveal their protein-polysaccharide complex nature.

The methods of obtaining fraction IVD, possibly released little quantity of antigen to be detected by tests for either protein or carbohydrate moities. Thus fractions I, III, IIA, IIC, IID, IVD and VA are primarily of proteins moity of different molecular weight (Albumin type or Gelatin type) and fractions IIB, IVA, IVB, IVC and VB are protein polysaccharide complex. Test for lipoids have not been undertaken which could possibly designate if these later antigens were lipoprotein polysaccharide complex. These observations are in line with the earlier findings of Baker *et al* (1952) with 2.5% NaCl extraction for isolation of protein from *Pasturella pestis* , 45%phenol extraction of Westphal *et al* (1952) on Gram negative bacteria and 80% phenol extraction method of Middlebrook and Dubos (1948) for isolation of protein polysaccharide complex from *M. tuberculosis.*

For critical analysis of immunobiological reactivity, purification of such antigen was essential. Therefore, the column chromatography of SAS and SSS through Sephadex G-25 column was attempted which eluted maximum protein concentration (peak) in 10th and/or 11th collection of fractionsof 5ml each. The proteins thus eluted were of low molecular weight allowing the proteins to be separated through this column.

The electrophoretic mobilities of the soluble, antigens of *L. donovani* were studied on 10% polysaccharide gel with marker proteins of 50-52 K.D., 42 K.D and 12 K.D. The molecular weight of the antigens varied from over 12,000 to less than 60,000. Slutzky and Greenblatt (1979) isoalated an excretory factor from *L. tropica* and studied the SDS-PAGE, when they obtained lower molecular weight immunologically active substance from sonicated organisms which they considered as precursor of the higher molecular weight material. However, no earlier work on this line on *L. donovani* was available for comparison with the present findings. Ray and Ghose (1985) noted considerable electrophoretic heterogeneity in immunoelectrophoresis, SDS-PAGE with crude soluble antigen and polysaccharide rich antigen of *L. donovani.* The minimum molecular weight of the basic structural unit was stated by Ray and Ghose (1985) to be around 2×10^4 daltons, which are confirmed in this study.

The particulate antigens isolated for identifying the role of surface or membrane or flagellar antigen in immunostimulation were analysed for their agglutination property.

Agglutination is a type of antigen— antibody reaction in presence of electrolyte where the antiogen is in particulate form. Agglutination reaction maty be employed either to detect the presence of specific antibodies in a serum by using a known antigen or an unknown antigen may identified when reacted with a known antiserum. The test may also be employed to determine the concentration and type of antibodies in the serum. The three reagents required for agglutination test, are antigen, antiserum, and electrolyte and the reaction can take place at varying incubation temperature and periods. Reactions at higher temperature takes place at a faster rate than at lower temperature. The test may be conducted either in the test tubes where settling of agglutinated mass is noted or on a glass slide or a plate. For quick diagnostic results as in field survey work, plate or slide test is employed with concentrated coloured antigens. The type of antigen used in agglutination test is therefore of utmost importance for rational interpretation of visible reactions due to various immunoglobulins.

Tube Agglutination Test (TAT) performed in this study, revealed agglutination of *L. donovani* antisera at a higher titre with heat-killed promastigote cells (carbolysed) than "H" antigen and "HK" antigen. Again the *L. donovani*-whole cell "HS" antiserum contained the maximum titre of antibodies than "H" antiserum and "HK" antiserum. It is possible that higher temperature of incubation for agglutination of the antigen antibody system might have reflected higher titre with flagellar antigen or antiserum which however was not used in this study. In this case, the agglutinated cells settled in a spread out fashion making a smooth carpet in the entire concave bottom of the test tube, like a flaky appearence. Control or non-agglutinated cells settled in the form of a compact bottom.

Earlier, tube agglutination and plate aglutination tests were evaluated by Senekjie and Liewis (1944) in leishmaniasis; which were however of lower advantage as mentioned by Allain and Kagan (1975). However, no earlier report on TAT with so many leishmanial antigen-antibody system is available in the literature. It is of interest to note that carbolised antigen and carbol-saline diluent increased the sensitivity and specificity of TAT as was recommended by WHO for slow Agglutination Test (SAT) for diagnosis of brucellosis prticularly in bovines (Alton *et al,* 1975). From the observations made in this study, it can be recommended that carbolised antigen be only used when TAT is applied for serodiagnosis, of leishmaniasis which could detect both 'H' and 'O' antibodies at higher titres.

