INTRODUCTION
1. Historical Review

Leishmanias are flagellated protozoan parasites, they are the causative agents of a variety of human diseases termed leishmaniasis. The leishmaniasis today appears to be a far more prevalent disease having a much greater public health importance than what was previously thought. Rough estimates based on extrapolations of the scarce data available indicate that some 350 million people in the world are at risk of acquiring leishmaniasis and that approximately 12 million people per year are currently infected (Report of WHO Expert Committee, 1990, Olliaro and Bryceson, 1993).

Visceral leishmaniasis is caused by *Leishmania donovani* and spread by the bite of infected sand flies. The etiological agent, *L. donovani* is named after the discoverer, Leishman and Donovan, both of whom reported on the organism simultaneously; Leishman first published his report from London (UK) in May 1903, while Donovan reported from Madras (India) in July 1903 (Chatterjee, 1982).

The parasite Leishmania was first seen in 1885 by Cunningham while examining a histological section from an oriental sore in India. This organism at that time was thought to be a Mycobacterium and subsequently, in 1989 Brovosky established the protozoan nature of the parasite. As mentioned earlier, it was Leishman (1903) who for the first time described this organism as a causative agent of visceral
leishmaniasis in man. Rogers (1904) and Nicolle (1908a) established the trypanosomatid nature of *L. donovani* and *L. tropica*. Also, Nicolle (1908b) mentioned about the causative agent of infantile kala-azar and named them as *L. infantum*. It is more commonly found in the Mediterranean countries, as also in other parts of Asia, Europe and Africa. In 1951 Yokimoff and Scholhov described *L. tropica major* as the causative agent of zoonotic cutaneous leishmaniasis. It was Bray in 1977 who established the name *L. aethiopica* for the causative agent of cutaneous as well as more diffused cutaneous leishmaniasis in Europe and Kenya. Muniz and Medina in 1946 described and discovered *L. enriettii* in guinea pigs in Panama State and Brazil. Biagi 1953 and Floch in 1954 referred to *L. tropica mexicana* as the causative agent of "Chiclero's ulcer", the name that stems from the time that leishmaniasis was an occupational disease of gum (chile) collectors. It is currently associated with farming and a variety of forestry activities. *L. tropica guyanensis* and *L. tropica braziliensis* are responsible for causing cutaneous leishmaniasis and mucocutaneous leishmaniasis, respectively (Zuckerman and Lainson, 1977).

However, mucocutaneous leishmaniasis is a name currently applied only to New World disease known as "espundia", which is caused by *L. braziliensis* and *L. panamensis* (both species of the subgenus Vianna) (Santrich
et al, 1990). So far, efforts have been made to identify Leishmania only at the species and subspecies levels, as originally defined by Lainson and Bray (1978, 1987). The methods and tools developed for the purpose include: isoenzyme profiling (Le Blanq et al, 1987), monoclonal antibodies (Grimaldi et al, 1987), chromosomal separation (Gainniani et al, 1986; Spithill and Samaras, 1985), k-DNA and genomic DNA restriction analyses (Lopes et al, 1984; Barker et al, 1986; Barker, 1987; Beverley et al, 1987; Ramirez and Guevara, 1987). More recently, Uliana et al (1991) have described a molecular probe that recognized the genus Leishmania, regardless of the subgeneric taxa. Furthermore, this probe clearly distinguishes Leishmania from Trypanosoma, and could prove very useful for clinical diagnosis and epidemiological surveys.

2. Taxonomy

The taxonomy of the species of the genus Leishmania represents a diverse group of kinetoplastid protozoan parasites grouped with Trypanosomes as 'Haemoflagellates'. The species of Leishmania - like parasites of lizards are now grouped in the genus Sauroleishmania. Since there are few morphological differences among the various members of the genus Leishmania, therefore they are classified according to the clinical disease they produce. The different clinical forms given below have been taken from Report of a WHO Expert Committee, 1990.
2.1 Old World Visceral leishmaniasis

Visceral leishmaniasis is caused by parasites of the *L. donovani* complex. In visceral leishmaniasis, the disease tends to the chronic, and children especially are affected. The common symptoms are fever, malaise, shivering or chills, weight loss, anorexia and discomfort in the left hypochondrium. Cough and diarrhoea occur less frequently. The common clinical sign are non-tender splenomegaly, with or without hepatomegaly, wasting and pallor of mucous membranes; lymphadenopathy may be present. In West Bengal, lymphadenopathy may be the only clinical manifestation. Darkening of the skin of the face, hands, feet and abdomen is often found in India (Chowdhury et al., 1993). Frequently, from Sudan and occasionally from East Africa, the occurrence of a cutaneous nodule or ulcer or a mucosal lesion, containing leishmanial parasites has also been reported (Perea et al., 1991). Cases of visceral leishmaniasis are now more frequently reported from people having some kind of immunosuppression due to acquired immunodeficiency syndrome (AIDS) or chemotherapy or whatever.

2.2 Old World cutaneous leishmaniasis

Clinical features of cutaneous leishmaniasis tend to differ between and within the different geographical regions, reflecting the different species of parasites or the type of zoonotic cycle concerned, as also perhaps due to the
genetically determined responses of the patient. A “classical” lesion starts as a nodule at the site of inoculation. A crust develops centrally, which may fall away exposing an ulcer which heals gradually, leaving a depressed scar with altered pigment. Satellite nodules at the edge of the lesion are common.

2.3 Old World diffuse cutaneous leishmaniasis

Diffused cutaneous leishmaniasis is caused by *L. aethiopica* and is characterized by widely disseminated thickening of the skin in plaques, papules or multiple nodules, appearing on face and exterior surfaces of the limbs and sometimes resembling like lepromatous leprosy nodules.

2.4 New World visceral leishmaniasis

The etiological agent of New World visceral leishmaniasis is *L. chagasi*. The major symptoms are similar to those of Old World visceral leishmaniasis.

2.5 New World cutaneous leishmaniasis

People in endemic areas have indigenous names for some forms of the disease like “Chiclero’s ulcer” and “Pin-bois” caused by *L. mexicana* and *L. guyanensis* respectively. In Chiclero’s Ulcer, lesions occur generally on the face and ears (60%); mucous membranes are very rarely affected. A significant percentage of ulcerative lesions of the ear cause extensive destruction of the pinna. In Pian-bois, there is
multiple ulcerative lesion in 80% patients; these often metabolize along the lymphatic channels producing multiple ulcers, which can stimulate sporotrichosis, spontaneous healing is extremely rare, and relapses are frequent.

