1. INTRODUCTION

1.1 Historical Review

Leishmaniasis is one of the top ten parasitic diseases identified by World Health Organisation for causing an estimated 1.2 million cases of infection per year.

The Leishmanias are the causative agents of a variety of human diseases termed "Leishmaniasis" (Stauber, 1970). A 1985 report states that there were 150,000 cases of Kala-azar during 1977 to 1982 resulting in hundreds of deaths. About 400,000 new cases of disseminated leishmaniasis are reported almost every year from endemic areas of the world (Lainson and Shaw, 1978). Although there is no quantitative evaluation at the moment but rough estimates based on extrapolation 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 are currently influenced by it (W.H.O. Tech. Rep. Ser. 1990).

The human leishmaniasis was first reported by Cunningham in 1885, when he observed, but misinterpreted, the parasites in the tissues of a Delhi boil in Calcutta. The organism was mistaken as Mycobacteria. Later, in 1898 Borovovsky established the protozoan nature of the parasite (Zukerman and Lainson, 1977). But in 1903, it was Leishman who first described this organism as a causative agent of
'Visceral Leishmaniasis' after having examined the spleen of a British soldier with kala-azar. The Britishman was stationed at 'Dum Dum' near Calcutta, hence the name 'Dum Dum fever'. Later in the same year, Donovan also identified the parasite in splenic tissue. The organism is often called *Leishmania donovani* or L.D. body. In 1904, Roger observed the conversion of amastigotes to promastigotes in culture. Promastigotes were also found in sandflies by Adler and Odor in 1925. Transmission of oriental sore by infected sandfly bite was observed by Wenyon in Baghdad (1917) and Sergent in Algeria (1921).

The Leishmania Skin Test was described by Montenegro in 1926 and in 1942. The British Commission in India experimentally transmitted Kala-azar to human volunteers through sand fly bite. Zuckerman and Lainson (1977), described the causative agent of Zoonotic Cutaneous Leishmaniasis as *L. tropica major*. Bray *et al.*, (1973) established the name *L. aethiopica* for the parasite causing Cutaneous, as well as more diffused leishmaniasis in Ethiopia and Kenya. Guteremala and Yucatan held *L. tropica braziliensis* and *L. tropica guanensis* responsible for causing mucocutaneous leishmaniasis in Brazil and Cutaneous Leishmaniasis in Panama and Costa Rica respectively. Zuckerman & Lainson (1977) and Lainson and Shaw (1972) classified leishmaniasis into two major groups, the *L. mexicana* and *L. braziliensis* complexes.
1.2 Taxonomy

The various causal agents of leishmaniases belong to the genus *Leishmania* whose systematic position is as follows:

(W.H.O. - TDR/ LEISH - SEM/ 80.3)

- **Phylum**: Protozoa
- **Sub phylum**: Sorcomastigophora
- **Order**: Kinetoplasticida
- **Sub order**: Trypanosomatina
- **Family**: Trypanosomatidae
- **Genus**: *Leishmania*

The genus *Leishmania* has a wide distribution in the tropics and subtropics but is apparently absent in South-East Asia and the Pacific.

1.3 Life Cycle and Morphology

*Leishmania* have a relatively simple lifecycle. Promastigotes in the proboscis of a female sandfly are introduced into the skin of a vertebrate host during a blood meal. The promastigotes invade reticulo-endothelial cells, transform into amastigotes, multiply within macrophages and invade other reticulo endothelial cells. Further, sandflies feeding on infected individuals ingest parasitized cells and the amastigotes transform into promastigotes which multiply in the gut and migrate to the proboscis, thus completing the cycle.
1.3.1 Amastigote Stage

The amastigote found within reticuloendothelial cells in the vertebrate host, is a round or oval organism measuring 2 to 5 μm in greatest diameter. In Wright or Giemsa-stained preparations, the pale blue cytoplasm is surrounded by a plasma membrane and contains a large dark-purple nucleus and a small purple kinetoplast. A delicate thread connects the kinetoplast to a dot like basal body from which an axoneme arises and extends to the anterior end of the organism. Multiplication is by binary fission and mitotically dividing forms may be seen in spleen smears.

1.3.2 Promastigotes Stage

The promastigote found in culture and in the sandfly digestive tracts measures 1.5 to 3.5 by 15 to 20 μm and has a single free flagellum 15 to 28 μm long. Old cultures may contain short, broad and rounded forms 4 to 5 μm in diameter with long flagella centrally directed to form rosettes. Ultrastructurally, the organism is surrounded by a trilaminar plasma membrane, beneath which is a row of microtubules. The cytoplasm contains a large central nucleus, ribosomes, rough and smooth endoplasmic reticulum, a golgi body, various vesicles and a single mitochondrion. The kinetoplast is a complex body and appears as an electron dense granular band with a distinct fibrillar pattern, lying within an extension of the mitochondrion. The axoneme, which arises from the
basal body and a paraxial rod are contained within the flagellar sheath.

1.4 Ultra Structure of Leishmania Parasite

There are two developmental stages in leishmania, the promastigote in the sandfly host and the amastigote in various species of mammals and reptiles.

The body and flagellum of the promastigotes are each approximately 20 μm long, and their organelles have staining characteristics similar to those of amastigotes. Electron microscopy has revealed that both forms are surrounded by typical trilaminar unit membrane 10-12 μm thick and have a sub-pellicular microtubular system. The microtubular system functions as a cytokeleton imparting shape and flexibility to the parasite and perhaps also serves to anchor intrinsic plasma membrane proteins. The flagellar pocket, although not a true cytoplasm, has coated vesicles which contain supercoiled DNA (kDNA) that exists as a network of catenated minicircles and maxicircles. The kDNA divides before nuclear DNA but the nature of the proteins encoded by kDNA are in general unknown.

Attempts to identify the role of kDNA have utilized drug induced dyskinetoplastic parasitic forms. Comparison between normal and dyskinetoplastic forms have inferred that kDNA may encode for mitochondrial proteins. Reproduction starts with the division of the kinetoplast and
blepharoplast. The kinetoplast lengthens, without change in structure, then divides, but the dividing cells have two blepharoplasts, one at the base of flagellum while the other develops later and apart from the flagellum. Division of the nucleus has been said to involve spindle formations. The structure of the nuclear membrane remains unchanged during the divisions.

The ultra structure of amastigotes generally resemble that of promastigotes, except for the overall shape and the absence of a free flagellum.

1.5 Geographical Distribution and Epidemiology

The Leishmaniasis have an immense geographical distribution in the tropics and subtropics of the world. The world wide annual incidence of leishmaniasis varies from 0.4 to 12 million cases (TDR, 1989).

1.5.1 Visceral Leishmaniasis (VL, Kala azar)

Visceral leishmaniasis or infantile kala-azar is widely distributed in Mediterranean Portugal, France, Italy, Greece, Yugoslavia, North Africa, Lebanon, Iraq, Iran, Saudi Arabia, Yemen, Southern Russia, Central Asia and North China.

Indian Kala-azar occurs in several parts of India (Assam, Bengal, Bihar, Uttar Pradesh, Madras and Sikkim) and Bangladesh. African kala-azar is common in Kenya and Sudan but sporadic cases occur in Chad, Upper Volta, Central African Republics, Zambia and Ethiopia.
Precise incidence figures are not available but it is likely that tens of thousands of cases of Visceral Leishmaniasis occur each year throughout the world.

