1. INTRODUCTION
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1.1 General Introduction

Leishmaniases are a group of related diseases caused by the protozoan parasite of the genus *Leishmania* (family: *Trypanosomatidae*). Leishmanial infection is transmitted by the blood sucking sandflies belonging to the family *Phlebotomidae* of the genera *Phlebotomus*, *Lutzomyia* and *Psychodopygus* (Lewis and Ward, 1987). Leishmanial infections are both anthroponotic and zoonotic in nature (Bryceson, 1996). The wild and domestic mammals such as, rodents, edentates or canines, gerbil etc. are the normal vertebrate hosts and also form reservoirs for human infection (Ashford and Bettini, 1987; Shaw and Lainson, 1987). Leishmanial organisms may exist in as many as 30 different species forms of which over a dozen are associated with various clinical forms of leishmaniases (Lainson and Shaw, 1987). The clinical manifestation of different forms of the disease depends primarily on the infecting species of the parasite, genetic background and immunological response of the host, reservoir, vector and their intimate relationship with a particular ecological setting which exists in different zoogeographical areas around the world (Bryceson, 1996). Thus, human leishmaniasis may actually be considered as 'variety of syndromes' instead of a single disease that are quite 'complex' and 'cosmopolitan' (Granham, 1987).

About 12 million people are estimated to be infected with different forms of leishmaniasis and about 350 million
people live in *Leishmania* endemic areas around the world (Modabber, 1993). The problem has been magnified more seriously as many of the leishmaniasis cases have been reported to be associated with the HIV-infection (Bryceson, 1996). The importance of leishmaniasis as a global public health problem justifies its inclusion as one of the major five parasitic diseases in the WHO Tropical Disease Research Programme. Several excellent books, reviews, and articles covering various aspects of leishmaniasis have recently become available (Chang and Bray, 1985; Peters and Killick-Kendrick, 1987; Chang, Chaudhuri, and Fong, 1990; Warren, 1993; Schnur and Greenblatt, 1995; Bryceson, 1996).

1.2 Historical Perspective

The earliest reference on visceral leishmaniasis (VL)- or kala-azar (KA)- like illness dates as far back as in 1824 when 'malarious intermittent fever' with splenomegaly were reported from the Jessore district (now in Bangladesh) (Sanyal, 1985). The disease spread in the epidemic form in Western and Central Bengal during the middle of the nineteenth century (1850-1875) when it was termed as 'Burdwan fever' (Rogers, 1897). Similar type of epidemic fever was prevalent in the Purnia district of Bihar as well as in the hill areas of Assam in India during the period ranging between 1869 and 1900 (Rogers, 1897). The fever was given the name 'kala dukh' (meaning 'black misery' or the 'fatal misery') or kala-azar (meaning 'black fever' or 'fatal
fever') (Ross, 1899). Rogers (1897) observed a close resemblance in the clinical and epidemiological features associated with 'kala-azar' and 'Burdwan fever'. The real cause of the fatal disease kala-azar or Burdwan fever remained unknown till 1903 when Leishman (working in London) and Donovan (working in Madras, India) simultaneously, although independently, reported the identification of the causative organism of the fatal disease kala-azar. The parasite was named as *Leishmania donovani* and included in the genus *Leishmania* (Laveran and Mesnil, 1903; Ross, 1903). Subsequently, similar parasites were demonstrated in infants suffering from a disease resembling kala-azar in the Mediterranean region (Tunisia) and given the name *Leishmania infantum* (Nicolle, 1908a). Chagas (1936) demonstrated that the leishmanial parasites were of quite common occurrence in the liver of patients suffering from yellow fever in South America. The parasite was incriminated as the causative organism in South American kala-azar and named as *Leishmania chagasi*. Existence of kala-azar in Africa (Neave, 1904), China (Marchand, 1904; Aspland, 1910) and Paraguay (Migone, 1913) was also reported earlier.

The existence of cutaneous forms (oriental sore-like illness) of leishmaniasis was probably known to the medieval Moslem scholars (Mohammad and Muna Al-Taqi, 1981). However, it was not until the early 20th century that *Leishmania tropica*, the causative agent of oriental sore was identified (Wright, 1903; Luhe, 1906). *Leishmania braziliensis* (Carini,
were reported from different parts of the world as the causative agents of mucocutaneous leishmaniasis.

U. N. Brahmachari (1922) in India first reported 3 human cases having non-ulcerative nodular skin lesions in which *L. donovani* was demonstrated. These persons had earlier suffered from kala-azar and were fully or partially treated with antimony drug. Brahmachari termed this phenomenon as 'dermal leishmanoid'. Later on, Action and Napier (1927) termed this clinical condition as 'post kala-azar dermal leishmaniasis'.

Rogers (1904) first successfully cultured *L. donovani in vitro* at 22°C in citrated salt solution. The culture form (leptomonad or promastigote) of the parasite was elongated in shape, contained a polar flagellum and differed from the intracellular leishmanoid form (amastigote) of round or oval body having no flagellum (Fig. 1.1). Subsequently, leishmanial parasites were cultured in 'NNN' medium, a medium developed by Novy, McNeal and Nicolle (1908b).