Primary cutaneous lesions are caused by *L. braziliensis* where lymphatic spread and involvement of lymph glands are frequent, and spontaneous healing is rare. Reinfection is not common but it may occur; some patients develop a second cutaneous lesion at a different site after their primary infection has healed. Mucosal involvement is the most serious complication.

2.6 New World diffuse cutaneous leishmaniasis

New world diffuse cutaneous leishmaniasis is clinically and pathologically similar to Old World dermal cutaneous leishmaniasis. It has been associated only with membranes of the *L. mexicana* complex.

2.7 New World mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis is caused by *L. braziliensis* or *L. panamensis*. The characteristic of mucocutaneous leishmaniasis caused by these species is its propensity to metabolize the mucous tissues by lymphatic or haematogenous dissemination.

3. Life Cycle

The parasite has two stages in its life cycle, the amastigote form occurring in the mononuclear phagocytes of
the reticuloendothelial system of the mammalian host and the promastigote form occurring in the digestive tract of sandfly vector, Phlebotomus sp. The amastigote of L. donovani is a small non-flagellated spherical or ovoid body 2 to 4 μm in length and 1-2 μm in width. It contains a central nucleus, a rod shaped kinetoplast and a rudimentary non-functional flagellum (Zukerman and Lainson, 1977; Chang, 1983). The promastigotes are long slender spindle-shaped bodies, measuring 15 to 20 μm in length by 1 to 2 μm in breath. Kinetoplast lies transversely near the anterior end, the nucleus is situated centrally. The flagellum may be of the same length as the body or even longer, projecting from the front. The flagellum does not curve round the body of the parasite and therefore there is no undulating membrane (Chatterjee, 1982).

The amastigote form while residing in the cells of reticuloendothelial system, multiplies by binary fission. Multiplication goes on continuously till the cell becomes packed with the parasite. The host cell is thereby enlarged and eventually ruptures. The parasites liberated as a result of the rupture into the circulation, invade fresh cells and the cycle is repeated. In this way the entire reticuloendothelial system becomes progressively infected. In the blood stream, some of the free amastigotes are phagocytosed by the neutrophilic granulocytes and
macrophages. A blood sucking fly draws these free amastigote forms as well as those within the macrophages during its blood meal. These amastigote forms develop into promastigote forms which again multiply by binary fission producing an enormous number of flagellates. Multiplication proceeds in the mid-gut of the sandfly and the flagellates tend to spread forward to the anterior part of the alimentary canal. A heavy pharyngeal infection of the sandfly is usually observed between the 6th and the 9th day of its infective blood meal. The transmission is thereby effected through the bite of the infected sandfly (Chatterjee, 1982).

4. In vitro Cultivation

The parasites, Leishmania, have been cultivated, in vitro, in liquid, semisolid or biphasic media enriched with blood or serum. The first culture medium of citrated blood for L. donovani was reported by Rogers in 1904. The NNN medium is a modified form given by Novy, Mac Neal and Nicolle (Nicolle, 1908a, b). This medium has been used in clinical laboratory practice for the last eighty years. It consists of blood agar and distilled water without peptone and beef extract.

In 1928 Mayer and Ray used a defined Noller medium for cultivation of various leishmania parasites (Ray, 1932). This medium contained agar-agar and slightly alkaline Liebig's broth. Ray's medium developed in 1932 is still very popular
because it is relatively simple in composition and, as such, easy to prepare and preserve. It contains an energy source, glucose, amino acid source, peptone, and an osmotic pressure stabilizer, sodium chloride.

Another medium successfully employed for isolation and maintenance of various *Leishmania* species is blood beef agar and Locke’s diphasic ‘NIH medium’ of Tobie et al, (1950) as modified from Salle and Schmidt (1928) and Senekje (1939). The NIH medium contains 30% defibrinated rabbit blood and is used for isolation of parasite from infected humans and other mammals.

Contella (1968), Borysia (1968) and Clinton (1969) removed the cellular and other particulate components of blood by centrifugation or filtration through millipore filters, in order to get a liquid medium with high yield of clean promastigotes for antigen preparation and biochemical studies. Further modification of NIH medium was carried out by using 50 gms of bacto-beef instead of 25 gms and Hank’s Balanced Salt Solution instead of Locke’s Solution (Decker-Jackson and Honigberg, 1978). A few semi-defined and non-defined media, such as brain heart infusion (BHI) (Gaughan and Krassner, 1971) and NIH blood agar medium (Tobie et al, 1950) are also widely employed for obtaining large number of parasites required for metabolic studies. Semi or non-defined media are often used because they support the growth
more than the chemically defined media (Berens et al, 1976).

*L. donovani* and *L. braziliensis* have been successfully grown on defined culture medium (REI) (Steiger and Steiger, 1976). Cultures of *L. donovani* in REI medium are initiated with amastigotes from infected hamster's spleen or from promastigotes initially maintained in REI + 10% fetal bovine serum. The generation time of *L. donovani* and *L. braziliensis* is reported as ~21.5 and 25 hours. The mean maximal cell yields are generally obtained on day 6 (Stationary phase) ~ 6x10⁷/ml for *L. donovani* and 3.5x10⁷/ml for *L. braziliensis*. These parasites can also be grown in medium REI + 2.5 to 10% inactivated dialyzed fetal bovine serum. This medium was also found to support excellent growth of *Trypanosoma rhodisiense* and *T. brucei* (Steiger and Steiger, 1976).

Another defined medium (HOSMEM – II) is a modification of semi-defined HOMEM medium. The 10% fetal calf serum component of HOSMEM was replaced with vitamins, purine base, albumin mixture based on the defined medium REI (Steiger and Steiger, 1976), and H x 25 (Cross and Manning, 1973). The HOSMEM- II supports continuous growth after repeated subcultures. The medium is easy to prepare compared to REI medium. The growth characteristics of the parasite in this medium are identical to those reported for HOSMEM (Berens and Merr, 1978).
Hendricks (1975) reported the development of a simple culture method using monophasic medium. Eleven haemoflagellate species including *L. braziliensis* and *L. donovani* were cultured in the tissue medium to which 30% fetal calf serum was added. The tissue culture medium contained 199 Grace's insect tissue culture medium and Schneider's Drosophilla medium. Schneir's insect culture medium, however, was simpler to use and was found helpful in the diagnosis of cutaneous leishmaniasis. It was shown to be more successful than NNN medium (Hendricks and Wright, 1979). The Schneider's insect culture medium with 30% (v/v) heat inactivated fetal bovine serum plus 100 I.U./ml penicillin and 100 µg/ml streptomycin was simpler to use in the diagnosis and management of visceral leishmaniasis in Kenya (Hockmeyer et al, 1981).