1.5.2 Mucocutaneous Leishmaniasis (MCL)

It is primarily found in South America although cases have also been reported in Africa, notably from Ethiopia and Sudan.

1.5.3 Cutaneous Leishmaniasis

Cutaneous leishmaniasis is the most prevalent form of leishmaniasis found in Africa, Latin America, the Indian subcontinent, South West Asia and parts of the Mediterranean Basin and Soviet Union.

Cutaneous leishmaniasis infection is characterized by nodular and ulcerative skin lesions caused by *Leishmania tropica, Leishmania major* or *Leishmania aethiopica*.

[A] Leishmania tropica infection

Urban cutaneous leishmaniasis is a natural infection of dogs and mainly caused by *L. tropica*. The parasite has been demonstrated in cutaneous lesions on the ears, lips, nose and inner canthus of the eyes of dogs in Iran, Iraq and India, where it is transmitted by *P. sergenti*. The infection was formerly common in many large cities of the Middle East, namely Baghdad, Tehran, Aleppo and Damascus, Southern Italy and Greece. Pakistan and North Western India are also affected.
[B] Leishmania major infection

The cutaneous leishmaniasis caused by *L. major* is found in a wide area of sub-Saharan Africa between the tenth and thirteenth parallels north, from Senegal in the west to Sudan in the east.

The Nile rat (*Arvieanthis mitoficus*) is the major host, although infection has also been demonstrated in other rodents. Human disease is also a rural zoonosis and the main vector is *P. papatasi* in Sudan. River et al., (1993) suggested that CBA mice were can be protected from virulent *L. major* challenge by subcutaneous administration of radioattenuated parasite.

[C] Leishmania aethiopica infection

Cutaneous leishmaniasis in Africa can also be caused by *L. aethiopica*. The parasite is found in mountain valleys of the rift in Ethiopia and Kenya. It infects the rock hyrax (*Procasia lebessirica*) and the tree hyrase (*Heterohyrax brucei*) and man is infected when his house stands on rocks on deforested mountain slopes.

The situation in Namibia superficially resembles to that in Ethiopia and Kenya because sporadic cases of cutaneous leishmaniasis occur and leishmania have been isolated from hyrase and from *P. rassi* near hyrase borrows. However, the parasites from man and *P. rassi* are identical.
1.6 Clinical Forms

Leishmaniasis, the disease caused by the infection of flagellate protozoan parasite leishmania occurs in three major clinical forms, Visceral, Mucocutaneous and Cutaneous.

1.6.1 Visceral Leishmaniasis

Visceral leishmaniasis or kala-azar is a clinical condition manifesting itself due to the infection of reticuloendothelial cells by *L. donovani* parasites. The resultant disease is characterized by a lengthy incubation period an insidious onset and a chronic course attended by irregular fever, enlargement of spleen and liver, leucopenia, anaemia and progressive wasting. The mortality is high, death occurs in untreated cases in two months to two years.

Post Kala-azar Dermal Leishmaniasis (PKDL)

It is an important sequelae of Kala-azar found in India, China and Sudan. It develops when visceral infection persists for a year or so. The condition is characterized by hypopigmented or erythematous macules, papules and nodules. The skin lesions contain amastigotes of leishmania. A large number of *L. donovani* can be recovered from such skin lesions.

1.6.2 Mucocutaneous Leishmaniasis (MCL)

Mucocutaneous leishmaniasis or Espundia of South America is a clinical condition due to infection of reticuloendothelial cells, initially of the skin and
subsequently of the mucosae of the mouth and nose following *L. braziliensis* infection. It is characterized by the appearance of a variety of skin lesions; a papular lesion commonly becomes nodular and then ulcerates. Sometimes metastatic lesions may develop in the mucosae of the upper respiratory tract and the mouth, which extensively erode the adjacent tissues.

### 1.6.3 Cutaneous Leishmaniasis (CL)

Cutaneous leishmaniasis can present as hard, indurated plaques, nodules, scabby papules or warty tumor like lesions. Characteristically however, a lesion on an exposed area of the body begins as a red papule; enlarges to form a shallow ulcer with raised red margins and then heals with scarring. A healed lesion has a flat border with a slightly depressed area in the center that is completely reepithelialized with thin scar tissue. The infection may disappear spontaneously leaving disfiguring scars, or persist for a very long period causing multiplications.

### 1.7 Leishmania Antigen

Like the other protozoan parasites, *leishmania* consists of complex series of antigens (Crook *et al.*, 1969; Afchain *et al.*, 1972; Clinton *et al.*, 1972). Some antigens are species specific (eg. Oelerich 1973); some genus-specific (eg Oelerich 1973); and some are shared with related Generas, such as trypanosomas (Goble 1970, Neal & Miles 1970,
Afchain et al., 1972; Kagan 1973) or Striogomonas (Ranque et al., 1969).

In leishmaniasis, the antigens play a very important role pertaining to immunodiagnosis, immunopathology and protective immunity. Preston and Dumonde (1971), proposed that leishmania may contain two types of antigens; those which stimulate specific resistance to infection (particulate antigen) and those which depress specific resistance to infection (soluble antigen). Crook et al., (1969) reported that leishmania parasites constitute an antigen mosaic of eleven antigens in a lysate of *L. mexicana*. In a comparison between *L. braziliensis* and *L. donovani*, there was much antigenic overlapping among the organisms.

Differences in the antigenic composition between amastigote and promastigote stages of the parasites are documented by earlier studies. Although the above two forms certainly share some common antigen (Dwyer, 1973, Chang, 1981). Dwyer (1973) not only confirmed the presence of shared antigens in clones of amastigotes and promastigotes of the same strain but also found stage specific antigens in each form.

Antibody cross-absorption experiments showed that both the amastigote and promastigote stages shared some cross reactive antigens (Dwyer, 1976) among which four antigens
were common to promastigote and amastigote forms. These antigens were detected in cell free extracts of *L. donovani* (Simpson, 1968, Ghatak et al., 1982).

Previous studies have shown a cross reaction between leishmania and other protozoans in immunological tests (Martareshe et al., 1975; Roffi et al., 1980). Such cross-reactivity is also reported between leishmaniasis sera and mycobacterial antigens (Smrkovski and Larson, 1977). Infection with non-pathogenic or mildly pathogenic strains of *Mycobacterium* in normal human beings and laboratory animals can induce the formation of antibodies reactive with leishmania at low serum dilution (Allain and Kagan, 1975, Bardana et al., 1973).

It was also reported that both promastigotes and amastigotes of *L. donovani* share antigens that cross-react with components of blood cells from normal individuals of all ABO system type (Decker - Jackson and Honigberg, 1978). Adler and Gunders, (1964) differentiated these antigens among *L. tropica*, *L. donovani*, *L. braziliensis* and *L. mexicana*. There are also certain antigens shared between different species of Leishmania as shown in the immunofluorescent and ELISA tests (Edrissian and Darabian, 1979). Strains of *L. tropica*, *L. donovani* and *L. braziliensis* possess common antigens as shown by positive reaction in Kala-azar.
Since cross-reactivity between *T. Cruzi* and Leishmania species is well known in serological tests (Decker - Jackson and Honigberg, 1978), it was suggested that extracts from *L. donovani chagasi* promastigotes should be the antigen of choice for diagnosis of visceral leishmaniasis by means of IFA and IHA tests. These tests were considered more suitable for areas with known endemicity for trypanosomias is (Badarlo et al., 1983).