The mode of transmission of leishmanial infection received considerable attention from earlier workers in this field (Patton, 1907; Basile, 1911; Franchini, 1911; Mackie, 1914; 1915). Napier (1925) noted a correlation between the distribution of the sandfly *Phlebotomus argentipes* and kala-azar in India. Sandflies were found to be readily infected when fed on kala-azar patients (Knowles et al, 1924; Christopher et al, 1925). Finally, Swaminath et al (1942) successfully transmitted leishmanial infection to human
Fig. 1.1 Ultrastructure of *Leishmania donovani*. (A) promastigote and (B) amastigote.
volunteers by the bites of *P. argentipes* preinfected with *L. donovani*. Indian Kala-azar Commission had earlier identified the sandfly *P. argentipes* as the possible transmitter of Indian kala-azar (Report of the Indian Kala-azar Commission, 1932). Studies carried out elsewhere implicated *Phlebotomine* sandflies as the possible vector for infection caused by other *Leishmania* species (Sergent et al, 1921; Ar gao, 1922; Adler and Theodor, 1926). In fact, Adler and Ber (1941) transmitted 'L. tropica' (probably *L. major*) to man by the bite of infected *P. papatasi*.

A trivalent antimonial, tartar emetic was first used by Vianna in 1912 in Brazil (Bryceson, 1987) and, simultaneously, in India to treat the KA cases with undesirable side effects. A remarkable advance in the fight against the fatal disease KA followed through the introduction of a pentavalent antimony compound ‘urea stibamine’ discovered by Brahmachari in 1922. Treatment with this compound reduced the mortality rate from about 95% in untreated cases to less than 5% in the treated cases.

1.3 Review of the Literature

Different species of the parasite, disease spectrum and geographical distribution

Different species of *Leishmania* cause various forms of leishmaniases which are epidemiologically diverse and complex. Table 1.1 summarizes different forms of clinical manifestations, geographic distribution of the parasite, their reservoir and transmitting vectors.
Table 1.1.
Clinical manifestations, geographic distribution of the parasite, reservoir and vectors

<table>
<thead>
<tr>
<th>Clinical syndromes</th>
<th>Parasite</th>
<th>Reservoir</th>
<th>Vector</th>
<th>Geographic areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visceral leishmaniasis</td>
<td>L. donovani</td>
<td>Humans</td>
<td>P. argentipes</td>
<td>North East India, Bangladesh, Burma</td>
</tr>
<tr>
<td>(Kala-azar)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(involvement of reticuloendothelial system e.g. spleen, liver, bone marrow etc.)</td>
<td>L. infantum</td>
<td>Dogs, foxes, jackals</td>
<td>P. perniciosus, P. ariasi</td>
<td>Mediterranean basin, Middle East, China, Central Asia</td>
</tr>
<tr>
<td></td>
<td>L. chagasi</td>
<td>Foxes, dogs, opossums</td>
<td>Lutzomyia longipalpis</td>
<td>Central America, Northern South America, esp. Brazil, Venezuela</td>
</tr>
<tr>
<td>Post kala-azar dermal leishmaniasis</td>
<td>L. donovani</td>
<td>-</td>
<td>P. duboscqi</td>
<td>Kenya, possibly Ethiopia and Somalia</td>
</tr>
<tr>
<td></td>
<td>L. donovani</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L. major</td>
<td>Rodents</td>
<td>P. duboscqi</td>
<td>Middle East, Central Asia, North Africa, Indian subcontinent</td>
</tr>
<tr>
<td></td>
<td>L. tropica</td>
<td>Humans</td>
<td>P. sergenti</td>
<td>Mediterranean region, Middle East, West Asia, Indian subcontinent</td>
</tr>
<tr>
<td></td>
<td>L. aethiopica</td>
<td>Hyraxes</td>
<td>P. longipes, P. pedifer</td>
<td>Ethiopian highlands, Kenya</td>
</tr>
<tr>
<td></td>
<td>L. mexicana</td>
<td>Forest rodents</td>
<td>Lu. olmeca</td>
<td>Mexico, Central America, South America</td>
</tr>
<tr>
<td></td>
<td>L. amazonensis</td>
<td>Forest rodents</td>
<td>Lu. flaviscutellata</td>
<td>Tropical forests of South and Central America</td>
</tr>
<tr>
<td></td>
<td>L. brazilensis</td>
<td>? Forest rodents, peridomestic animals</td>
<td>Psychodopygus wellcomei et al, Lutzomyia spp., Lu. umbratilis</td>
<td>Panama, Costa Rica, Colombia</td>
</tr>
<tr>
<td></td>
<td>L. guyanensis</td>
<td>Sloths, Anteater, Forest rodents</td>
<td>Lu. trapidoi et al</td>
<td>Peru, Western Andes, Argentinian highlands, Ecuador</td>
</tr>
<tr>
<td></td>
<td>L. panamensis</td>
<td>Sloths, Monkeys, Dogs</td>
<td>Lu. verrucarum, Lu. peruenis</td>
<td>Ethiopia highlands, Kenya</td>
</tr>
<tr>
<td></td>
<td>L. peruviana</td>
<td>Dogs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse cutaneous leishmaniasis</td>
<td>L. aethiopica</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(lesion spreads through bloodstream to all over the skin)</td>
<td>L. mexicana</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L. amazonensis</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucocutaneous leishmaniasis</td>
<td>L. braziliensis</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(involvement of skin and dermis)</td>
<td>L. panamensis</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adopted from Pearson, 1993; Bryceson, 1996
Cutaneous leishmaniasis. The classic form of cutaneous leishmaniasis (CL) is the 'oriental sore' which is caused by *Leishmania tropica* (major) in rural areas of the middle East, Central Asia, North Africa and the Mediterranean basin (Table 1.1) (Lainson and Shaw, 1987; Jeronimo and Pearson, 1989; Pearson and Sousa, 1990). Typically, cutaneous lesions start as papules, enlarge and then ulcerate. Ulcers heal after variable periods of time, leaving flat atrophic scars as evidence of the disease.