The microbes stimulate the production of antibodies by their host against the antigens primarily present on the surface- be them the surface envelop antigen, capsular antigen or the antigen distributed on the surface of the flagella. The *L. donovani* promastigotes being flagellate, stimulate the production of antibodies corresponding to the antigen (s) present on the flagella. These antibodies would react with the corresponding flagellar antigens and neutralise the motility caused by flagella, eventually making the organism immobilise. The slide agglutination test carried out in the determining the 'H' antigen of Salmonella is the principle which has been utilised in studying the antibodies against *L. donovani* promastigotes. Immobilisation of movements of Leptospires by specific antibodies observed under microscope has been accepted as a standard reference procedure for diagnosis under the name Microscopic Agglutination Test/MAT (WHO, 1967), where surface somatic antigens participate in 'MAT'. In this study, similar tests with live promastigote and antibodies to live cultare, formolised antigen, heat-killed antigen were performed noting the reactions under the microscope, and therefore the immobilisation reactions have been designated as Microscopic Agglutination Test (MAT). It is postuated that subsequent to conversion of promastigotes following inoculation by vectors the amastigotes of *L.donovani* in definitive host contain the precussor of flagella, the kinetoplast as observed under electron microscopy by Chang (1956).

The present study revealed that healthy rabbit serum or healthy human serum when tested by MAT, after heating at 56°c for 30 minutes,did not produce MAT reaction even in undiluted state (neat); thus indicating the removal of nonspecific heterophilic antibdies or natural antibodies (Gibson 1930, Ulrich *et al* 1968) in these sera, if any.

The whole cell (HS) antiserum showed higher titre by MAT than the 'H' antiserum or 'HK' antiserum, which could be explained by the fact that the whole promastigote cell contain the intact flagella compared to the formolised 'H' antigen when the flagella could not possibly cover totally the surface of the cell, to stimulate better 'H' antibody production. The low titre 'HK' antiserum, was however anticipated. From all these information it can be concluded that the MAT has the potentiality to be a simple, sensitive, diagnostic test for seroepidemiological studies of *L.donovani* infection.

The specificity of the test can be assessed from the Microscopic Agglutination Inhibition Test (MAIT), in which it was observed that the formolised 'H' antigen inhibited the MAT at the highest dilution and similar inhibition was also caused by soluble Autoclaved Superanatant (SAS) or Fraction I, which indicated the spatial location of the antigen participation in the test to be on the surface of the flagella. Other soluble antigen of particulate antigens could

not inhibit the MAT, indicating their absence on the surface of the flagella. Thus, this test (MAT) proves to be not only sensitive and simple but also specific.

The agglutination test being the easiest of all the serum test, several modifications of the agglutination test have been tried to make the visible reaction quicker, easily perceptible and sensitive under different conditions. The antigen, used has therefore been coloured by different dyes or strains. In bacterial diseases coloured antigens have been used for quick plate agglutination test (PAT) in detection of Salmellonosis, Brucellosis, as well as for the detection of Brucella antibodies in milk. In leishmanial infection, Allain and Kagan (1975) used antigen stained with Evan's Blue for direct agglutination test (DAT). Harith *et al* (1986) utilised the promastigote cells coloured with Coomassie Brilliant Blue stain. Taking the clue of utilising vital staining with 2, 3, 5 triphenyl-tetrazolium chloride of Brucella cells for antigen preparation in milk ring test in this study an antigen has been developed by staining promastigotes with the same dye.