In an attempt to determine the role of T-lymphocytes and the nature of antigens that activate them, or antigen specific murine T-lymphocyte clone which proliferates in response to antigens present on the membrane of intact *L. donovani* promastigote were propagated. One such line cross reacts with membrane antigens on seven other species and to a lesser extent with antigens on African Procyclic Trypanosomes. These antigens were found to contain both mannose and galactose ligands. The antigen activity was also absorbed using extensively cross reactive monoclonal antibodies to parasites (Sheppard et al., 1983).

In a preliminary investigation on the surface antigen profiles of promastigotes from *L. donovani* complex, isolated in Brazil and Africa, no difference was observed. In addition, a glycoprotein of 65,000 molecular weight recognized by human sera from different geographical areas was the main component in both subspecies (Lepay et al.,
1983). A polysaccharide rich antigen was also isolated from the parasite (Ghose and Roy; 1983, Majumder, 1992). In an early study, soluble antigenic substances liberated by amastigotes of *L. donovani* were shown to be present in the sera of infected dogs (Nattan – Larrier and Gremard Richard, 1983). Adler and Gunders (1964) also demonstrated the release of exoantigens by *L. tropica* and *L. mexicana* amastigotes and promastigotes. These exoantigens were able to elicit skin response in ensitized persons (Sergevie and Shuikina, 1969). Clinton et al., (1972) 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., 1972). It was demonstrated that antigenic determinants are shared by promastigotes. This EF and the EF from the homologous amastigotes, are serologically specific for individual leishmanial strains. It is therefore, possible to define leishmania serotypes with the aid of EF (Schnur et al., 1972). The EF of *L. mexicana* was used as an antigen for coating wells for modified ELISA and sheep red cell agglutination assays. The ELISA as tested against sera of LD positive patients, was found much more sensitive than tanned red cell haemagglutination tests (TRCH).
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). The existence of leishmanial exoantigens was suggested by positive immune peroxidase staining in the cytoplasm of giant cells (infected with *L. major*) and non-parasitized epithelial cells (Sells & Burton, 1981).

Soluble immune complexes containing anti-leishmanial antibodies were demonstrated in human serum in visceral leishmaniasis (Casali & Lambert, 1979). It was also found that there are other substances released by *L. donovani* living promastigotes into the tissue for example, blood of infected hamsters. These substances differ from the excretory factors in their antigenic and biochemical characteristics. They were given the name of Antigenically Active Glycoproteins (AAGP). The AAGP were found to consist of 6-7 antigenically distinct components, which give reaction of identity and partial identity with human red cells and *Mycobacterium butyricum*. They also share certain antigens with a carbohydrate rich extract of *T. cruzi* (Decker-Jackson and Honigberg, 1978).

1.8 Antigen Preparation

Killed promastigotes have been used by many workers as antigen (Holbrook et al., 1981a; El-Amin et al., 1985). The
promastigotes may be killed by formalin treatment or by freezing and thawing. For the formalin treatment, the promastigotes are harvested during log phase growth by centrifugation (900 x g for 30 minutes), washed three times in Earle's balanced salt solution (EBSS) and killed by suspending in 0.1% formalin for 30 minutes at room temperature. Formalin killed promastigotes (FKP) are kept at 4°C overnight, washed three times, counted in a haemocytometer and resuspended in EBSS at the desired concentration (Holbrook et al., 1981a; Holbrook and Cook, 1983; Cook & Holbrook; 1983).

For preparing freeze - thawed antigen, the promastigotes are first suspended in EBSS and frozen at -70°C and thawed at 37°C. This procedure is repeated three times. The criterion for the determination of promastigotes death is the absence of parasite motility (Dwyer, 1976; Decker - Jackson & Honigberg, 1978; Cook & Holbrook 1983). The other method of antigen preparation includes disintegration of promastigotes to get a cell free extract. For such preparation the promastigotes are obtained from 72 hour old cultures, washed twice, by centrifugation and disintegrated in Mickle's disintegrator for 30 minutes. The obtained homogenate is further clarified by centrifugation (Ghatak et al., 1982). The parasites may also be disrupted by sonication at 30 MHz for one minute burst for five times in a Branson's
cell disruption. A complete disruption of cellular integrity may be verified by light microscopic examination (Rassam and Al-Mudhaffar, 1980, Cook and Holbrook, 1983).

Soluble and particulate fractions are also separated by centrifugation of the sonicated promastigote preparation at 4000 x g for 20 minutes. The supernatant is passed through 0.2 um millipore membrane filters. The particulate pellet is washed three times with EBSS and suspended to the original volume (Cook and Holbrook, 1983).

Rassam and Al-Mudhaffar (1980) prepared the soluble antigen by keeping the ultra sonicated promastigote suspension at 4°C for two hours and then centrifugating it at 7000 x g for one hour in a refrigerated centrifuge. The supernatant fraction representing the soluble antigen was dialyzed for 20 hours against distilled water at 4°C and then freeze dried.

The soluble and particulate fractions are also prepared by repeated centrifugation and washing of promastigotes followed by osmolysis in sterile distilled water at room temperature. The lysed suspension is centrifuged at 12,000 x g for 30 minutes in a refrigerated centrifuge (Jalees et al., 1981). Another method reported by Obaid et al., (1985), utilizes the osmolyzed parasitic suspension which is further centrifuged at 105,000 x g for one hour to obtain soluble and the particulate antigen.
fractions. The supernatant left after the last centrifugation represents the soluble antigen fraction (Ghatak et al., 1982).

1.9 Host Parasite Relationship

In contrast to the situation in many other protozoan diseases most patients who overcome the first bout of cutaneous or visceral leishmaniasis do not harbour a latent infection (Neal et al., 1969).

While, antibody is formed particularly in visceral leishmaniasis, it is not thought to be of critical value in protection and immunity in leishmaniasis. It is generally considered to be predominantly cell mediated (Zuckerman, 1975). Leishmaniasis may indeed serve as a prototype for a disease whose immune mechanism is dominated by cell-mediated reactions (Dumonde, 1973). The basic leishmanial lesion is the proliferating infected macrophage; itself a cell associated with immunity.

Intracellular symbiosis in leishmaniasis represents the ability of amastigotes to live in macrophages. For establishing such an intracellular symbiosis, the sequented events are important: recognition, intra-cellular entry, survival and multiplication (Moulder, 1979). Under certain conditions leishmaniasis may exert either synergistic or antagonistic effects on the host’s reaction to the other organisms or their antigenicity.
Leishmania macrophage interaction has been studied by infecting peritoneal macrophages in-vivo and in-vitro with *L. donovani* (Miller & Twhoy, 1967; Akiyama and Mcquillen, 1972; Ebert et al., 1976; Change and Dwyer, 1978).