American cutaneous leishmaniasis (ACL) is widespread in Central and South America. The disease manifestations range from single, small, dry, crusted lesions (caused by *L. mexicana* subspecies) to large, deep, mutilating ulcers (more common with *L. braziliensis* subspecies) (Pearson, 1993).

Diffuse cutaneous leishmaniasis (DCL) occurs in a small proportion of cases with *L. aethiopica* (Bryceson, 1970) and *L. amazonensis* (Diaz et al, 1985). The primary lesion does not ulcerate but, after a period of months or years, slowly spreads locally and to other parts of the skin, producing nodules, plaques and hypopigmented macules that may resemble lepromatous leprosy and cause grotesque deformity (Convit and Kerdel-Vegas, 1965; Bryceson, 1969).

Following resolution of cutaneous lesions due to *L. braziliensis* (Jones et al, 1987) or *L. guyanensis* or *L. panamensis* (Grimaldi et al, 1989), a significant percent of the cutaneous leishmaniasis patients develop chronic mutilating mucosal lesions of the nose, face or oral pharynx.
leading to a disfiguring condition known as 'espundia' (Marsden et al, 1991).

**Visceral leishmaniasis or kala-azar.** Visceral leishmaniasis (VL) or kala-azar (KA) is characterized by intermittent fever, anemia, leucopenia, hypergammaglobulinemia with reversal of albumin/globulin ratio and wasting syndrome (Chatterjee and Sengupta, 1970; Wilcocks and Manson-Bahr, 1972; Carvalho et al, 1987; Schnur and Greenblatt, 1995). The disease is usually fatal, if untreated. However, patients recovering from the infection, either spontaneously or through chemotherapy, subsequently become immune to another attack of the viscerotropic form of the disease (Napier, 1924; 1946).

Marked hyperplasia of the lymphoid-plasma cell system is a common feature in visceral leishmaniasis (Lloyd and Paul, 1928; Brahmachari, 1928) which is associated with typical cellular and biochemical changes in the blood and tissue pathology (Bryceson 1996). Parasites (L. d bodies) are demonstrable in the splenic or bone marrow aspirates during the active form of illness which forms the basis for confirmation of clinical diagnosis. Various immunodiagnostic techniques including enzyme-linked immunosorbent assay (Hommel et al, 1978; Anthony et al, 1980; Fargeas et al, 1996), direct agglutination test (Harith et al, 1988) and immunoblot assay (Bogdan et al, 1990; Hoerauf et al, 1992; Cardenosa et al, 1995) have been developed and used for the detection of antileishmanial antibodies. Recently, detection
of leishmanial parasites in the clinical samples of patients with various forms of leishmaniases has been made possible by using specific DNA probes and polymerase chain reaction (PCR) techniques (Howard et al, 1991; Adhya et al, 1995; Laskay et al, 1995; Mathis and Deplazes, 1995; Schaefer et al, 1995). These techniques have also been found useful to detect asymptomatic and subclinical forms of infection caused by *L. donovani* which are now believed to be of common occurrence amongst people living in KA endemic areas (Heyneman, 1971; Pampiglione et al, 1974; Badaro et al, 1986; Alvar et al, 1989). Individuals suffering from this asymptomatic or subclinical form of infection may recover spontaneously within a few months or years; while others, especially malnourished or immunosuppressed ones may eventually develop classical VL (Cerf et al, 1987).

Kala-azar patients can usually be successfully treated with various preparations of pentavalent antimony (Bryceson, 1987; 1996; Berman, 1988; Modabber, 1993). Patients, unresponsive to the antimonial treatment (Bryceson et al, 1985; Berman, 1988), can usually be successfully treated with pentamidine or other chemotherapeutic agents (Bryceson et al, 1985; Badaro et al, 1990; Chang et al, 1990; Giri and Singh, 1994; Jha et al, 1995; Schnur and Greenblatt, 1995; Seaman et al, 1995; Bryceson, 1996; Russo et al, 1996; Sundar and Murray, 1996).

Post kala-azar dermal leishmaniasis. The post kala-azar dermal leishmaniasis (PKADL) is a sequel to VL as the disease
is seen in about 20% of Indian KA cases, usually after 1-2 years of an apparent cure (Brahmachari, 1922). Interestingly enough, the disease is rarely encountered in other KA endemic areas around the world except in East Africa where 2-5% of KA cases develop PKADL (Bryceson, 1996). In India, PKADL lesions usually appear as hypopigmented macules over chin, lips, neck, extensor surfaces of arms etc. and eventually spreads all over the body. The onset of the lesions, sometimes, may take as long as 20 years (Munro et al, 1972) following apparent cure of the viscerotropic form of the disease. The skin lesions develop into non-ulcerating nodules which may persist for a prolonged time in the chronic form. In all these cases, L. d bodies can be demonstrated in biopsy materials from affected areas, but not from the visceral organ. No apparent difference between the L. donovani strains isolated from KA and PKADL cases has been clearly established so far. Thus, it appears that the viscerotropic character of L. donovani changed to dermatropism, probably due to the altered immune status of the host (Adler, 1964). The PKADL cases have been implicated as the human reservoir for transmission of L. donovani infection in the Indian subcontinent (Ashford and Bettini, 1987; Shaw and Lainson, 1987).