The coloured antigen particles of uniform size and shape were used for easilyy readable diagnosis. The non-aggtlutinated cells settled to the bottom of the cup as a clear red "button" while agglutinated red cells settled as a pink spread-out zone called "mat" formation. The coloured particulate antigen prepared, developed and standarised in this study is a new antigen and the test performed was also a new technique using haemagglutination perspex plates utilised for haemagglutination test in New Castle Disease. The test was therefore, designated as Plate Agglutination Test (PAT). For this new technique, the diluent for the antigen and serum as well as the concentration of cells for detection of highest antibody titre were to be standardized. To that effect it was noted that the PAT antigen when diluted with only carbol-saline or carbol-saline containing 1% peptone showed the agglutination ("mat form" settling of antigen) at 1:640 dilution with *L.donovani*- whole cell ("HS") antiserum when only carbol-saline was used as diluent. However, the controls did not work well. The antigen diluted with carbol-saline containing 0.2% peptone showed agglutination at 1:320 dilution only with "HS" antiserum. The PAT antigen at its 1:5 dilution in carbol-saline with 1% peptone was selected for this study because of the highest serum titre detected by this dilution of antigen. Further dilution of antigen detected lower serum titre. The standardization of PAT antigen was thus achieved by initial fixing of cell concentration at 20% followed by 1:5 dilution in carbolsaline with 1% peptone. An incubation period of minimum 1 hour at 37°c temperature, was found to be satisfactory to us. However, the storage of the test plate in refrigerator (4°c temperature) for 18 hours facililated most convincing matting

("mat" formation) in test series with "button" formation in control well. The promastigote being of large particle of 15 to 25 μm in size, have the tendency to settle down on incubation for one hour. The use of "U bottom" holes in perspex plates allows the agglutinated promastigote to settle down in the manner of erythrocytes settling in positive haemagglutination test in such plates. The "HS" serum dilution of 1:640 was considered as one "PAT unit" for inhibition test.

It is pertinent to mention here that the PAT has definite advantage as a diagnostic test for its (1)sharp notable end titre as compared to control, (2)less time requirement for incubation to note the result, (3)non-reactivity with malaria convalescent sera and (4)antigenic specificity adjudged from inhibition (PAIT) tests. However, the cross reactivity of PAT antigen with serum from patient suffered from other flagellated haemoparasites could not be ruled out, since such human cases are not available and inexistence in India.

The observations suggests that the PAT is primarily dependent on the surface antigen (flagellar or somatic) and is a modified type of Tube Agglutination Test (TAT) which is easy to perform with perceptible conclusive end titre of the serum because of colouring the antigen and pattern of settling of cells.

Eisenberg and Volk as early as 1902 showed that when a constant amount of bacterial suspension was allowed to react varying concentration of an agglutinating serum, proportionately more agglutinin was absorbed from more dilute serum. The immunological specificity of a test is dependent upon chemical groups presnt on the surface. Though the stereo-chemical differences in structure of different fractions were not determined in this study, absorption experiments with thsese fractions isolated were attempted to determine the immunological specificity by plate agglutination inhibition (PAIT) test.