At a critical point in the development of an immune response, foci of infected macrophages become surrounded by lymphocytes and plasma cells which attack and destroy emerging parasites or infected macrophages or both (Anonymous, 1968). Ruskin et al., (1969) summarized the fundamental cell-mediated immune mechanism against intracellular parasites as probably involving the specific sensitization of lymphoid cell by parasitic antigen and the delivery of a parasiticidal message of macrophages, which thereby become activated both against the specific homologous antigen and in some cases, against nonspecific heterologous antigens as well. In order for sensitized lymphocytes to arrest the development of a leishmanial focus, they must therefore establish intimate contact with the surface of infected macrophages (Garnham and Humphrey, 1969). Such contact releases agents leading to the secondary accumulation and activation of macrophages (Frankenburg et al., 1993). Interaction in vivo, between sensitized lymphocytes and macrophages infected with Leishmania may possibly be cytotoxic to the macrophages and parasiticidal to the liberated parasites (Bryceson et al., 1970a). The
lymphocytes being the active, and the macrophages being the relatively passive, partner in the interaction. Specificity inheres in the long lived, thymus dependent sensitized lymphocytes recognizing and interacting with its homologous antigens.

Cell-mediated immunity develops slowly with the clonal replication of such specifically sensitized lymphocytes (Garnham and Humphrey, 1969). Lymphoblast transformation was more intense in the presence of homologous leishmanial antigen than in the presence of antigens from heterologous species. Kutish and Janovy (1981) showed that the infective stage of *L. donovani* suppresses macrophage function beyond the time at which initial lysosomal fusion with the parasitophorous vacoule occurs. Light and electron microscopy revealed that in the presence of lymphokines, macrophages are activated and intracellular Leishmanias develop specific, subcellular lesions in the kinetoplast mitochondria (Chang and Chiao, 1981a).

Leishmaniasis, with the extensive changes it imposes on the lymphoid macrophage system, may non specifically affect the immune response to a host to other organisms or antigens. Human Polymorphonuclear cells (PMN) can ingest and kill *L. donovani* by the $\text{H}_2\text{O}_2$ - peroxidase halide system and may be capable of providing host defense against the invading promastigotes (Pearson and Steigbigel, 1981). The promas-
tigotes were found to be susceptible to killing, \textit{in-vitro} by $\text{H}_2\text{O}_2$ and other intermediates (Haidaris and Bonventre, 1982).

1.10. Experimental Leishmania Infection in Animal Model

A considerable amount of information has been gained from experimental studies of immune responses in animal models in the past few years. The choice of laboratory animals models for human leishmaniasis has generally been mice and hamsters. Monkeys are the recent addition to this menagerie (Wolf, 1976). It is evident that results from animal experimentation do not necessarily provide direct insight into specific human situation as the precise relationship between infection of laboratory animals and the corresponding human diseases is always difficult to establish. Laboratory experiments usually bypass the natural route of infection and a given animal model with respect to inoculation of a particular parasite, in different ways according to various factors such as age, dose and site of inoculation, genetic background etc. In view of these shortcomings, however, it is also evident that in the absence of more appropriate means, animal experimentation remains the only rational approach to precisely understand the complex immunological reactions that may be elicited by the host as a result of leishmanial infection. The usefulness of any animal model of human leishmaniasis is clearly determined by the degree of similarity of such models to the human disease, particularly its immune responses and immunoprophylaxis.
Infection with *L. donovani* in mice do not mimic the fatal visceralizing diseases caused by the parasite in man. However, the broad spectrum of responses to this infection in mice provide an appropriate system for analyzing the innate and acquired resistance to non-lethal systemic infections (Blackwell, 1982).

Since last few years hamsters have been proved to be a better animal model for *L. donovani* (Stauber, 1963). Stauber (1963) reported 50 fold increase in parasite numbers in first week with an enormous parasite load in liver and spleen leading to death, following an intra-cardial inoculation of amastigotes in hamsters. However, protection against reinfection is said to be present after cure (Heyneman, 1971). These models thus provide appropriate systems for analyzing protective capacities of antigenic preparations. They have specially helped us to define the basic properties required for a vaccine against leishmaniasis, as also for the *in vivo* and *in vitro* tests of biological function needed to identify, define and assay such candidate vaccines.

### 1.11 Immunity in Visceral Leishmaniasis

Interest in the immunology of leishmaniasis has been sporadic, since leishmaniasis could have important applications for the other intracellular infections as well. In addition a number of considerations support the desirability and feasibility of a vaccine for disease
control. These include the limited ability to control transmission with insecticides or by destroying animal reservoirs in many areas as well as through the use of chemoprophylactic drugs. The experimental leishmaniasis model in rodents has provided interesting insights into the disease and can potentially be exploited in developing novel immunological approaches to disease control in humans.

When a leishmania infected sandfly attempts to take a blood meal from its mammalian host, promastigotes are inoculated. Although leishmania exists only transiently in the promastigote stage, the initial interaction of promastigotes with the mammalian host defense mechanism is critical to the development of infection. Based on in vitro studies with human serum and polymorphonuclear leukocytes, it appears that promastigotes must escape potentially lethal humoral and cellular host defense mechanisms in order to gain entrance into macrophages. But once the leishmanias transform into amastigotes within macrophages, the resolution of infection depends predominantly on cell-mediated immune mechanism (Mauel & Behin, 1974; Rezai et al., 1978). Inspite of the above studies, a role of humoral immunity against leishmanial infection cannot be ruled out, usually humoral responses accompany cellular response in leishmaniasis as observed in laboratory animals.
1.11.1 Cell-mediated Immunity

The protective immune response to leishmaniasis is primarily cell mediated. Several animal models and in-vitro systems have been used to investigate the role of cell mediated immunity in host defense against leishmania (Zuckerman, 1975; Graham and Humphrey, 1969; Turk and Bryceson, 1971). Lymphokine containing supernatants can induce leishmaniacastic and leishmanicidal activity in amastigote infected mice peritoneal macrophages (Behin & Louis 1984). Reed et al., (1984) showed that mice infected with L. donovani and treated with lymphokine rich supernatants (encapsulated in liposome) have significantly fewer liver parasites than the control group, demonstrating an in vivo activity of lymphokines against an infectious organism.

Solubly (1972) suggested that infected macrophages in a human lesion may be targets for sensitized lymphocytes or for their secreted lymphokines. The crude lymphokines which prime the human monocyte derived macrophages to generate H₂O₂, exerting a microbicidal activity against intracellular L. donovani were found to be rich in interferon (IFN) gammas. It was suggested that IFN gamma is the key macrophage activating molecules present within human lymphokines. It can enhance both the oxygen dependent and independent antipariteal mechanisms of human mononuclear phagocytes (Murray et al., 1983). In the active stages of
leishmaniasis cell mediated immunity was found absent or not prominent. But after drug induced healing, generally a vigorous delayed response appears and the individual becomes solidly immune (Manson Bahr, 1961; Rezai et al., 1978). Allison et al., (1973) suggested that if infection macrophages should be inhibited *in-vivo* by the presence of sensitized lymphocyte secreting a migration inhibition factor, this would tend to confine the area of infection. Impairment of cell mediated immunity in kala-azar appears to be of generalized lesion (Halder et al., 1983). Some other workers have also shown that immuno supression in visceral leishmaniasis is both specific and non specific (Carvalho et al., 1981; Ho et al., 1983).