NNN medium overlayed with Schneider's medium was found more sensitive than either NNN or Schneider's medium alone for culturing *L. donovani*, especially from splenic aspirates (Githure et al, 1984). This medium was also found more sensitive than Schneider's medium alone for detecting *L. donovani* in cultures made from peripheral blood (Chulay et al, 1985).

For cultivation of *L. donovani*, Rassam and Al-Mudhaffar (1980) used Panmede medium (Paines and Byrne Ltd.). It is composed of glucose, NaCl, KCl, distilled water, fetal calf
serum with penicillin and streptomycin sulphate. Ray and Ghose (1980) developed a liquid culture medium (LDLC - II) for bulk cultivation of *L. donovani* promastigotes, *in vitro*. This medium was found to be relatively easy to prepare, yielding a number of parasites \(4.3 - 10^5 \times 10^7/\text{ml culture}\). The parasites grown in this medium were found free of any contamination and appeared suitable for immunological and related studies on leishmaniasis. The LDLC - II medium is composed of peptone, Hank's balanced salt solution, liver extract, normal rabbit serum and rabbit blood cell lysate to which streptomycin and penicillin are added. The culture time is around 10 -13 days.

5. The Parasite antigen

Parasite antigens are employed in the laboratory for various purposes such as vaccination, immunodiagnosis, analysis of immunopathology, and for quantitation of various immune responses in infected, vaccinated, or naturally resistant hosts. In the absence of defined antigens, little progress can actually be made towards understanding the immunological aspects of the host-parasite relationship. Hybridoma technology has provided a powerful new tool for the analysis and identification of parasite antigens (Mitchell and Cruise, 1981), and hopefully the use of recombinant DNA techniques for the production of protein antigens will be a major activity in the next decade.
Cook et al, 1969, have reported that Leishmania parasites constitute an antigenic mosaic. They demonstrated 11 antigens in a lysate of L. mexicana. Similar results were obtained with L. donovani by Clinton et al, 1972. Dwyer (1976) has shown in his antibody cross absorption experiments that both promastigote and amastigote stages share common or cross reacting surface membrane antigens. Each parasite form is known to contain unique-stage-specific, surface membrane antigens. Alongwith these cross-reacting surface antigens, four antigens common to promastigote and amastigote forms are also known to exist. These antigens were detected in cell free extracts of L. donovani (Simpson, 1968; Ghatak et al, 1982).

Subcellular fractions of cell-free extracts showed that the maximum amount of such common antigens are present in the soluble supernatant. These supernatants were found to contain three types of antigens distinguished on the basis of their thermostability. These antigens donot share antigenic relationship with Mycobacterium and blood group antigens (Ghatak et al, 1982).

Furthermore, Clinton and his colleagues recognized for the first time the existence of a soluble factor in the used medium from cultures of L. donovani promastigotes. This excreted factor (EF) was shown to be immunologically active in sensitized guinea pigs, as it produced a delayed skin
reaction and was capable of precipitating anti-Leishmania antibodies raised in rabbit against living homologous promastigotes (Schnur et al., 1972a). Related and cell-membrane-bound carbohydrate determinants have been cited as being important constituents of leishmania parasite (Jaffe et al., 1990, McConville et al., 1990; Singh et al., 1990, Dc Majumdar, 1992). Surface carbohydrates of leishmania promastigotes are important for attachment to the macrophages (Blackwell, 1985; Turco, 1988) and sand fly digestive tract (Molyneux et al, 1986). Panels of lectins have revealed inter and intraspecific differences in promastigote surface sugar moieties (Schottelius, 1982; Schnur and Jacobson, 1989). Released carbohydrates have also been shown to vary antigenically at the inter and intraspecific levels, using polyclonal and monoclonal antibodies (Schnur, 1982; Greenblatt et al., 1983).

The EF of L. mexicana was used as an antigen for coating wells for modified ELISA and sheep red blood cell agglutination assays. The ELISA as tested against sera of LD positive patients was found much more sensitive than tanned red blood cell haemagglutination test. The EF also showed some cross reaction against sera of patients with malaria and amoebiasis. Thus the crude EF of L. mexicana which can be obtained in large quantities can serve as a suitable and convenient antigen for large scale epidemiological surveys (Arora et al., 1985). Moreover, the existence of leishmanial exoantigens was detected by positive immunoperoxide staining.
in the cytoplasm of Giant cells (infected with *L. major*) and non-parasitized epithelial cells (Sells and Burton, 1981).

More recently, Jacobson and Schnur (1990) have shown that promastigotes derived from the same clone of a well documented strain of *L. major* (LRC-L137) exhibit different carbohydrate configurations on their surface membranes and excrete different amounts, and possibly “types” of glycoconjugates in liquid and diphasic medium.

The preparation of Leishmania antigen has been made in a diversified way according to their use and need, like some investigators have used whole parasite antigen, some particulate and some soluble antigen fractions (El-Amin et al, 1985; Obaid et al, 1989; Scott et al, 1987). For preparing freez-thawed antigen, the promastigotes from the log phase are first suspended in HBSS and frozen at -70°C and then thawed at 37°C. This procedure is repeated for three times. The criterion for the determination of promastigote death is the absence of parasite motility (Dwyer, 1976; Decker-Jackson and Honigberg, 1978; Cook and Holbrook, 1983).

The soluble and particulate antigen fractions are prepared by repeated centrifugation and washing of promastigotes followed by osmolysis in a sterilized distilled water at room temperature. The lysed suspension is centrifuged at 12000 x g for 30 min in a refrigerated
centrifuge (Jalees et al, 1981). Another method used by our laboratory (Obaid et al, 1985) utilizes the osmolysed parasite suspension, which is further centrifuged at 105,000 x g for one hour, to obtain soluble and particulate antigen fractions. Subcellular fractions are obtained by successive centrifugation of the cell free extract at 2000 x g for 10 min, 12000 x g for 15 min and 105,000 x g for 60 min. The sediments in this method include cell debris, nuclear fraction, mitochondrion, golgi apparatus etc.

The detergent-free soluble fraction of promastigotes was prepared in the following way by Scott et al, 1987. The log phase promastigotes were sonicated in Tris buffer (pH 8.0) with protease inhibitors and centrifuged at 100,000 x g for one hr. The soluble fraction was then collected and dialyzed against phosphate buffered saline. Since this antigen preparation was made to assess the protective immunity against *L. major* in BALB/c mice, therefore the candidate immunogens within the soluble antigen fraction were fractionated by anion exchange chromatography on Mono Q column where nine molecular fractions were obtained. Fractions 1 and 9 were capable of inducing protective immunity.