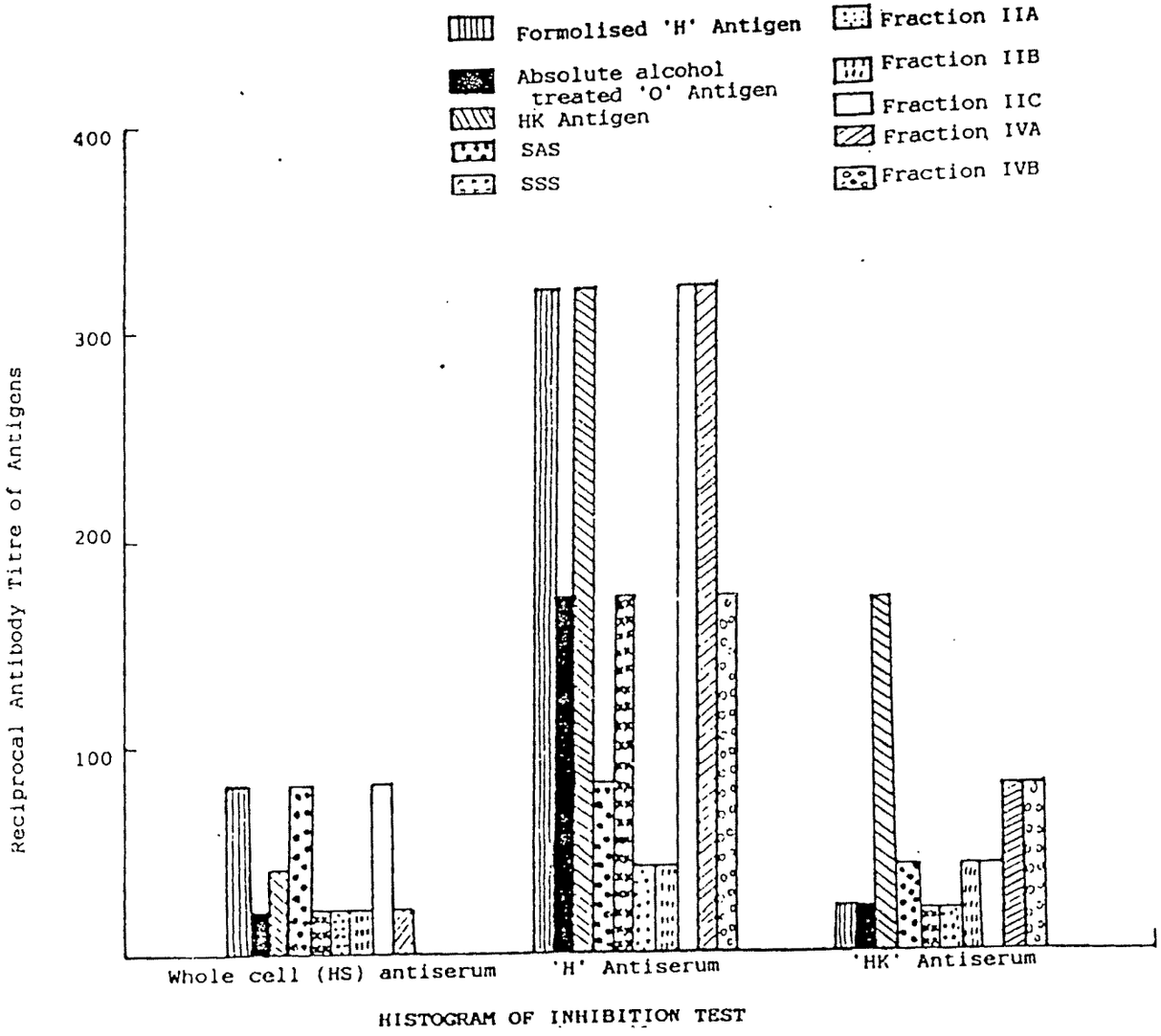
The plate agglutination inhibition test (PAIT) showed the highest titre of Heat-Killed ("HK") antiserum with "HK" antigen at 1:160 dilution, and fraction IVA and IVB inhibited the PAT at 1:80 dilution with the same antiserum. However, other antigens showed lower titre at 1:40 or 1:20. Thus, the PAIT with 'HK' antiserum, revealed the localisation of 'HK' antigen on the surface of the cell, and fraction IVA and IVB were also on the cell surface but in smaller quantity.

The PAIT with whole cell (HS) antiserum showed lower inhibition titre (1:80) than "HK" antiserum. The highest inhibition titre was caused by 'H' antigen, SAS antigen and Fraction IIC, i.e. 2.5% NaCl treated fration of Autoclaved pellet (AP). This would indicate that when whole cell *L.donovani* is injected only the surface antigen of the flagella and the cell surface of

promastigote, were exposed for antiobody production and these antigens reacted in very low quantity with the antiserum. The PAIT with formolised 'H' antigen showed highest inhibition (1:320) titre with homologous antiserum ('H' antiserum) and similar inhibition titre were also noted with 'HK' antigen and Fracrtion IIC and IVA. The PAIT titre with homologous system was always higher than the other whole cell antigens or fractions thereof (Fig. VIII), indicating the specificity of the Plate Agglutination Test (PAT). The relative spatial localisation on the basis of PAIT with flagellar system indicated with presence of Fractions IIC, IVA as well as Fractions III and IVB on the surface of promastigote probably distributed sparingly on both flagellar and somatic region except Fraction III which seems to be of flagellar origin. The observations with whole cell ("HS") serum however indicate autoclaved antigen (Fraction I) and 2.5% NaCl extract therafter (Fraction IIC) also to be located on the surface adsorbing the cell surface acting agglutinins resulting to inhibition of PAT at higher titre.