Life cycle of the parasite

Leishmanial organisms are transmitted between the long-lived vertebrate hosts and short-lived sandflies and have independent developmental cycles in each of the hosts (Fig.
The parasite exists in two morphologically distinct forms; the nonmotile, round shaped, aflagellated amastigote form (maximum diameter 2.5-6.8 μm) within the macrophage of the vertebrate host and the motile, flagellated, long-slender promastigote (10-20 μm x 1.5-3 μm) form in the sandfly gut or in cell-free culture medium (Alexander and Russell, 1985). Following a blood meal from an infected reservoir by the sandfly, amastigotes transform into promastigotes and divide in the sandfly gut. Thereafter, these migrate towards the pharynx, possibly chemotactically attracted by sugars from the fly crop (Bray 1983), developing into infective, metacyclic forms. Metacyclic promastigotes are inoculated into the host with the sandfly saliva which increases their infectivity (Titus and Ribeiro, 1988; Lima and Titus, 1996). The inoculated parasites are engulfed by the host macrophages and transformed into amastigotes which multiply intracellularly by binary fission. On rupture of the macrophage, the amastigotes are released and taken up by other macrophages derived from locally recruited monocytes (Schnur and Greenblatt, 1995).

Differentiation of amastigotes into promastigotes and vice versa involve qualitative as well as quantitative changes in the expression of several important biomolecules of structural as well as functional importance (Morrow et al., 1980; Fong and Chang, 1981; Iovannisci et al., 1984). Fong and Chang (1981) have shown that tubulins of leishmaniae are developmentally regulated proteins as their biosynthesis increases rapidly during amastigote-to-promastigote
Fig.1-2: Life cycle of *Leishmania* in sand fly and mammalian hosts
differentiation and vice versa. Expression of stage-specific molecules (epitopes) and genes has been demonstrated using monoclonal antibodies (Kahl and McMahon-Pratt, 1987) and differential cDNA hybridization techniques (Kidane et al, 1989; Saramas and Spithill, 1989). However, developmentally regulated molecules/genes specific for the amastigote stage are yet to be shown to play important role(s) in leishmanial virulence. Several surface molecules such as a major surface glycoprotein (gp63), the lipophosphoglycan (LPG) and the acid phosphatase have all been found in both stages of the parasite (Chang et al, 1990).

Host-parasite interaction

Establishment of any parasitic infection in the vertebrate host is dependent upon a series of molecular and cellular interactions which occur prior to the development of specific type of immune response (Wakelin and Blackwell, 1993). Leishmanial infection is also no exception to this rule as the initial susceptibility of the host to leishmanial challenge depends primarily on the ability of the parasite to get inside the host macrophages, evade nonspecific killing mechanisms and multiply intracellularly (Mauel, 1990). Within the vertebrate host, the leishmanial promastigotes must, however, survive the complement mediated damage before their entry inside the macrophages (Sacks et al, 1993). Leishmanial attachment to the macrophage membrane is receptor mediated and is a prerequisite to phagocytosis. Receptors involved in the process may include the complement receptor (CR3)
(Russell and Wright, 1988), receptors recognizing arg-gly-asp (RGD) sequence or integrin type receptors for the gp63 molecule of the parasite (Hynes, 1992), receptors recognizing fucose-mannose containing ligand on leishmanial cell surface etc (Blackwell et al, 1985; Russell and Wilhelm, 1986; Wilson and Pearson, 1986; 1988; Quaissi, 1988; Russell and Wright, 1988). Interestingly, amastigotes may utilize different receptors than those used by promastigotes for their entry inside the cell (Russell and Wilhelm, 1986; Wozeneraft et al, 1986; Soteriadou et al, 1992; Love et al, 1993; Straus et al, 1993). Once inside the host cell, survival of intracellular parasites depends on the genetic factors which regulate host cell's biochemistry and physiology. These factors probably determine the host's ability to kill intracellular parasites through respiratory burst and concomitant production of high levels of degradative enzymes and reactive metabolites such as, superoxide anion \( \left( \text{O}_2^- \right) \) (Murray, 1981a), hydrogen peroxide \( \left( \text{H}_2\text{O}_2 \right) \) (Murray, 1981b), nitric oxide (NO) (Green et al, 1990; Mauel et al, 1991) and other nitrogen derivatives which have been shown to be toxic to all stages of leishmanial parasites (Murray, 1981b). Studies carried out in inbred strains of mice have been able to identify and map a genetic locus (called 'Lsh gene') on mouse chromosome 1 which possibly regulates receptor-mediated priming and activation of macrophages leading to parasite destruction (Blackwell et al, 1989). Currently available evidences suggest that Lsh operates subsequent to ligand binding to macrophage receptors.
at the level of signal transduction and/or regulatory gene (DNA binding protein) control (Blackwell et al., 1989) and is independent of immune lymphocyte mediated protection. Studies designed to find human homolog of \textit{Lsh} gene have already identified a conserved gene sequence on the human chromosome 2 (Blackwell et al., 1988; Schnur et al., 1989a; 1989b). The existence of \textit{Lsh} gene equivalent in human is also suggested by recent epidemiological studies on leishmaniasis which highlight the existence of asymptomatic individuals infected with leishmanial parasites (Blackwell et al., 1989; Schnur et al., 1989b).