The available *in vitro* data indicate that the intracellular fate of amastigote depends upon the capacity of T-cells to activate macrophages (Zuckerman, 1975, Pompeu et al., 1992, Lytton et al., 1993). Restoration of specific T-cell responsiveness aided by antileishmanial drug therapy results in the reduction of antigenic load through parasite destruction along with decrease in the circulating antibody levels, particularly that of Immunoglobulin G class (Halder et al., 1983). Immunosuppresion of host guinea pigs by the administration of antilymphocyte screen (Bryceson and Turk, 1971) or cyclophosphamide, by whole body irradiation (Lemma & Yau, 1973), or by the induction of tolerance (Bryceson et
al., 1972) exacerbated infection and at the same time reduced cell mediated reactions, without necessarily affecting antibody synthesis. Since plasma cells continued to abound. The role in protection of each of the individual reactions that are the hallmarks of cell-mediated immunity is still conjectural (Blewett et al., 1971; Allison et al., 1973).

The fact that cell-mediated immunity is somehow involved in protection is strongly suggested by the failure of protection, when cell-mediated immunity is suppressed. However, even though cell-mediated immunity may contribute to resistance, the degree of resistance is not always commensurate with the extent of delayed hypersensitivity (Dumonde, 1973; Targett, 1973). The immunosuppressed guinea pig with L. enriettii is considered analogous to the human being with diffuse cutaneous leishmaniasis (Bryceson, 1973).

Whereas immunosuppression of cell-mediated immunity in guinea pig clearly damaged the protective mechanism against L. enriettii similar experiments in mice with human leishmaniasis were equivocal. The lesions of L. tropica major were exacerbated in thymus-deprived CBA mice (Preston et al., 1972) and in other mouse lines (Targett, 1973), but neonatal thymectomy did not modify the susceptibility of Balb/c mice to L. donovani (Schmunis & Herman, 1971).

It was also found that affected human and rodent horts commonly show abnormalities of T - cell function which may
be related to change in macrophage physiology due to intracellular parasitism. Macrophages infected with *L. donovani* product increased amounts of arachidonic acid metabolites with the potential for influencing cellular immune functions and the inflammatory response to infection (Reiner and Malemud, 1985). It was further suggested that the protection by specific cell-mediated immunity against *L. donovani* infection is not quite absolute. Such protection is probably dependent on other host related factors leading to the destruction of parasite and recovery of patient (Halder et al., 1983).

1.11.2 Humoral Immunity

In the course of human visceral leishmaniasis the immunoglobulin titre rises very sharply, chiefly in the IgG and IgM compartments (Turk & Bryceson 1971, Manson - Bahr 1971, Bray 1972). Gamma globulin may reach 50gm/ litre and may comprise 50% of the total serum proteins (Stauber 1970). Most of this product is not specific antibody, and is not protective (Clinton et al., 1969; Neal et al., 1969), but is attributed to the continuing synthetic activity of a greatly hypertrophied and damaged lymphoid macrophage system. The product may only be the antibody produced in response to damaged host cells (WHO/LEISH/687.7). This mass of globulin in the plasma can be coagulated, flocculated, or precipitated by a variety of reagents, and its presence is the
basis of formol gel and the antimony tests. These tests are simple to perform in the field (Cahill 1970, Chowdhury et al., 1993). Despite the non-specific nature of the globulin, these tests are diagnostic of active kala-azar, and a plea has recently been accepted to retain them for field use.

The view that antibody produced in human leishmaniasis is non-protective is a widely accepted concept (Neal et al., 1969; Stauber, 1970). However, many have noted the frequent presence of plasma 'cell', in leishmanial lesions denoting the probable local production of antibody at the focus of infection (Stauber 1970). Furthermore, in lymphatic tissue of guinea pigs with *L. enriettii* infection both germinal centers (associated with humoral immunity) and procortial regions hypertrophied (Bryceson and Turk, 1971).

Similar hypertrophy of germinal centers occurs in mice with *L. tropica* infection and is accompanied by a marked rise in circulating antibody titer. Thus humoral responses accompany cellular responses in leishmaniasis in laboratory hosts. (Preston et al., 1972). It is suggestive that antibody is somehow associated with the healing process. This view is supported by the fact that cyclophosphamide, which damages the host's ability to produce antibody, interfered with healing when administered to mice with developed infections of *L. tropica* (Preston 1973). A factor in immune rabbit serum inhibiting the development of promastigotes *in vitro* was
ineffective in vivo (Rezai et al., 1970). Antibody from guinea pigs immune to L. enriettii was also found cytotoxic to promastigotes in vitro. Mauel (1973) suggested that the same antibody may contribute to parasite destruction in vivo. The possibility thus still exists that some protection may be potentiated by antibody in leishmaniasis (Allison et al., 1973).

It was recently observed in hamsters with L. donovani that there exists a relationship between the increase in the number of antibody secreting cells per spleen and the increase in the number of parasites in this organ. These observations suggest that the hypergammaglobulinaemia present in infected hamsters may be the result of polyclonal activation of B-cells induced by components of this parasite (Campos-Neto and Bunn-Moreno, 1982). It was also suggested that hypergammaglobulinaemia may be the consequence of an imbalance of regulatory T-cells, possibly associated with direct stimulation of hamster B-cells by L. donovani components (Bunn-Moreno et al., 1985). Hoover et al., (1985) showed that infection of individuals with L. donovani resulted in the production of a new quantitatively and qualitatively distinct immune mechanism directed against amastigote forms of the parasite.

The immune mechanism was termed as antibody-directed and an alternate complement pathway-mediated cytotoxicity.
The resistance of *L. donovani* to screen complement factors may be the primary reason for its ability to escape from the site of inoculation and cause discriminated disease (Hoover *et al.*, 1984). Pearson and Steigbigel (1980) showed that promastigotes are killed by activation of the classical pathway. In contrast Chang (1981b) reported that *L. donovani* promastigotes activate the alternate pathway.

*In vitro* experiments showed that amastigotes enter macrophages without being opsonized by antibodies. Within the host macrophages amastigotes are protected from antibodies as also from other circulating substances that might be harmful to the parasite (Report of WHO expert committee, 1984).

Natural IgM antibodies in the sera of a rodent and lagomorph hosts agglutinate and lyse leishmanial promastigotes, but not amastigotes (Schmunis and Herman 1970). In nature, this would tend to destroy most of the parasites inoculated by sandfly bite. Such extensive destruction has been observed with hours of syringe inoculation in hamsters receiving *L. tropica* promastigotes (Zuckerman 1953). Anti leishmanial antibody frequently produced is often cross reactive with trypanosoma, particularly with *T. Cruzi*, and is non-protective by passive transfer (Neal *et al.*, 1969; Stauber 1970; Heyneman 1971).

In neoplasia, antibody may possibly mask antigenic determinants, thus blocking the induction of a cell-mediated
response and leading to enhancement of infection (Clinton et al., 1969; Turk and Bryceson 1971). After cure, the production of antileishmanial antibody is interpreted as a signal of the continuing presence of parasites or parasitic amastigotes in the host. Since antibody titres are not altogether nil on cure; eradication of leishmanial disease from a given region by chemotherapy or other means can be monitored (Bittencourt et al., 1968; Ambroise 1970). When antibody titers remain elevated despite apparent clinical recovery, continuing occult infection rather than radical cure is suspected (Walton 1970).