The Direct Agglutination Test (DAT) initially developed and utilised by Allain and Kagan (1975) was made to be more sensitive by Harith *et al* (1986, 1987) by staining promastigotes with Coomassie stain. In recent past, this modified DAT has been universially advocated for diagnosis of *L. donovani* infection or kala-azar (Anon, 1989). Hati (1994) commented that research was going on in wrong direction. EIISA and DAT were being evolved without proper standardization besides the fact that these were falacious not suitable in field conditions. With the development of a new coloured antigen stained with 2,3,5, tri-phenyl-tetrazolium chloride for plate agglutination test (PAT) in this study, it has become necessary to compare PAT with DAT under identical experimental condition by parallel tests. The observations with whole cell ("HS") antisera using both the antigens revealed DAT titre to be 1:256 as compared to PAT titre of 1:1024 (with 1:10 dilution of antigen)at 22°c for 18 hour. Since the PAT antigen can detect the *L. donovani* antibodies in higher titre than the DAT antigen, the PAT is considered to be superior to the DAT under our conditions. It may apparently occur that the "HS" serum showed highest titre at 1:640 but in this parallel test with DAT, titre of 1:1024 was obtained with PAT antigen. This may explained to be due to — (1)the time temperature combination used in this parallel study (18 hr. at 22°c temp.).(2)doubling dilution of PAT antigen (1:10 instead of 1:5).The higher sensitivity of the test which detects specific agglutinin would be the choice for field diagnosis of *L. donovani* infection. Therefore this test has been tried on human and animal sera in this study.

Agar-gel double diffusion test applied by Schnur *et al* (1972, 1973, 1974) and Schnur and Zuckerman (1977) in differentiating serotypes of *L. donovani* in Sudan, Kenya and Ethiopia did not prove to be of any value in differentiating



HISTOGRAM OF INHIBITION TEST

the antigen fractions isolated in this study due to absence of any positive reactions by gel diffusion test. The preparation of gel plate probably did not allow the separation of antigens in this study. However, the immunoelectrophoresis made in this study enabled to identify the purity of different fractions.

Immunoelectrophoresis (IEP) is a very useful technique to study antigen-antibody reaction. It has the advantage over simple gel diffusion technique in that the different fractions of the electrophoresed protein are separated in an electric field and gives better precipitation reaction. Electrophoresis involves the migration of charged protein particles in an electric field, as seen in the migration of the gamma globulin fraction of the serum. The electrophoretic properties of antibodies are distinguished from other serum proteins by precipitation of the antiserum with homologous antigen.

In this study, the IEP pattern of five antigens viz. SAS (Fraction I), SSS (Fraction III), ESA (Fraction V) and peak fraction in Sephadex G-25 fraction of SAS and SSS, with "HS" antiserum, showed precipitation bands on either side of the hole and a faint trailing zone of precipitation band continuing to the anodal end. The more purer proteins (Sephadex fraction of SAS and SSS) showed more intense band with "HS" antibody. The study thus indicated that the fractions I, III and V are all homogeneous antigenically and column chromatography in Sephadex G-25 further concentrated the antigen in peak fraction. No difference in antigen components between the fractions could be revealed.

Antigens prepared by freezing and thawing was utilised for CIEP by Mansueto *et al* (1977) while Rezai *et al* (1977) utilised antigen obtained by ultrasonication. In this study, it has been noted that the autoclaved supernatant, sonicated supernatant and extracellular secretory antigen could detect antibodies in whole cell antisera while only the Sephadex G-25 purified peak fraction of Fraction I and III could detect antibodies in "H" and "HK" antiserum. It is possible that the type of antigen and their molecular structure would determine the universal efficacy of counter-immunoelectrophoresis in detecting leishmanial antibodies (Desowitz *et al* 1975, Aikat *et al,* 1979c).