Studies on the interaction of \textit{Leishmania} with host macrophages have identified the phagolysosome as the final intracellular site in which parasites survive and multiply (Mauel, 1990). Therefore, intracellular survival of the parasite may depend on its ability to thrive in the acidic environment of the phagolysosome (Mukkanada et al., 1985; Glaser and Mukkada, 1992) as well as to resist destruction by the lysosomal enzymes and toxic metabolic products (Murray, 1981b; Evans et al., 1996). Several mechanisms have been proposed by which leishmanial amastigotes can achieve these objectives. Some of the key molecules involved in the process may be the lipophosphoglycan (LPG) present on the parasite surface (Handman et al., 1987; Turco, 1988; Panaro et al., 1995; 1996), the excretory factor (related to LPG) released by the parasite in the soluble form (Sacks et al., 1993), surface protease glycoprotein gp63 (Chang and Chang, 1986; Russell and Wilhelm, 1986; Kink and Chang, 1987; 1988; Wilson
and Hardin, 1988; Wilson et al, 1989), cysteine proteases (Lockwood et al, 1987; Glew et al, 1988; Mallinson and Coombs, 1989), acid phosphatase (reviewed by Dwyer and Gottlieb, 1985; Glew et al, 1988), Mg-dependent ATPase (Zilberstein and Dwyer, 1988) etc.

**Immunology of leishmaniasis**

a) In man. Humoral immune response in visceral leishmaniasis or kala-azar is marked by the production of both specific and non-specific antibodies in circulation (Bray, 1976; Ghose et al, 1980). Antileishmanial antibodies have been shown to be primarily of immunoglobulin G (IgG) class (and to a lesser extent of IgM class) (Ghose et al, 1980). Production of many autoantibodies to various proteins, haptens (Galvo-Castro et al, 1984), cytoplasmic and nuclear antigens (Argov et al, 1989) in the sera of KA patients is likely to arise as a result of polyclonal B-cell activation. Complement levels are reduced (Ghose et al, 1980) and immune complexes are present in serum in high titres, notably cryoglobulins and rheumatoid factors (Pearson et al, 1983). Antileishmanial antibodies are also found in the sera of fresh as well as chronic PKADL cases (Haldar et al, 1981).

Antileishmanial antibodies could be detected in the sera of majority of patients with CL, the rising titres being correlated with the severity of the disease symptoms (Ranque and Quilini, 1970; Anthony, 1980). Presence of high titres of circulating antibodies has been demonstrated in the sera of DCL patients (Bray and Lainson, 1967; Bittencourt and
Guimaraes, 1968; Convit and Pinardi, 1969). Circulating immune complexes and anti-Ig antibodies are also detectable, particularly in cases with multiple mucosal lesions (Desjeux et al, 1980).

A profound immunosuppression in the cell-mediated immune component is a characteristic feature of classical VL during the active stage of the disease (Turk and Bryceson, 1971). This is manifested in KA patients in the form of lack of their T cell responsiveness to leishmanial antigens both in vitro and in vivo (Carvalho et al, 1981; Haldar et al, 1983; Ho et al, 1983; Sacks et al, 1987; Neogy et al, 1988). A reduction in the peripheral blood CD4+/CD8+ cell ratio has been documented in Kenyan (Koech, 1987), Brazilian (Carvalho et al, 1992) and Indian (Ghosh et al, 1996; Rohtagi et al, 1996) KA patients while no such variations are noted in Mediterranean VL cases (Cillari et al, 1991). Furthermore, lack of mitogen induced lymphoproliferative response has been documented in African (Ho et al, 1983) but not in South American (Carvalho et al, 1981) and Indian KA patients (Haldar et al, 1983; Sacks et al, 1987). While exploring the cellular basis of this unresponsiveness in VL patients, Carvalho et al (1989) failed to restore antigen-specific responses of their lymphocytes by depletion of macrophages, B cells, Fc receptor high-avidity cells or CD8+ and CD4+ cells. Kala-azar patients, on the other hand, regain their normal cell-mediated immune function (Carvalho et al, 1981; Haldar et al, 1983; Sacks et al, 1987; Neogy et al, 1988) several
months after their apparent recovery, which may make them immune to the viscerotropic form of the illness.

Antileishmanial antibodies are also demonstrated in the sera of PKADL patients. Peripheral blood mononuclear cells (PBMC) of these patients show variable degrees of proliferative response in vitro to leishmanial antigens (Haldar et al, 1983) and their circulating CD4⁺/CD8⁺ cell ratios remain within the normal limits (Ghosh et al, 1995).