More recently, a method for rapid purification of amastigotes of *L. major* and *L. donovani* was described by Glaser et al, 1990. They claim that this method is very
simple, gentle, rapid and gives complete purification of amastigotes of \textit{L. major} and \textit{L. donovani} from mouse lesions and hamster spleens, respectively.

\textit{L. major} amastigotes were purified from tail lesions in nude mice approximately after 4 to 4.5 weeks of infection. About four lesions were removed in 10 ml PBS-EDTA, Glu (PEG) buffer at 4°C (Jaffe et al, 1984). The PEG containing the lesions was poured over a fine wire mesh and a single cell suspension was made by gently forcing the material through the mesh with the flat end of a plunger from a 10 ml disposable syringe. The cell suspension was transferred to a glass Dounce homogenizer with a clearance of 60-90 \(\mu m\) and the amastigotes were released from host cells with seven thrusts. Centrifugation at 80xg for 5 min at 4°C pelleted large cellular debris. The supernatant was then centrifuged at 1300 x g for 10 min at 4°C to pellet amastigotes. Pellets were resuspended in 10 ml of a fresh solution of ammonium chloride (168 mM) and were incubated at room temperature for 10 min to lyse red blood cells. The ammonium chloride solution was diluted five folds in PEG and the parasites were collected by centrifugation as above. Amastigotes were resuspended in 10 ml PEG per two lesions and were sequentially filtered through polycarbonate filters of pore sizes 8.5 and 3 \(\mu m\) using a gentle vacuum. The vacuum was adjusted to allow a flow of 50 ml aqueous solution through a 25 mm filter with a 3 \(\mu m\) pore size in 30 sec. Host cells and
cell debris were retained on the filters and parasites were collected in the filtrate. Amastigotes were washed twice with PEG before use. In these experiments $10^9$ amastigotes were obtained from five lesions.

The following variations were used for the purification of *L. donovani* amastigotes. PSGEMKA buffer (Hart et al, 1981) was used instead of PEG. Up to two spleens from 8 weeks infected Syrian golden hamsters were excised and diced. The material was transferred to a glass dounce homogenizer in a 30 ml buffer and the parasites were released from macrophages with seven thrusts. After the centrifugation steps, red blood cells were lysed by incubation for 7 min in 0.05% saponin in PSGEMKA at 4°C. After one wash, amastigotes were resuspended in buffer (10 ml per spleen) and were filtered as described for *L. major*.

6. Vaccination Studies

Numerous attempts at experimental immunization against leishmanias have been made. Inocula used for the purpose included parasite materials such as ultrasonicated promastigotes (Preston and Dumonde, 1976), dead promastigote associated with adjuvant (Holbrook and Cook, 1981), promastigotes without an adjuvant (Holbrook and Cook, 1983), Crude antigen-antibody complex (Greenblatt, 1980), soluble antigen (Scott et al, 1987), live promastigotes McGurn et al., 1990; Rivier et al., 1993) and chemically mutagenized avirulent
promastigotes (McGurn, et al, 1990). But satisfactory protection using any of the above formulation has not yet been achieved. However, 94% protection was observed with two intraperitoneal injections of $5 \times 10^7$ irradiated (150 Krad) promastigotes, the animals were capable of completely healing or controlling a challenge infection with *L. major* (Scott et al, 1987). Here the soluble antigen was prepared by centrifugation of sonicated log phase promastigotes at 100, 000 x g for 4 hr. To identify the candidate immunogen within soluble antigen, the soluble antigen was further fractionated by anion exchange chromatography. In these experiments fraction 9 was most protective. This fraction contained a highly negative charged group of molecules, which represented less than 1% of the total protein found within fraction 9.

An important question in vaccine development is how to accomplish preferential induction of the right antigen. Specific, CD4$^+$ T cell and CD8$^+$ T cell subsets were observed to enhanced the antileishmanial activity of macrophages. Whilst receptor-mediated endocytosis of parasite antigens might readily lead to processing in acid endosomes and binding to major histocompatibility antigen complex (MHC) class II for presentation to CD4$^+$ T cells, since endocytosed antigen cannot be presented by MHC I molecule. Hence it is clear that antigen-specific CD8$^+$ T cells are generated during leishmanial infection and are important in the curing
response (playfair et al, 1990). The demonstration by Engelborn and co-workers (1990) that IFN-r producing T cells generated during cure of leishmanias are able to recognise a broad range of leishmanial antigens suggesting that even though single molecule vaccines (eg, GP63, LPG) have met with some success in inbred mice, the search for a single major protective antigen for vaccination against leishmaniasis in man may be fruitless. In any case, polymorphism for MHC class I and class II molecule in genetically diverse human populations makes single antigen vaccine unattractive since some molecules may fail to bind the antigen for presentation to T cells. A recombinant vaccine combining T cell epitopes for an array of protein antigens might therefore be more effective.

The Iranian group has admitted using 'leishmanization' because all other attempts to control the disease in some communities had failed. 'Leishmanization' is performed either by exposing certain areas of skin to sandfly bites or by scratching the skin area with material from active lesions. Technique was standardized when methods for culturing promastigotes became available but variability in size and duration of lesions resulting from the inoculation of virulent strains has caused the practice to be discontinued in Israel and Soviet Union. Any way, the spread of human immunodeficiency virus infection and the widespread
use of immunosuppressive drugs mean that prospects for a live vaccine are not good (Greenblat, 1988).

Immunization trials with killed or attenuated leishmania parasites were also carried out in laboratory animals with variable reasons. Lainson and Bray (1964) found that intradermal injection of mice and hamsters with formalin killed *L. mexicana* amastigotes with Freund's complete adjuvant failed to elicit resistance against parasite challenge. Lemma and Coole (1974) reported that injection of eradiated *L. enteriettii* promastigotes in guinea pigs failed to elicit protective immunity against challenge with untreated organisms. In contrast, Countinho (1954) found that injection of killed promastigotes produced a limited degree of protection in guinea pigs against *L. enteriettii*. Particulate fraction of *L. enteriettii* in Freund's complete adjuvant conferred a resistance against challenge in guinea pigs, while cell sap fractions did not stimulate protective immunity (Preston and Dumonde, 1976). Immunogenic enhancement with appropriate immunomodulating agents, which especially enhance the host defense mechanisms, may thus be required to elicit protective immunity.