The primary interaction of antigen with antibody and sensitized lymphocytes causes some *in vitro* or *in vivo* reactions, which are basis of any diagnostic tests, and are also the basis of the pathogenetic mechanism of hypersensitivity diseases. The intercellular microbes are of special importance in stimulating the host to respond to both the types of (antibody associated and sensitised lymphocytes associated) reactions, as in the case of Tuberculosis, Brucellosis or viral infections, and parasitic infections, particularly the protozoan diseases. The intercellular infective agent leads to the stimulation of sensitised

lymphocytes besides the formation of antibodies --- IgA, IgG, IgM, IgD, IgE etc. The allergized lymphocytes liberate lymphokines when they encounter the corresponsding antigens and these lymphokines attract the cells, the macrophages to the site of actions resulting to the pathological conditions. The post-kala-azar dermal leishmaniasis indicate the participationm of celll mediated immunity which led the earlier workers (Sengupta amd Mukherjee, 1962, Southgate and Manson-Bahr, 1967) to utilise intradermal test or leishmanin skin test forthe diagnosis of kala- azar. Ghose *et al* (1983) postulated that *L. donovani* infection in man stimulates the production of specific suppressor T-cells for the effective suppression of T-lymphocytes responsiveness to leishmania antigen. The CMI response to leishmania antigen was noted in majority of kala-azar patient. In active kala- azar there is a supppression of delayed type of hypersensitivity (DTH) and emergence of positive DTH response to leishmanin test was nopted in cured kala-azar patients. It is known that DTH are the manifestations of the T-cell effector arm of immune system. Cells involved in DTH reactions include antigen specific DTH-effector-T-lymphocytes, monocytes, regulatory B cells and basophills. Studies by earlier workers Abdalla *et al* (1975), Saran *et al* (1991), indicaetd that the antigens prepared by different methods - freezing and thawing, ultrasonication of whole cell - evoked reactions dofferently. In this study, Soluble Autoclaved Supernatant (SAS), Soluble Sonicated Supernatant (SSS), Excretory Secretory Antigen (ESA or Fraction V), fractions obtained after sodium chloride or phenol treatment of Sonicated Pellet (SP) and the antigens concentrated by Sephadex G-25 column were tested as allergen in rabbits previously sensitised with live culture of *L. donovani* and it was revealed that SSS or Fraction III and SAS or Fraction I induced the maximim reactions in delayed type of hypersensitivity persisting for 48 hrs or more. It is felt that the antigen liberated by sonication would be the choice for trial in kala-azar patients.