Lymphocytes from CL and MCL are characterized by vigorous proliferative response when exposed to leishmanial antigens. Patients with MCL have abnormally low numbers of CD4⁺ cells in their periphery and have altered CD4⁺/CD8⁺ cell ratios (Castes et al, 1988). Patients with DCL show a state of unresponsiveness which is more of leishmanial antigen-specific than generalized in nature (Turk and Bryceson, 1971).

b) In mouse. Chronic L. donovani infection in BALB/c mice has been reported to lead to massive hepatosplenomegaly and hypergammaglobulinemia as seen in clinical cases. Antibody response to promastigote antigens may be detectable in L. donovani-infected BALB/c mice only during the late stage of infection (Reiner, 1982). Specific humoral response has also been detectable in murine cutaneous leishmaniasis (Nguyen et al, 1984). Chronic infection with L. donovani is associated with T cell unresponsiveness towards both leishmanial antigen and mitogens (Murray et al, 1982; Reiner and Finke, 1983; Nicol and Bonventre, 1985; Kaye et al, 1991) and a lack of
DTH reaction in vivo (Reiner, 1982). Decreased IL-1 production is also associated with murine visceral leishmaniasis (Reiner, 1987). Several observations suggest that the ability of chronically infected BALB/c mice to reduce their parasite burden at the late stage of infection is mediated by leishmanial antigen-specific T cells (Blackwell et al, 1980; Blackwell, 1983).

c) Mechanisms of protective immunity. The course of leishmanial infection in mouse model appears to be determined by the pattern of lymphokines produced by leishmania-reactive CD4$^+$ T cells (Coffman et al, 1991). Murine CD4$^+$ cells can be divided into two functionally distinct subsets, i.e., the T helper type 1 (Th1) and type 2 (Th2) (Mosmann et al, 1986). The Th1 cells produce IFN-gamma, IL-2 and are functionally related to delayed type hypersensitivity (DTH) reactions. Th2 cells, on the other hand, produce IL-4, IL-5, IL-6 and IL-10 and have functionally been associated with B cell activation (Mosmann et al, 1986). IL-4 produced by Th2 cells is shown to be involved in the disease aggravation process while IFN-gamma (produced by Th1 cells) is responsible for protective immunity (Scott et al, 1989; Boom et al, 1990; Coffman et al, 1991; 1995; Kemp et al, 1993; Locksley, 1995). At the cellular level, it has been found that IFN-gamma inhibits the function of Th2 cells and IL-4 inhibits Th1 cell functions (Coffman et al, 1991; 1995; Locksley et al, 1995).

The possibility that Th2 activation may be involved in progression of the disease, in human VL cases, has been
suggested by the demonstration of elevated levels of IL-4 in KA sera (Zwingenberger et al, 1990), isolation of Th2-like T cell clones from KA patients (Kemp et al, 1993) and demonstration of mRNA for IL-10 in bone marrow cells (Karp et al, 1993), lymph nodes (Ghalib et al, 1993) and peripheral blood mononuclear cells (PBMC) (Carvalho et al, 1994) from active VL patients. Evidences are available which suggest a role of IFN-gamma and IL-2 in the restoration of T cell responsiveness. These include an increase in the expression of MHC class II molecules on antigen presenting cells (APC) (Reiner et al, 1988), down-regulation of IL-4 and IL-10 production (Mossman and Coffman, 1987; Chomarat et al, 1993), increase in the macrophage activation (Nathan et al, 1983; Liew et al, 1989). Tumor necrosis factor (TNF) has been suggested to play an important role in mediating host protection against experimentally induced CL (Titus et al, 1984; Liew et al, 1985). Recently, IL-12 has been reported to be involved in man as well as in experimental animal models through the restoration of IFN-gamma production and cytotoxic cell response leading to control of leishmanial infection (Ghalib et al, 1995; Bacellar et al, 1996; Guler et al, 1996). Although various other mechanisms contribute towards the induction of resistance or susceptibility of the host to leishmanial infection, the balance between Th1 and Th2 may be of profound importance in the pathogenesis of leishmaniasis (Kemp et al, 1994a).
Antigens of leishmanial parasites

The structural components located on the surface membrane of leishmanial parasites are expected to play a key role in interaction of the parasite to its immediate environment. This is true for both the promastigote and amastigote forms of the parasite. Further, parasite's ability to adapt themselves to the different environments is also likely to be reflected in their differential expression of certain cell surface moieties, both qualitatively as well as quantitatively (Schnur and Greenblatt, 1995).

Leishmanial surfaces are known to contain several proteins (or glycoproteins) of different molecular weights, some of which are conserved amongst various species of *Leishmania* (Dwyer et al, 1974; Lepay et al, 1983; Ramasamy et al, 1983; Gardiner et al, 1984). The major surface glycoprotein of *Leishmania* is described to be a zinc metalloprotease (Etges et al, 1992) of subunit molecular weight 63 kD (gp63) (Lepay et al, 1983; Gardiner et al, 1984; Etges et al, 1986; Bordier, 1987; Bouvier et al, 1989; Chaudhuri et al, 1989). The protein appears to be a conserved one as its presence in different *Leishmania* species (Lepay et al, 1983; Ramasamy et al, 1983; Gardiner et al, 1984; Bouvier et al, 1995) in both amastigote and promastigote forms of the parasite (Frommel et al, 1990). The abundance of gp63 appears to be correlated with the infectivity of the parasite (Kink and Chang, 1987; 1988; Kweider et al, 1987; Wilson et al, 1989; Schnur and Greenblatt, 1995) and immunization with native/recombinant(r) gp63 has been shown to protect
susceptible mice from leishmanial challenge (Handman and Mitchell, 1985; Russell and Alexander, 1988; Jardim et al, 1990; Yang et al, 1990). Sera from active VL patients in Brazil, Sudan and Kenya have been reported to contain anti-gp63 antibodies (Shreffler et al, 1993; Kurtzhals et al, 1994). However, conflicting results are available in literature in respect to the ability of gp63 to induce T cell proliferative response in vitro (Nascimento et al, 1990; Mendonca et al, 1991; Russo et al, 1991; Kemp et al, 1991; 1994b; Kurtzhals et al, 1994).