1.12 Pathology of Visceral Lesihmaniasis

Kala-azar is defined as reticulo endothelialosis resulting from the invasion of the reticuloendothelial system by *L. donovani*. Following inoculation by the sandfly promastigotes enter reticuloendothelial cells and multiply. At the site of inoculation a granuloma develops, consisting of histiocytes filled with amastigotes and surrounded initially by epithelioid cells and later by giant cells as well. Parasites spread to local lymphnodes and homatogenously enter macrophages and then to the liver, spleen and bone marrow. They stimulate a granulomatous cellular immune response that results in subclinical disease and spontaneous resolution or when they multiply further they cause the clinical syndrom of kala-azar (Report of WHO Export
Committee, 1990). Once in the host tissue, the parasite is taken up by phagocytic cells and disseminates throughout the reticuloendothelial systems. It then resides and multiplies in macrophages as an obligate intracellular amastigote. Over a period of weeks to months, the host develops progressive hepatomagaly, splenomegaly and cachexia, and often dies from bacterial superinfection.

Massive splenomegaly is common (1000 to 3000 g) and hypersplenism is believed to be a major cause of anaemia, neutropenia and the thrombocytopenia of kala-azar. Spleen is the most affected organ. It is grossly enlarged and the capsule is frequently thickened due to perisplenitis. It is soft and friable and cuts easily without resistance due to absence of fibrosis. The cut section is real or chocolate in colour due to the dilated and engorged vascular spaces. The trabeculae are thin and atrophic. Microscopically, the reticular cells are thin and increased in numbers and are loaded with LD bodies (Aikat et al., 1979; Chulay et al., 1985).

The pathological changes which occur in the liver in kala-azar are that the kupffer cells are affected and become enlarged and parasitized. The reticulo endothelial tissue invades the lobules and separates the hepatic parenchymal cells. In the central zone, the capillaries are dilated and the parenchymal cells are compressed and may be atrophied.
There is usually some fatty change in the parenchymal cells. At a later stage the reticulo-endothelial tissue is partly replaced by fibrous tissue, producing lobular cirrhosis (Napier, 1951). These infected kupffer cells have deformed mitochondria disrupted endoplasmic reticulum and localized rupture of cell membrane. The hepatocytes in the vicinity of damaged kupffer cells also show degenerative changes within electron dense mitochondria. The cut surface may show a nutmeg appearance (Pampiglione et al. 1974).

Usually, two types of lesions are observed microscopically in the liver: a granulomatous reaction consisting of histocytes and lymphogranulocytic cells with some eosinophils. The other type of lesion shows extensive areas of haemorrhagic necrosis, representing disappearance of hepatocytes, erythrocytic congestion, extravasation and reticular collapse (Pampiglione et al., 1974).

The bone marrow is heavily infiltrated with parasitized macrophages which may crowdout haemopoietic tissues. Peripheral lymph nodes and lymphoid tissues of the nasopharynx and intestine are hypertrophic due to infiltration with the parasitised cells, though this is not frequently seen in Indian cases (Report of WHO Expert Committee, 1990).

Anaemia occurs as a result of infiltration of the bone-marrow as well as by the increased destruction of
erythrocytes due to hypersplenism. Auto antibodies to the red cells may contribute to haemolysis. Leucopenia with marked cells may contribute to haemolysis, leucopenia with marked neutropenia, and thrombocytopenia are frequently seen. Polyclonal hypergamma globuinaemia is a common finding (Aksoy et al., 1970; Bray and Wilson, 1972). Other changes include mucosal haemorrages due to thrombocytopenia and prothrombin depletion, intestinal ulceration, myocardial damage, amyloidosi and cloudy swelling of the liver (Pampiglione et al., 1974).

Experimental leishmaniasis in golden hamsters results in splenomegaly and hepatomegaly. Anaemia and leukopenia with marginal lymphocytosis, and having no increase in monocytes, are also some of the other features (Jalees et al., 1981). Hamsters inoculated intraperitoneally with L. donovani show progressive testicular atrophy. Spermatogenic cells of the seminiferous tubules show vacuolar degeneration with a decrease in the number of sperm cells leading to a total azospermia. Lymphoplasmacytic infiltrates with macrophages containing leishmania parasites in the inter tubular spaces were observed. Testicular amyloidosis is thought to be a pathogenic mechanism related to a dysfunction of the plasma cells. The stimulation of the RES is attributed to the antigenic character of parasites (Gonzalez et al., 1983).
1.13 Immunological Adjuvant

Adjuvants are defined as substances that are incorporated into or infected simultaneously or concomitantly with an antigen. These substances could stimulate cell mediated (delayed-type hypersensitivity) or allergic responses and autoimmune reactions against homologous or heterologous tissue antigens (WHO Tech. Rep. Series 595, 1976; Elderman 1980).

A good immunomodulating adjuvant must conform to the following attributes.

1. It should not induce hypersensitivity reactions to the host's own tissues or to itself.
2. It must not contain cross-reactive antigens with human tissues.
3. It should not be carcinogenic, teratogenic or abortogenic.
4. It should not be contaminated with substances that might stimulate other immunological events not involved in the specific response.
5. It must be biodegradable in human body.
6. It must never produce harmful nodules or abscesses if administered intramuscularly (im) or subcutaneously.
7. It should not be unstable in the manufactured vaccine for at least a period of two years.
So far various adjuvants have been used in combination with leishmania antigen for immunization purposes. Adjuvants which were found in useful immunotherapy, or which have been major subjects of experimental investigations have been elucidated and discussed here.

1.13.1 Freund's Adjuvant

Freund's et al., (1948) was the first to demonstrate the importance of using Freund's complete adjuvant (FCA) in combination with parasitic antigen material for achieving protective immunity. Subsequent vaccination in avian, rodent and simian malaria further confirmed the above findings (Cohen et al., 1985, Maheswari et al., 1989).

Freund's complete adjuvant consists of dead mycobacterium suspended in mineral oil with an added emulsifier. It induces a powerful cell-mediated response, humoral immunity, breaks tolerance and potentiates tumor rejection. Freund's incomplete adjuvant (ie oil emulsion adjuvant) is used in veterinary vaccines but because of its tendency to cause sterile abscesses at the site of injection it has been abandoned for human use. In malaria, many investigators used FIA in their vaccination studies (Freunds et al., 1948, Targett and Fulton, 1965, Siddiqui 1977; Perrin et al., 1984; Siddiqui, 1987 and Holder, 1988). Although the FCA is a very effective adjuvant but because of
its toxic side effects it is totally unacceptable for human use.

1.13.2 Aluminium Salts

Recently, alum hydroxide, the only adjuvant approved for human use, is employed particularly to raise the immunogenecity of toxoids in the development of antibiotic antisera. The first application of alum as an adjuvant was reported by Gleny et al., (1926), who used diphtheria toxoids precipitated with potassium alum KAI (SO₄)₂. 12H₂O) filtered and emulsified with water in experimental vaccination of guinea pigs.