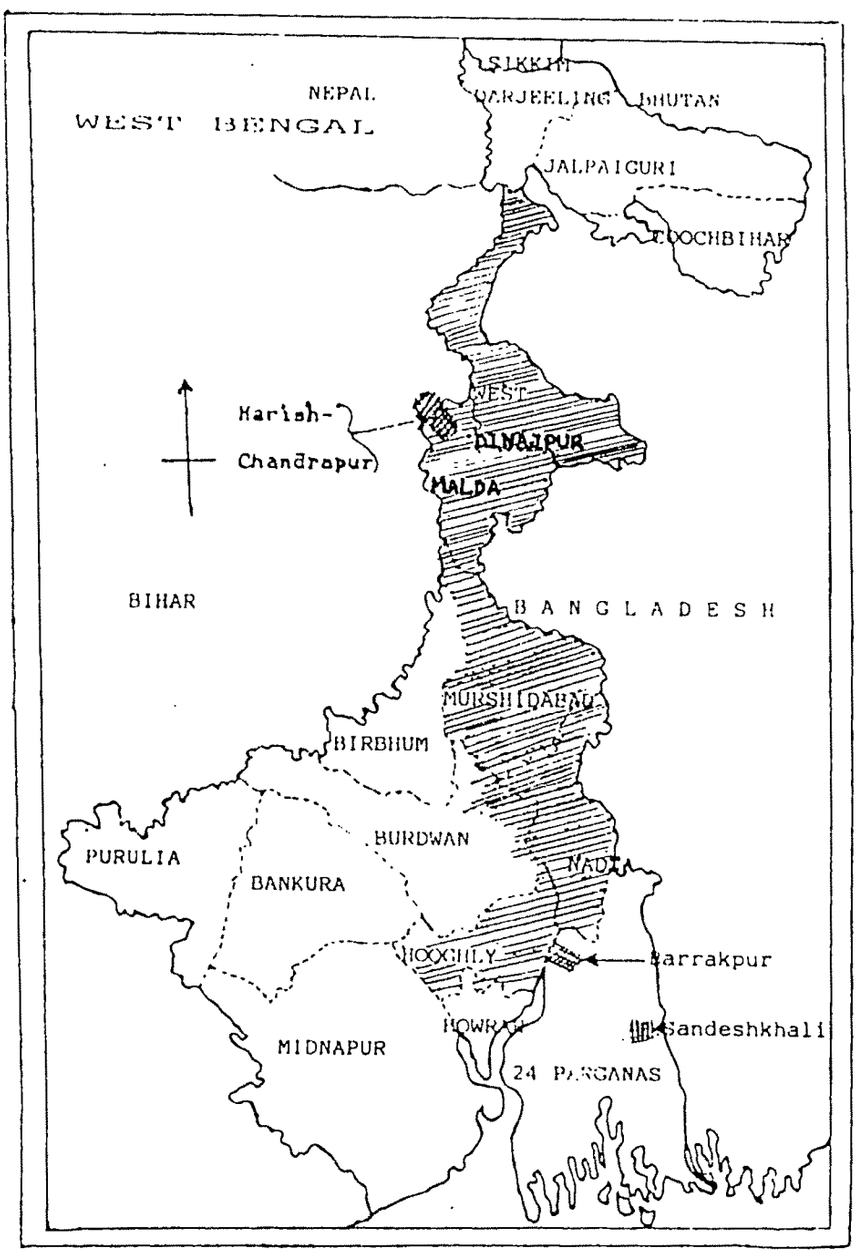
Since the defence mechanism of the animal body against infection is due to the cellular and humoral agents, the two main groups of phagocytic cells macrophages pand microphages, are greatly involved in defence mechanism, among which microphages particularly the neutrophiles are active. Phagocytosis of certain infection take place in absence of opsonic antibody or opsonin, which coats the infection surface, thereby facilitating the phagocytosis. Chang (1981) reported the leishmanicidal mechanisms of human polymorphonuclear phagocytosis. This principle has been utilised in this study to detect opsonocytophagic index. In this study the opsonocytophagic index is very low or insignificant.

It is well accepted that lymphocytes are the key cells in immunology. Among the two classes of lymphocytes, the T-lymphocyte cells when sensitised by pre-immunization with antigen; losses its ability to migrate when exposed later to this antigen. Nandy *et al* (1983) observed that there was initial depressed cell mediated immune response in the form of depression of number of circulating T-lymphocytes, negative allergic reaction and low leycocyte migration index. Following successful treatment, the number of circulating T-lymphocytes and Migration- Inhibition increased steadily with increased leishmanin test positivity.

In this study too, it is noted that inoculation of live promastigotes to rabbits to initial decrease of lymphocytes on 48 hr. and 72 hrs. which returned to its optimum after 96 hours and the rabbit gave positive reaction to intradermal skin test after a month. These observations led to utilise the leucocyte migration inhibition test as the parameter for hypersensitivity (Soborg and Bendixen 1967, Aikat *et al,* 1979). The *in vitro* leucocyte migration inhibition test indicated the role of SAS, SSS and Sephadex peak fractions of SAS and SSS in cell mediated immunity. While Aikat *et al* (1979c) performed the test with BCG, Nandy *et al* (1983) conducted LMIT with sonicaetd antigen. Since they utilised the test for screening human kal-azar patients sera as a diagnostic tool, the observations made in this study were not comparable. The present observations only indicated that cellular immunity to Leishmania in rabbits resembled the cellular immunity described as a factor of resistance to several other microorganism which parasitize macrophages such as toxoplasma, salmonella, Brucella or Tubercle bacilli (Miller and Twohy, 1969).