The soluble acid phosphatases of *L. donovani* promastigotes, of approximately 110 kD and 130 kD sizes (Bates and Dwyer, 1987), play an important role towards the survival of *Leishmania* in the sandfly gut as well as in the macrophage lysosomal environment by imparting resistance to toxic metabolites (Saha et al, 1985). Antibodies to this enzyme may conceivably contribute to the pathobiology of leishmaniasis within the host (Glew et al, 1988; Gottlieb and Dwyer, 1988).

Apart from gp63 and acid phosphatases, several other proteins/glycoproteins have been shown to induce humoral immune response in VL patients. Dos Santos et al (1987) identified two *L. chagasi* polypeptides of 123 and 119 kD that are shown to react with all VL sera but not with *T. cruzi*-infected patient sera. Jaffe and Zalis (1988a) identified and purified two *L. donovani* membrane proteins of molecular masses of about 70 and 72 kD and developed (1988b) an assay.
system for the diagnosis of VL cases. Another major membrane component of 80 kD has been isolated and purified from L. donovani which appears to be useful in the serodiagnosis and immunoprophylaxis against VL (White and McMahon-Pratt, 1988). Immunization of mice using glycoproteins of molecular weights of 46 and 97 kD from L. amazonensis and L. major respectively, has been reported to induce protection through elicitation of high levels of antibodies (Champsi and McMahon, 1988; Frommel et al, 1988). Reed et al (1990) have purified two proteins from L. chagasi of molecular weights 30 and 42 kD. Antibodies against these proteins have been reported to be present in majority of VL sera. Further, a surface glycoprotein of 42 kD (gp42) from L. amazonensis has been shown to react strongly with sera from patients with different forms of leishmaniasis as well as Chagas' disease. In addition, gp42 has also been reported to stimulate the proliferation of T lymphocytes obtained from several leishmaniasis patients (Burns et al, 1991).

A 36 kD glycoprotein (gp36) has been identified in L. donovani strains and shown to contain major fucose-mannose ligand (FML) containing carbohydrates (Palatnik-de-Sousa et al, 1993). The FML containing fraction is used to detect human VL cases in ELISA with 100% sensitivity and 96% specificity (Palatnik-de-Sousa et al, 1995). It is also found to be immunogenic and protect mice from leishmanial challenge by about 85% reduction in liver parasitic burden (Palatnik-de-Sousa et al, 1996).
A recombinant 24 kD protein of \( L. \text{major} \) has been shown to protect susceptible mice from infection with \( L. \text{major} \) when administered as a vaccine with IL-12 (Mougneau et al, 1995). The recombinant product (rk39) of 39 amino acid repeats, encoded by a kinesin-like gene of \( Leishmania \) species, has been reported to have potential diagnostic value in KA and PKADL cases (Singh et al, 1995; Badaro et al, 1996). Another recombinant antigen (Lcr1) of molecular mass >200 kD from \( L. \text{chagasi} \) has been isolated and shown to partially protect mice against challenge with \( L. \text{chagasi} \) (Wilson et al, 1995).

Suffia et al (1995) partially characterized two proteins of 14 kD (p14) and 18 kD (p18) isolated from \( L. \text{infantum} \). These proteins are shown to be useful in the seroepidemiology of visceral leishmaniasis cases. Over 80% of asymptomatic individuals presenting positive DTH reaction have been reported to contain antibodies against p14 and/or p18 (Marty et al, 1994). These proteins also induce proliferative response of primed PBMC in vitro (Suffia et al, 1995). A kinetoplast membrane protein (KMP-11) has been identified to be an abundant 11 kD surface glycoprotein of all leishmanial promastigotes (Tolson et al, 1994; Stebeck et al, 1995). It is a LPG-associated protein found in both life stages of \( Leishmania \) (Jardim et al, 1995) and has been shown to be capable of eliciting a strong T cell proliferative response in vitro (Jardim et al, 1991; Mendonca et al, 1991; Kemp et al, 1991; Russo et al, 1992; Tolson et al, 1994, ). A 94 kD component of \( L. \text{infantum} \) has been shown to be recognized by all VL sera in immunoblot assay suggesting its importance in
the serodiagnosis of VL (Rolland-Burger et al, 1991).