Jarecki - Black in 1988 demonstrated that immunoprophylaxis is feasible using a combination of killed promastigotes and aluminium hydroxide in a subcutaneous immunization. It can successfully induce resistance against *L. donovani* infection. The mechanism responsible for the success of aluminium hydroxide as an adjuvant still remains to be identified. It is known that cellular responses to the infection of antigen aluminium gel include granuloma formation and the influx of effector cells such as macrophages and plasma cells.

1.13.3 Bacillus Calmette Guerin (BCG)

BCG as a potent immunostimulant is presently the most commonly used non specific stimulating substance in human
therapy. It enhances the production of antibodies and increases the number of the spleen antibody producing cells. It also increases delayed type hypersensitivity for particulate and soluble antigen (Pinhata et al., 1993). It accelerates the rejection of skin allograft and transplanted tumors in mice and inhibits carcinogenesis. It also tends to modify the action of chemotherapeutic, such as cyclophosphamid (Mackaness, 1970). In respect to toxicity, the BCG may act synergistically or antagonestically with certain chemotherapeutic drugs (Milstein and Gibson, 1990).

1.13.4 Muramyl Di Peptide (MDP)

Another adjuvant, muramyl di peptide has been known for more than a decade to be a minimal structure capable of replacing mycobacteria in Freund's complete adjuvant (Ellouz et al., 1974). The compound has been shown to enhance antibody response to a variety of natural or synthetic antigens even when administered only in saline (Chedid et al., 1976). When incorporated into liposomes MDP is effective in increasing cellular immunity (Masek et al., 1978). Several investigators examined the relationship between chemical structure and adjuvanticity (Chedid et al., 1976; Azuma et al., 1976).

1.13.5 Trehalose Di Mycolate (TDM)

The first well defined immunoactive mycobacterial glycolipid was identified as a cord factor - a mixture of 6-
6-di esters of D trehalose with natural mycolic acids, which is purified from mycobacteria and other microorganisms is known to have adjuvant activity. It has antiinfectious, anti parasitic and anti tumor (Lederer 1982) activity. Intra-peritoneal injection of TDM in oil emulsion was able to protect against an intraperitoneal challenge by Salmonella typhi and Salmonella typhimurium. (Yarkoni & Backierkunst, 1976). Oil emulsion of TDM is also active against tumors (Old et al., 1980, Sakuvai et al., 1989).

TDM emulsion in the presence of oil is toxic, since the toxicity largely depends on the percentage of oil used. Aqueous emulsions are very stable, they are active in mice against Klebsiella pneumoniae and Listeria monocytogenes (Parant et al., 1977) and can protect mice against Babesia microti (Clark, 1979), Schistosoma mansoni (Olds et al., 1980), Taxoplasma gondii (Masihi et al., 1979) and Trypansoma cruzi (Leon et. al., 1983). Protection studies with TDM were also carried out by Kumar and Ahmad (1984) and Lederer (1986, 1988) in the mouse model infected with P. bergei. They observed good protection in mice.

In vitro experiments with mouse macrophages pre treated with an aqueous suspension of TDM showed enhanced phagocytosis of liver glutaraldehyde - killed Trypanosoma cruzi and latex particulates indicating that TDM molecules modulate macrophage functions by augmenting both
internalization and intra cellular destruction (Kierszenbaum et al., 1984).

1.14 Biochemical changes
1.14.1 In Liver

Ever since the discovery of *L. donovani* in the viscera of humans by Leishman & Donovan in 1903, extensive research on various aspects of the parasite including metabolic processes, membrane structure and function, enzymatic machinery and their chemotherapy and immune system of host have been carried out. But even till date comparatively little is known about the sequential changes that occur in the various biochemical markers such as macromolecules and enzymes of the host during the course of *L. donovani* infection. In other protozoal infections such as trypanosomiasis and malaria, however, extensive researches have been made on metabolic processes and enzymatic machinery of the host.

In kala-azar the liver is enlarged and considerable biochemical and pathological changes in the organ take place (Napier 1951). Parasites mostly derive their nutrition from the host material and liberate metabolic end products in the host system. Upto a certain limit no deleterious effects manifest in the host, though host-immune system does get stimulated, when the balance breaks down. Ill effects of parasitism are observed in the form of pathological lesions
and nutritional deficiencies. Significant changes in biochemical parameters occur comprising of different enzymes and chemical constituents of liver.

Common histological feature of the liver tissue in leishmaniasis is that of the so called "fatty degeneration" in addition to centrilobular necrosis. Mercado and Von Brand (1958), who biochemically examined this aspect and observed a total increase in the hepatic lipid in animals infected with malaria. A similar observation was made by Riley and Maegraith (1962), who biochemically examined this aspect and found a total increase in the liver fats from 10-25% over control value. The finding by gas chromatography of the fatty acid contents of these lipids supported the above observation, besides showing a significant increase in unsaturated fatty acids, such as oleic acid and linoleic acids along with corresponding decrease in the saturated palmitic and steric acids.

In electron microscopy, simple lipid deposition in the form of droplets in close proximity to the damaged mitochondria was a common finding in liver from infected animals (Fletcher, 1964).

Gutierrez et al., (1976) have demonstrated increase in lipid contents of liver in Actus monkeys infected with P. falciparum. Maegraith (1966) has also observed significant increase in lipid contents of liver of rhesus monkeys.
infected with *T. knowlesi*. Mercado and Von Brand (1958) and Angus (1971) on histochemical examination reported the presence of lipid in the liver of mice and monkeys infected with *P. knowlesi* and *P. berghei* respectively. Further, Fletcher (1987), Rajvir et al. (1981) Saxena et al., (1981) and Gupta (1988) have also found raised levels of total lipid contents.

A close co-relationship between the cellular injury, peroxidation of membrane lipids and oxidative damage to cells have been reported by Sharma & Krishnamurty (1976). In consonance with observations on animals under the effect of toxic chemicals or pathological conditions, liver homogenates of golden hamsters infected with *L. donovani* produce higher amount of lipid peroxides and this increase is directly proportional to the number of amastigotes in the liver of golden hamsters.

Saxena et al., (1981) have shown that liver and spleen produce higher amounts of lipid peroxidation due to an increased susceptibility of these tissues to oxidative damage under the stress of the infection. In Vivo experiments have shown that *L. donovani* cells are killed by oxidative radicals. One mechanism of survival of the parasite in the host phagocytic cells may be the alternation in production of the toxic metabolites by leishmanial phosphatase activity.
Hypoglycaemia in malaria infections has also been described (Maegraith 1948). Srivastava et al., (1984) have reported that during acute \textit{P. knowlesi} infection in rhesus monkeys, glycogen was depleted for a considerable extent. This depletion was mainly due to excessive breakdown of glycogen by phosphorylase.

In the ecology of hypoglycaemia, the possibility of monokines acting as a significant mediators cannot, however, be ruled out (Clark et al., 1981). Clark and his co-workers have produced experimental evidence which according to them indicates a central role for these monokines with regards to protein metabolism. Significant decrease was reported in the protein contents which mainly causes hypoproteinamia. Von Brand (1973) has noted decreased protein synthesis during malaria. In our studies, we have also found a decrease in liver protein. This might be due to decrease in synthesis of nucleic acids.