A number of immunoadjuvants like Glucan (Obaid et al, 1989), BCG (Convit et al, 1987; Costa et al, 1988), Freund's complete adjuvant (Preston and Dumonde, 1976) and *Corynbacterium parvum* (Scott et al, 1987) have been used for
vaccination against Lesihamia with varying success. More recently, BCG has been used as a routine immunomodulator. BCG alters the balance of suppressor to helper cells, permitting greater macrophage activation and leading to rapid clearance of parasite. BCG increases the resistance of murine macrophages to Trypanosoma cruzi as demonstrated by in vitro test (Hoff, 1975). BCG has also been shown to induce complete protection against a fatal strain of Babesia (Clark et al, 1976). But induction of non-specific resistance against protozoal infections using BCG has not been uniform. For example, BCG failed to protect cattle against Babesia. When the strain of mice or species of malaria was varied different results were obtained. The induction of non-specific resistance is a complex process that depends on several characteristics of the 'inducers', the host, and the challenge organisms (Mahmoud, 1982).

Due to success of BCG as an immunomodulator, in Venezuela, Convit et al, (1987) have shown that intradermal inoculation of live BCG together with heat-killed leishmania promastigotes provided effective immunotherapy against localised cutaneous leishmaniasis. Three vaccinations over 32 weeks gave a similar cure rate (94%) to three 20-day courses of meglumine antimonate.
7. Immune Responses

Most clinical and experimental observations suggest that immune resistance to leishmaniasis is linked to cell-mediated immunological processes, although cell-bound antibodies may influence the expression of this cellular response (Bryceson 1970a). In man, the induction of cell-mediated immunity depends upon the immunological competence of the host and virulence of the infectious agent. Since, cell-mediated immune responses persist for many years following recovery from an infection (Wyler et al, 1979), therefore an increasing number of patients with acquired immune deficiency syndrome (AIDS) or other forms of immune deficiency are being reported to suffer from recrudescent leishmaniasis (Badaro et al, 1986; Antunes et al, 1987, Grammiccia et al., 1992). However, one of the immunological hallmarks of visceral leishmaniasis is a remarkable increase in serum immunoglobulins mostly of the IgG and IgM classes (Ghose et al, 1980), causing a reversal of the albumin/globulin ratio (Irunberry et al, 1968; Rezai et al, 1978). Furthermore, most previous studies on visceral leishmaniasis have been carried out in rodent hosts, and although significant advances have been made due to these studies, the precise mechanisms of protective immunity in visceral leishmaniasis remains unresolved and poorly understood (Gross et al., 1992; Frankenburg et al., 1993).
7.1 Cellular Immune Responses

Protection and immunity in leishmaniasis is generally conceded to be predominantly cell-mediated (Garnham and Humphrey, 1969; Zuckerman, 1975), and may be via different mechanisms. Furthermore, there is ample evidence that the mechanism of recovery against subsequent infection also has an immunological basis. The cutaneous ulcer caused by L. tropica usually remains confined to the skin at the site where an infected fly bites. Patients recovering from uncomplicated old world cutaneous infection or from similar infections caused by Leishmania species of the New World, achieve radial cure and acquire solid, long lasting immunity against reinfection by the homologous organisms (Heyneman 1971; Mackelt 1972). Rare cases of reinfection or relapse have been documented (Guirges, 1971), particularly after immunosuppressive therapy. Similarly, spontaneous or drug induced healing of the visceral infection lead to such an excellent degree of immunity that true second attacks are unknown (Manson-Bahr, 1961).

Nacy et al (1981) have demonstrated in vitro killing of macrophage infected L. tropica by lymphokines. Lymphokine treated macrophages developed two potent antimicrobial activities such as increased resistance to infection by L. tropica amastigotes and inhibition of replication and ultimate killing of intracellular parasite. Reed et al.
(1984) also showed that mice infected with *L. donovani* and treated with lymphokine-rich supernatants (encapsulated in liposomes) have significantly fewer parasites in the liver than the control group, demonstrating an *in vivo* activity of lymphokines against an infected organism.

More recently, Sypek and Wyler (1990) using lymph node lymphocytes of *L. major*-infected mice constructed and cloned 2 T-cell hybridomas that could activate macrophages to exert antileishmanial defense *in vitro*. One clone, 1D5, produced lymphokines (including gamma interferon) that induced these affects. Production of macrophage activating lymphokines and the protective effect of 1D5 were suppressed by the addition of Cyclosporin A to cultures. The other clone, 1B6, produced no detectable macrophage activating lymphokines, and its protective activity/ability was not suppressed by Cyclosporin A. Moreover, neither clone was cytotoxic to the infected macrophages.

Mouse peritoneal macrophages were infected with Leishmania parasites from different species and were exposed to different electron carriers like methylene blue (MB), toluidine blue (TB), phenazine methosulphate (PMS) and Crystal violet (CV). This led to killing of the intracellular parasites with no harm to macrophages. On a molar basis, the potency of electron carriers decreased in the following order: CV, TB, MB and PMS. MB and TB were more active against
intracellular compared to free parasites, suggesting that the macrophages themselves might play a role in the observed anti-parasite toxicity. No difference could be detected between macrophages from different mouse strains as regard to their capacity to kill intracellular parasites upon incubation with electron carriers. When macrophages from *L. tropica* susceptible (non-healer) BALB/c mice strain were infected with either *L. enriettii* or *L. major*, and then exposed to an activating lymphokine rich supernatant, destruction of only *L. enriettii* was achieved whereas *L. major* survived intracellularly. Incubation with MB, however, led to intracellular destruction of both parasites. Other Leishmania species could also be killed irrespective of the genetic background of the macrophages. These observations suggested that the triggering events in electron carrier and lymphokine mediated intracellular killing were different (Mauel 1984).

The destruction of parasites within macrophages is presumably mediated by toxic metabolites of oxygen, which may induce the superoxide anion (*O₂*), hydrogen peroxide (*H₂O₂*), singlet oxygen (*O₂*), or hydroxyl radicals (*HO*) (Johnston 1978). This idea has been well documented in the case of *Toxoplasma gondii* infecting mouse macrophages (Murray et al 1979). A similar situation may occur in Leishmania infection, as it is well evidenced by the experiments of some

7.2 Humoral Immune Responses

Ample evidences from clinical and experimental studies are available to suggest that antibodies are capable of inducing parasite phagocytosis by macrophages (Herman, 1980). The humans completely cured of visceral leishmaniasis develop a strong Arthus reaction at the intradermal site where *L. donovani* are reinoculated. Parasites are not recovered at the site of challenge inoculum, and this reaction rapidly disappears (Manson-Bahr 1961). The high titres of class-specific antibodies and the enhanced CMI responses (Dennis 1984) after reinoculation of *L. donovani* into squirrel monkeys are marked with subsequent decrease in parasite densities in the liver.