One of the objective in this study was to develop any simple, sensitive and quick test to detect *L. donovani* antibodies in early stages of infection. Serological investigation by Ghose *et al* (1980) in Indian Kala- azar revealed significant increase in the serum IgG and IgM levels and the detection of circulating antibodies at the early stages of KA can be achieved by various serological tests (Ghosh *et al* 1983, Nandy *et al* 1983). In the present satudy, two new serological tests Microscopic Aggluitnation Test (MAT) and Plate Agglutination Test (PAT) have been developed which proved to be very easy to perform without requirement of sophisticated laboratory or equipment and found to be sensitive and highly specific by inhibition experiments with various antigens. Normal agglutinins in human or rabbits sera were also not reacted upon by the specificied antigens. The various serological tests for KA reviewed in earlier chapters only indicated DAT to be the choice for periferal field laboratories or centres. Observations with MAT on 15 KA patient sera and three healthy human sera and two each of malaria patient and normal rabbit serum indicated the utility of MAT in diagnosis of KA with ++ reactions at 1:4 dilution of sera. Though the test appear to be specific in detection of early

AREAS OF RECENT KALA-AZAR RESURGENCE



cases of KA, the low serum titre for positive diagnosis calls for search for more sensitive test.

In this context, the PAT developed in this study applied on eight KA-patient sera indicated the test to be more sensitive than MAT having positive titre of 1:80 considerd as diagnostic. Similar test designated as DAT (Harith *et al,* 1986) however did not reveal in this study to be as sensitive and specific in parrallel tests with PAT. Nandy *et al* (1983) noted also the superiority of ELISA over DAT and Mahajan *et al* (1983) recommended that the use of DAT for some day serodiagnosis in the field should be further explored and their tests should be adopted to improve field diagnosis of leishmaniasis. Hati (1994) considered research on ELISA and DAT to be falacious. Further, they talked about the need for low cost, simple sero-diagnostic methods. In this context, the present study provide unique solution with PAT having more sensitivity than DAT, usable by periferal laboratories yet specific, sensitive and of lower cost.

Investigation on dogs as reservoirs for visceral leishmaniasis by Heisch (1954), Kirk (1956) and Southgate and Oriendo (1962) confirmed kala-azar as zoonotic infection in African countries (Ngoka and Mutinga, 1968, 1977). In India too, the reappearance of kala azar after a lapse of 10-15 years suggested the possibility of an animal reservoir for *L. donovani.* Srivastava and Chakravarty (1984) detected low levels of antibodies by ELISA in six dog sera out of 125 dogs examined. To this effect, search for zoonotic reservoir was made in dogs with the best serological test developed in the course of this study the Plate Agglutination Test (PAT).

Recent resurgence of kala -azar in West Bengal amongst human has been recorded in many districts (Fig IX). It is possible the dogs belonging to homesteads where human cases existed surve as reservoir of infection (Mutinga *et al,* 1980). The Indo-UK Workshop on leishmaniasis dealing with animal reservoirs and epidemiology (Lainson and Ashford, 1983) recommended continuation of serological survey of dogs for possible existence of reservoirs of *L. donovani* infection. In this study, serum samples from 50 dogs from the areas of recent kala-azar were subjected to the PAT.

From the observations that one dog had 1:40 titre, four had 1:20 titre and five had 1:10 titre, it becomes evident that antibodies corresponding to somatic antigens of *L. donovani* were present in them. The hypothesis made by Srivastava and Chakravarty (1984) of possible cross-reactions with other dog-specific flagellate does not appear to hold good in these observations since adsorption test with 'H' antigen revealed absence of any 'H' antibodies in these sera. The interesting feature of these observations are presence of specific low

titre, indicative of past exposure to the antigen instead of active infection in which case 1:80 PAT titre was considered as diagnostic. It is possible that since the study was made from the endemic areas where transmission of infections takes place betweem man-vector-man, it could not be ruled out that these dogs had exposure to the accidental bites of the vector *Phlebotomus argentipes* on some past occassion, harboured the infection in quiscernt state to stimulate somatic antibody formation. However neither the obseravions prove the dogs as the reservoir in absence of cultural isolation nor it prevents to hypothesise the role of dogs in inter-epidemic period of kala- azar (Lainson and Ashford 1983; Srivastava and Chakravarty, 1984.)