An important macromolecule present on the cell surface of *Leishmania* is the unique lipophosphoglycan (LPG) (Handman et al, 1984; King et al, 1987) moiety. The LPG preparation show variable molecular weights ranging between 5 and 40 kD (McConville et al, 1987). Structurally, *L. donovani* LPG is a polymer of repeating phosphorylated disaccharide units of Gal(β1-4)Man α1 linked via a carbohydrate core to a novel lyso-alkylphosphotidyl inositol lipid anchor (Orlandi and Turco, 1987; Turco et al, 1987). The LPG moieties are present in different leishmanial species (Handman et al, 1984; Handman and Goding, 1985; McConville et al, 1987) and in both the leishmanial forms (reviewed by Chang, Chaudhuri and Fong, 1990). The molecule is released as an excretory factor (EF) (Schnur et al, 1972; Decker-Jackson and Honigberg, 1978; El-On et al, 1979; Slutzky and Greenblatt, 1979; Semprevivo and Honigberg, 1980; Handman et al, 1984; Turco et al, 1984) which is shown to be immunogenic and has been used in the serological differentiation of leishmanial organisms (Jacobson et al, 1982; Schnur, 1982; Slutzky et al, 1984). Antibodies to LPG and/or EF have been demonstrated to be present in the sera of VL patients (Ray and Ghose, 1985; Kurtzhals et al, 1992). Further, PBMC from recovered VL patients have been shown to proliferate and produce IFN-gamma on stimulation with LPG in vitro (Kemp et al, 1991; 1993; Kurtzhals et al, 1994). However, subsequent studies have shown that the T cell proliferative induction capacity of LPG
is associated with tightly bound proteins that are copurified with LPG (Russo et al, 1992; Kemp et al, 1993).

Only limited information is available so far with leishmanial antigens that show humoral as well as cell-mediated immune responses in mice. Moreover, very little is known about the correlation between man and mouse model of leishmaniasis in elicitation of immune response against the *L. donovani* strain. Whether the variation in disease outcome is host-specific or parasite strain-specific, distributed in wide geographical regions, remains to be clearly understood.

**Immunological approaches towards the control of leishmaniasis**

Immunological approaches for the control of leishmaniasis include (a) early diagnosis of leishmanial infected individuals, (b) immunotherapy of leishmaniasis cases (primarily in the form of a combination therapy when used with antileishmanial drugs) and (c) development of immunoprophylaxis. Some excellent review articles are recently available on the subject of immunodiagnosis of leishmaniasis (Manson-Bahr, 1987; Sacks et al, 1993; Ghose et al, 1994; Schnur and Greenblatt, 1995). Studies on the immunotherapy of leishmaniasis are based primarily on the use of immunomodulating agents such as *Bacille Calmette-Guerin* (BCG) (Smrkovski and Larson, 1977), *Corynebacterium parvum* (Scott et al, 1987), glucan (Cook et al, 1980; Holbrook and Cook, 1983), muramyl dipeptide (MPD) (Adinolfi et al, 1985) etc. More recently, certain cytokines like IL-2, IL-12, IFN-gamma, granulocyte-macrophage colony stimulating factor
(GMCSF) have been used (alone or in combination with antileishmanial drugs) against leishmaniasis in man as well as in experimental animals with variable degrees of success (Badaro et al, 1990; 1994a; 1994b; Ruef and Coleman, 1990; Murray, 1991; Lieschke and Burgess, 1992; Scott, 1993; Murray and Hariprasad, 1995). Information already available in the literature suggest that immunoprophylaxis against leishmaniasis is feasible and may serve as a cost effective control measure. This is particularly true for visceral leishmaniasis as individuals recovering from the VL become immune to a subsequent attack of the viscerotrophic form of the disease. Two approaches are currently being pursued for the development of a vaccine against leishmaniasis: (a) the first generation vaccines composed of killed parasites with or without adjuvants such as BCG (Sharples et al, 1994) etc and (b) while recombinant molecules (Yang et al, 1990; Xu and Liew, 1994; 1995; Olobo et al, 1995) or genetically engineered organisms (Cruz et al, 1991) represent another class of vaccines that require some preclinical development. Availability of an experimental model of VL in BALB/c mice should provide an excellent approach to carry out studies on the development of vaccine.

1.4 Aims and objectives of the study

Immunological mechanisms appear to play an important role in determining the course of leishmanial infection in man as well as in experimental animals such as, in mice. The
existing literature indicate that considerable variations may exist in the host's immune response following leishmanial infection. Such variability may arise as a result of differences in the genetic background of the host belonging to different species (or even within the same species). Furthermore, variability within the parasite strains isolated from different geographical regions may also contribute towards the diversity in the host-immune response. Unfortunately, no information is yet available in the literature on the subject of variable antigen recognition capacities by a given infected host against different parasite isolates. Further, whether or not the same antigen(s) of a given parasite strain will be equally effective in the induction of immune response in different susceptible hosts remains to be investigated. Clearly, identification of parasite antigens involved in eliciting both humoral and cell-mediated immune responses in susceptible hosts during the course of leishmanial infection should provide valuable information from the diagnostic, prognostic as well as prophylactic points of view.

Based on the above considerations, detailed studies were undertaken with the following major objectives:

1. To determine humoral and cell-mediated immune responses in active and treated kala-azar patients. The study includes both specific and nonspecific immune responses against crude leishmanial antigens and mitogens respectively.
2. To identify leishmanial antigens/antigenic components that are reactive to sera from active and treated kala-azar patients. Special emphasis is given to identify antigens responsible for stimulation of different IgG subclasses of antibodies in infected and immune hosts.

Some of the experiments, outlined as above under items (1) and (2), are also carried out with PKADL patients.

3. To study longitudinally the state of suppression, if any, in the cell-mediated immune component of the infected host (BALB/c mice) and correlate the results with the degree of parasitemia as well as humoral immune response.

4. To identify leishmanial antigens or antigenic fractions inducing humoral and cell-mediated immune responses in BALB/c mice during the course of *L. donovani* infection as well as following antileishmanial therapy.

5. To evaluate protective efficacies of different leishmanial antigen preparations against challenge with *L. donovani* amastigotes in the BALB/c model.