In studies of Das et al (1989) it was demonstrated that the killing of *L. donovani* promastigotes is antibody mediated. Immune serum raised against flagellar fraction of *L. donovani* isolate, UR6, has profound lethal effect on the in vitro growth of parasite. Lethal effect of immune serum was also examined using two other isolates of *L. donovani*, namely DD8 and AG83. It was observed that immune serum is equally effective against UR6 and DD8, but has no effect on AG83 promastigotes. Furthermore, parasite killing is mediated by Leishmania specific antibodies in the absence of
complement or any other factor present in rabbit serum. These results also indicated that the lethal effect of immune serum was due to impairment of membrane function, leading to inhibition in the uptake of essential nutrients needed for growth and survival of parasites.

Several workers have assayed the elicited humoral immune responses following vaccination of animals (Scott et al., 1987; Jaffe et al., 1990), in order to measure the extent of immune protection afforded by such procedures. The antibodies appear to directly participate as immune effectors in vaccine-induced immunity since high levels of circulating antibodies are detectable after i.p. or i.v. immunizations (Howard et al., 1982). Scott et al. (1987) have detected antibodies against intracellular antigens in BALB/c mice immunized with \textit{L. major} soluble antigens. The soluble antigens were further fractionated in order to identify the candidate immunogen(s) within this antigen fraction. They indicated that antigenic moieties within fraction 9 of the soluble antigen, are recognized by antibodies in the immunized mice, suggesting a definite role of humoral immunity in protection.

In fact, some antibody mediated protection has been demonstrated with monoclonal antibodies that are directed against leishmanial surface antigens (Anderson et al., 1983). Hu et al., 1989 have raised monoclonal antibodies (McAb)
against membrane antigen of promastigotes of *L. donovani* canine isolate.

They reported 3 McAb which inhibit the growth of promastigotes in culture. Monoclonal antibodies such as 2B12-A8 and 2H6-E3 showed strong inhibiting effect in the presence of complement. The inhibiting range fluctuated between 68-95%, and this inhibition was further confirmed by means of macrophage inhibition test. Moreover, the results of ultrastructural localisation of *L. donovani* antigen by protective McAb, 2H6-E3, with immunogold technique showed the gold particles in clumps widely distributed on the outer side of promastigote membrane. According to the high density of localised gold particles, it is suggested that antigen is localised on the plasma membrane.

In visceral leishmaniasis specific antileishmanial antibodies are produced in humans (Badaro et al, 1983; Edrisson et al., 1981), Owl monkeys (Broderson, 1982), hamsters (Campos-Neto and Bunn-Moreno 1982) and mouse (Kripatrick and Farrell 1982). Low IgM and IgG titres of class-specific antibodies have been reported from some cases of human visceral leishmaniasis (Ghose et al, 1980). Also, studies of Dennis et al, 1986 on infected squirrel monkeys showed production of IgM and IgG class-specific antibodies. IgG titres were found to be higher than IgM titres in the plasma from these monkeys.
In human visceral leishmaniasis, the major increase in serum $\gamma$-globulins is nonspecific for Leishmania spp. No correlation is observed between the increase in serum $\gamma$-globulins and the production of specific antileishmanial antibodies (Zuckerman 1975). However, in studies of Dennis et al., 1986 on infected squirrel monkeys, a correlation was noted between increased $\gamma$-globulin levels and raised levels of immunoglobulins (IgM and IgG) but not between $\gamma$-globulin concentration and specific antileishmanial antibodies. Conversely, a correlation was observed between increased levels of $\gamma$-globulin (immunoglobulin), IgM and IgG levels, and the production of IgG class specific antileishmanial antibodies in squirrel monkeys challenged with *L. donovani*. Much of the increased IgG appears to be specific to *L. donovani*. The study was used to compare the primary infection against challenged animals.

8. Macrophage - Parasite Relationship

Intracellular parasitism is a common feature of several parasitic protozoa that cause tropical disease. A remarkable example is *Leishmania*, whose amastigote stage exclusively parasitizes macrophages (Mononuclear phagocytes), which is one of the principal cell types responsible for cellular defense against invading microorganisms. The ability of amastigotes to live in macrophages is due to an intracellular symbiosis of leishmania. For establishing such intracellular
symbiosis for sequential events are important: recognition, intracellular entry, survival and multiplication (Moulder 1979b). *Leishmania* - macrophage interaction has been studied by infecting eritoneal macrophages *in vivo* and *in vitro* with *L. donovani* (Olivier and Tanner, 1887; Olivier et al, 1989, Lytton, Mozes and Jaffe, 1993).

The binding of leishmania to macrophages was studied *in vitro* by light microscopy. It was found that a large number of promastigotes become attached to the cells in relatively short time (Chang, 1981a). This binding was suggested to be mediated by discrete membrane molecules but not through non-specific interaction (Chang, 1979). It was also found that binding of *L. donovani* promastigotes to hamster peritoneal macrophages is a ligand receptor interaction involving antigenic surface membrane proteins. Thus the receptor mediated gains entry into macrophages (Chang 1981a).

The entry of leishmania parasites into macrophages depends on the phagocytic activity of these cells and the motility of the organisms. There is no specific orientation during this entry (Chang, 1979). A number of studies suggested that parasites reside within a vacuole of host cell origin, and that lysosomes do fuse with these parasitophorous vacuoles soon after infection (Alexander and Vickerman, 1975, Chang and Dwyer, 1976; 1978).
Chang and Fong (1983) have also reported similar findings from \textit{L. donovani} hamster macrophages in an \textit{in vitro} system. Amastigotes are first lodged in phagosomes which are subsequently fused with the secondary lysosomes. The ability of \textit{leishmania} parasite to live in the secondary lysosomes of the macrophages may be due to certain intrinsic structures and dynamic properties of the parasite plasma membrane. \textit{Leishmania} parasites undergo plasma membrane related changes on entry into macrophages at the morphological, antigenic and molecular levels. Most of these changes probably reflect necessary steps for the transition of parasites from an extracellular to an intracellular life.

It was also proposed that survival of \textit{leishmania} parasites in macrophages must be based on the resistance to enzymatic degradation into lysosomes and to the microbicidal factors present in the intracellular macro-environment (Chang, 1983). There may be two possibilities for the resistance of \textit{leishmania} to lysosomal enzyme degradation: (I) \textit{Leishmania} may have a naturally resistance surface to lysosomal enzyme degradation (Chang and Dwyer, 1976). (II) \textit{Leishmania} may release factors which act as enzyme inhibitors (El-On et al, 1980; Theodo's et al., 1991), or change the intralysosomal pH (Coombs, 1982).

Studies related to the infection of various inbred strains of mice by \textit{L. donovani} have demonstrated that innate
resistance to this intracellular parasite seems to be controlled by a single gene (Lsh), which maps on chromosome 1 (Bradley et al, 1979). Many in vivo studies have demonstrated that T cells are ineffective in regulating the expression of this gene, which also controls infection by other intracellular pathogens (Bradley and Kirkley, 1972; O'Brien and Metcalf, 1982; Skamena et al, 1982; Hoermache et al, 1983; Ulezak and Blackwell, 1983). T-lymphocytes have not been shown to have any regulating effect on the expression of the Lsh gene. But the importance of T cells in the elimination of L. donovani in C57BL/c mice has been well documented by Skov and Twohy (1974). Recently, Reiner and Finke (1983) and Reiner (1987) showed that the production of interleukin (IL)-1 and IL-2 by BALB/c mice cells is inhibited during infection in in vitro and in vivo systems Murray et al. (1987) have reported that the infection reduces the capacity of animals to produce interferon (IFN)-gamma. These findings clearly demonstrate that the infection while suppressing several immunological functions of macrophages permits the establishment of the parasites. Murray et al. (1982) have also shown that level of parasitism seems to be inversely related to the ability of the organisms to stimulate the production of macrophage activating lymphokines by T cells.
9. Pathology

Despite the wide range of manifestations seen in leishmanial infections, all clinical and geographical varieties of the disease share a common histological feature, namely, the early accumulation of mononuclear phagocytic cells (or hyperplasia) in the invaded tissue. The dermatologic species induces an initial histiocytoma in the skin, while the viscerotropic species induces hyperplasia of reticuloendothelial cells of the organs invaded. The pathological changes that characterize the various clinical forms of the disease reflect the balance between parasite multiplication, the immune response of the patient and the resultant degenerative changes (Report of WHO Expert Committee, 1990). Visceral leishmaniasis, kala-azar, occurs in all age groups. It is characterized by fever, anaemia, reduced white cell count, wasting, splenomegaly and serum protein imbalances. It usually ends up fatally, if remains untreated. In Indian kala-azar, a post kala-azar dermal leishmaniasis occurs in some individuals after treatment (Zuckerman and lainson 1977).

Visceral leishmaniasis may be manifested as a mild, self limiting, asymptomatic or oligosymptomatic infection, and only a small fraction of infected individuals develop progressive disease (Gutierrez et al, 1984). Self limiting infections are followed by a long lasting immunity. As long
term immunity requires the presence of antigens, it is believed that Leishmania (or some antigens thereof) must persist for long time following the original infection. The hypothesis that live leishmanias persist in apparently normal individuals is supported by the observation that increasing number of immunosuppressive patients, such as those of AIDS or those undergoing immunosuppressive therapy for cancer, are also found to suffer from visceral leishmaniasis with no known history of the disease. Hence, a state of premunition (balanced immunity and concurrent infection) apparently exists following the initial infection, and the depression of protective immunity shifts the balance in favour of parasite. On the basis of these observations, along with the demonstration of live parasites in mouse long after spontaneous healing of the lesions, it has been suggested that in certain cases visceral leishmaniasis may be considered as an opportunistic infection (Report of WHO Expert Committee, 1990).

A histiocytic proliferation with increasing parasite load is responsible for the early phase of hepatomegaly. The addition of lymphoplasmacytic elements aggravate the situation of the later stages. Histopathological changes of these organs involve necrosis apparently due to the lysis of parasitized histiocytes and granuloma formation which, as in cutaneous leishmaniasis, may be a post-necrotic phenomenon.
All these changes apparently result from host immune responses (Mauel and Behlin 1982).

Pancytopenia is particularly marked by a reverse haemolytic anaemia which is considered to be the most important contributory factor for the fatality of kala-azar. Thrombocytopenia and leucopenia may be moderate to severe. The actual mechanism triggering these haemocytopathic changes is not well understood, hypersplenism and lysis through an autoimmune reaction have been proposed (Woodruff et al, 1972; Musumeci et al, 1974a; Russo 1977). Isotopically labelled red cells and platelets were shown to have reduced life span in kala-azar patients and hyperactivity of the spleen was thought to be responsible for their destruction. But the rapid reversal of haemolytic activity by antimony treatment is not quite compatible with this idea, since antimonials donot suppress haemolytic activity in the anaemic conditions such as thalassaemia, in which incureased activity of the reticuloendothelial system is also very evident (Musumeci et al, 1974b).

The histopathology of different organs involved in visceral leishmaniasis has been described by many authors (Ritterson 1955; Duarte et al, 1983; Duarte and Corbett 1984, 1987; Gutierrez et al 1984; Correa et al., 1992), but little is known about the cutaneous changes which occur after parasite inoculation by the sandfly. Susceptibility to lysis decreases during growth.
of the organisms in cultures or in the sandfly. The chances that leishmania parasites will enter the target cells are greatly increased because they bind to target cells at multiple receptor sites. It has been shown that the salivary glands of sandflies contain potent inflammatory agents which enhance the infectivity of promastigotes in the mouse (Report of WHO Expert Committee 1990). Finally, infective macrophages have been shown to produce colony stimulating factors which stimulate precursor cells thereby providing new target cells for the parasite.

The most important immunopathological feature of kala-azar is haemolytic anaemia (Aksoy et al, 1970; Bray and Wilson 1972), in which the life span of erythrocytes is reduced during an active febrile disease (Woodruff et al, 1970; 72). The anemia is thought to be of an autoimmune nature. Red cell destruction occurs mainly in the spleen. The splenectomized patient is not generally anemic (Woodruff et al, 1972; Woodruff 1973). Other changes include thrombocytopenia and prothrombin depletion, intestinal ulceration, myocardial damage, amyloidosis and cloudy swelling of the liver (Pampiglione et al, 1974). Leucopenia due to absolute reduction in all forms of granulocytes with moderate increase in lymphocytes and monocytes was found as a usual feature in kala-azar (Aikat et al, 1979; Manson-Bahr 1971).
The report of WHO Expert Committee (1984) explains that hypoalbuminaemia is associated with oedema and other features of malnutrition. In the advanced stage, intercurrent infection such as pneumonia, dysentery and tuberculosis are quite frequent. These are usually the common causes of death in leishmaniasis. However, experimental leishmaniasis in golden hamsters results in splenomegaly and hepatomegaly. Maximum average enlargement of the liver is, however, comparatively of a lesser order than the spleen. Anemia and leukopenia with marginal lymphocytosis, and without any concurrent increase in monocytes are some other features (Jalees et al 1981).

10. Drug Therapy

Although significant advances in the Chemotherapy of leishmaniasis have been made, but a large number of chemotherapeutic agents currently available have a number of drawbacks such as high toxicity, including a significant number of chemotherapeutic failures (Steck, 1972, 1974). Recently, the TDR leishmaniasis unit has documented the first ever large-scale clinical resistance of kala-azar to antimonial drugs. Clinical cases resistant to antimonials are reaching alarming proportions in India. There are indications from the state of Bihar in India, that about 10000 cases were unresponsive to antimony treatment out of 200 000 reported (TDR news, 1990). The availability of better
and newer chemotherapeutic or immunotherapeutic agents is obviously required for the treatment of infections caused by Leishmania parasites.

**Pentavalent antimony in the form of sodium stibogluconate (Pentostam) or meglumine antimonate (Glucantime)** is the chief chemotherapeutic agent which has been used since approximately 1945 (Berman and Grogl, 1988). Extensive studies have been carried out on different aspects of these pentavalent antimonial drugs. Convit et al., 1987 have reported 94% cure rate in humans by three 20-day courses of meglumine antimonate.

White et al., 1989 have indicated that the opossum is an animal model which is markedly susceptible to visceral leishmaniasis (Hanson et al., 1980). Due to this marked susceptibility, chemotherapy studies were performed to determine the efficacy of meglumine antimonate and WR 6062, an 8-aminoquinoline derivative, in the treatment of visceral leishmaniasis and, to evaluate the potential of this host in the chemotherapy of visceral leishmaniasis. These results indicated that both the drugs were effective in the suppression of amastigotes in the liver and spleen of opossums. Despite the marked suppression of parasites in the liver and spleen of infected opossums, the experimental disease was fatal in all the infected animals inspite of the therapy.
Pentamidine is clinically effective but has been regulated to the status of a secondary agent because of toxicity. However, pentamidine is a widely employed antimicrobial because it is also effective in *Pneumocystis carinii* infections and in African Trypanosomiasis. Both Pentostam and pentamidine are more active against intramacrophage amastigote than extracellular promastigotes (Berman et al., 1980, Thakur et al., 1993). Both these drugs, in general, are more active against amastigotes than mammalian cells.

Uptake of pentostam by cells can be via simple diffusion, facilitated diffusion, or active transport. The experiments of Berman et al., 1987 have demonstrated that amastigotes, compared to promastigotes and mammalian macrophages, concentrate more pentostam, suggesting a mechanism that may contribute towards the toxicity of these drugs to amastigotes compared to promastigotes or mammalian macrophages.

Darouti and Rubaie (1990) used a combination of intralesional stibogluconate infection and superficial cryotherapy in an attempt to improve the therapeutic efficacy of either of these two modalities when used in the treatment of cutaneous leishmaniasis. This combined therapy resulted in a 100% cure rate in 15 patients with 23 lesions of cutaneous leishmaniasis. The results obtained by combined superficial freezing and intralesional stibogluconate
injection were much more impressive than those obtained by each of the two modalities used singly.

Although it is generally accepted that pentavalent antimonials are the standard first-line drugs, there is much variation in their use. The two pentavalent antimonials, sodium stibogluconate and meglumine antimonate are chemically similar and their toxicity and efficacy in visceral leishmaniasis are thought to be related to their content of pentavalent antimony ($\text{Sb}^{5+}$). Meglumine antimonate solution contains about 8.5% $\text{Sb}^{5+}$ (85 mg/ml), whereas sodium stibogluconate solution contains about 10% $\text{Sb}^{5+}$ (100 mg/ml). Treatment with antimonials is well tolerated, but if serious side-effects arise it may be prudent to interrupt the course of treatment temporarily. If relapses occur, patients should first be treated again with an antimonial. If the response is still unsatisfactory, or in cases of primary unresponsiveness, the second-line drugs are amphotericin B and Pentamidine (Report of WHO Expert Committee, 1990).
AIMS AND OBJECTIVES

Leishmaniasis is a parasitic disease that poses a major health problem in a large number of countries. Around the world some 350 million people per year are at risk of acquiring the infection, whereas approximately 12 million people are already infected. Though enormous efforts have been made to eradicate the leishmanial infections through vaccination and chemotherapy but progress on this front is still not very satisfactory. On the one hand vaccines currently tested against several forms of leishmaniasis has so far shown no promise to rely upon, while on the other hand the chemotherapeutic agents commonly used for combating the disease have a number of side effects including toxicity and immunosuppression. In view of the rather gloomy future, the only alternative is that the search for a new candidate vaccine(s) must continue.

Present research represents one such step in that direction, where a preliminary effort has been made to search/identify some candidate vaccine(s) against visceral leishmaniasis.

The experimental details of the proposed plan of study included work involving some of the following aspects:

2. Isolation and purification of different antigenic fractions: whole, soluble and particulate antigen isolates.

3. Biochemical characterization of antigenic fractions by estimating their protein, carbohydrate and DNA concentrations. Immunologic characterizations were carried out by means of IFA, IHA and ELISA.

4. Physico-chemical characterisation/identifications were carried out by determining their molecular weights in SDS PAGE.

5. Vaccination studies were carried out by immunizing golden hamsters with purified and characterized whole, soluble and particulate antigens through intraperitoneal routes.

6. Various antigen fractions were immunopotentiated and then used for immunization of golden hamsters with purified whole, soluble and particulate antigen fractions in association with BCG (2 x 10^6 Bacille Calmette-Guerin).

7. Assessment of Humoral immune responses were carried out by means of IHA and ELISA assay techniques.

8. Immunoglobulin levels were assayed in immune and hyperimmune sera by using various radial immunodiffusion techniques.
9. The cell-mediated immune responses were also assayed in immunized and immunopotentiated animals by making use of *in vivo* DTH responsiveness or refractoriness.

10. Protection studies in several groups of experimental animals were carried out by challenging the test animals with fatal doses (1 x 10^6 promastigotes) of *L. donovani* (NICD: C II Strain).

11. Detection of humoral immune responses through assessment of antibody titres by means of IHA and ELISA were subsequently carried out on post challenged animals.

12. Post challenged animals were also tested for estimating their immunoglobulin levels.

13. Cell mediated immune responses were again assayed in the above animals by employing DTH responsiveness and macrophage migration inhibition tests.

14. The level of lysozomal enzymes (Acid Phosphatase) were estimated in the macrophages of immunized/immunopotentiated animal groups.

15. Therapeutic efficacy of the drug, Stibogluconate, was also tested by using smaller/larger doses of drugs in immunized/nonimmunized animal groups.