1.14.2 Serum Analysis

It is well documented that leishmaniasis infection impairs liver function. The liver damage caused by invading parasites during leishmaniasis can be detected by means of several immunological and serological tests. Sadun et al.,(1965, 1966) have observed a significant increase in transaminase (viz. SGPT, & SGOT) levels in serum, which is indicative of abnormal liver function. Lal & Hussain (1978) have reported
a significant decrease in the GPT and GOT activity in the liver of mice infected with *P. berghei*.

1.15 Current Treatment of Leishmaniasis (Anti leishmanial drugs)

Response to treatment varies considerably from one form of the disease to another and among different geographic areas when the same disease is involved. For example, in India, China and Brazil, kala-azar responds relatively well to one course of antimony treatment while in Sudan it is notoriously resistant. East African and Mediterrancan kala-azar may require three courses of the usual drugs or retreatment with secondary agents. Three courses of antimonials are often necessary in South American mucosal leishmaniasis. The initial cure rate is about 50%. The decision regarding treatment of cutaneous leishmaniasis depends upon the area of the world and the location of the lesion.

In the Eastern Hemisphere, Mexico and portions of Central America, where spontaneous resolution is the rule and the ulcer is not in sight that would produce a disfiguring lesion when healing occurs, treatment may not be necessary. In other areas, such as most of South America, where there is a high incidence of mucous membrane involvement, treatment with antimonials is recommended by many authors’ (Olliaro and Bryceson, 1993).
In the New World, the identification of the parasite is important in determining the need for treatment.

1.15.1 The Antimonials

[A] Tartar Emetic (Antimony Potassium Tartrate)

This was the first effective antileishmanial drug to be given clinical trial, when Gaspar Viannia successfully used it against mucocutaneous leishmaniasis in Brazil in 1912. Inspite of the toxicity of this chemical, it must have been heralded as a "miracle drug" by the inhabitants of India, where its subsequent use against kala-azar reduced the mortality rate by about 95%. Tartar emetic has also been extensively used against Mediterranean Kala-azar (L. infantum) and oriental sore (L. tropica).

[B] Stibophen (Sodium Antimony III) Bis Catechol-3,5-disulfonate)

The considerable side effects, of tartar emetic soon prompted the investigation of a variety of other antimonials, but all other trivalent agents proved to be only slightly less toxic. Stibophen (Fouadin,, Reprodral) is perhaps the best known of these and was widely used until the advent of the pentavalent drugs. It appears to be much more effective against cutaneous leishmaniasis than against kala azar.
The only antileishmanial chemotherapeutic agent with a clearly favourable therapeutic index is pentavalent antimony (Sb) complexed to carbohydrate in the form of sodium stibogluconate (Pentostam) or meglumine antimonate (Glucantime). Pentostam is manufactured by the Wellcome Foundation in the United Kingdom and Glucantime is manufactured by Rhone-poulence in France and is used in French and Spanish-speaking countries.

Pentavalent antimonials can be tolerated at much higher dose levels than the trivalent ones, and they are excreted more slowly. The discovery was a major step forward in the chemotherapy of leishmaniasis, and the pentavalent antimonials remain the drugs of choice for many in present day treatment of both cutaneous and visceral disease (Johnson, 1990).

The structures of the clinical antileishmanial agents are shown in (Figure 1). A commonly drawn structure of Pentostam shows two molecules of gluconate complexed via oxygens to two molecules of Sb, with three sodium atoms balancing the three negative charges on the rest of the molecule (Rollo, 1965). The molecular weight for this structure is 746, and the osmolarity of a solution of 100 mg of Sb/ml would be 1,644 mol.
Pentamidine

Ketoconazole

Amphotericin B

Allopurinol
Pentavalent antimonials such as urea stibamine stibosan and neostibosan became available in the 1920 (Shortt 1945). Pentostam was first manufactured and used between 1935 and 1945, (Kerk R. & Sti N.H.). There has been no valid comparison of efficacy between the preparation of (Pentostam and glucantime) and both are given by slow intravenous infusion or by intramuscular injection during a ten day course, Pentostam is given in a daily dose of 6 ml (0.6 gm of antimony). Glucantime is supplied as a 30% solution and given as 10 or 20 ml per day to a total dose of 200-250 ml. Indian visceral leishmaniasis usually responds to one course of therapy whereas the disease seen in Africa often requires three courses (one month).

Antimonials have toxic effects although less toxic than the trivalent compound. Vomiting may follow an intravenous infusion. Chulay et al., (1985) described in detail the cardiotoxicity in Kenyan kala-azar treated with pentostam. There may be abdominal pain and hypotension, as well as hepatic dysfunction. Renal insufficiency is not uncommon.

[D] Glucantime

Another well tolerated pentavalent antimonial, Glucantime has been found to be effective against most forms of leishmaniasis, It can be given intravenously but the intramuscular route is recommended. Compounded with Pentostam, it has the disadvantage of requiring much larger
amounts of inoculation. It is produced in ampules of 5 ml aqueous solution, each containing 1.5 gm of meglucantime antimonials. Half the initial dose is given as a sensitivity test followed by a big dosage from 5 to 10 ml for 10-20 days. Glucantime has been successfully used against canine kala-azar in France and Brazilian (Barrios et al., 1986).

[E] Neostibosan (Ethyl Stibamine)

The stibanitic and derivative is comparatively non toxic and is useful in the treatment of kala-azar. Its drawback is that, it is not very stable in solution and should be freshly prepared for each administration. It contains upto 42% antimony and can be given in 25% solution intravenously, usually on alternate days. For the adults, the initial dose is 0.1 gm, followed by 0.2 gm and 0.3 gm. A total of 2.7 gm is recommended in ten injections.

The drug has been extensively used in treating infantile kala-azar. In children under 1 year the total dose is 0.1 to 0.15 gm in a course of sixteen intravenous injections. 0.2 - 0.25 gm for children under two years, and 0.3 gm in older children.

[F] Urea Stibamine (Carbostibamine, Carbantine, Stiburea)

Early trials with this pentavalent antimonials gave exceptionally good results against Indian kala-azar. Unfortunately, batches seen to vary considerably in the mode of preparations, antimony content, toxicity and efficacy, as
an antileishmanial agent. This drug is often used in combination with Neostibosan. The drug is administered intravenously an aqueous solution. The total amount recommended is 3 grains given in doses of 100 to 200 mg on alternate days over a period of 4 weeks.

1.15.2 The Aromatic Diamindenes

If the treatment with antimony is unsuccessful the aromatic diamines may be used (Rees et al., 1985, Sand et al., 1985). They are very effective for the treatment of kala-azar but because of their many and frequent side effects they are considered drugs of second choice.

[A] Stibamidiene Isothionate

This compound was found to be one of the most efficient agents known against kala-azar, even against those obstinate cases of the Sudanese disease that resisted antimonial treatment. However, this drug has a very serious side effect. It was found that in Medeterranean region, the kala-azar cases treated with this drug developed a serious trigeminal neuropathy and these neuropathological signs have been noted for as long as twenty years after the treatment.

Now a days stibamidine is used mostly as a last resort in antimony resistant visceral leishmaniasis. It is given daily by the slow intravenous injections of a 10% aqueous solution. The initial dose for an adult is 0.025 gm and the
dose is increased very slowly to reach a total of 2.0 gram per kilogram body weight over a period of ten days.

[B] Pentamidine Isothionate

Among the aromatic diamidine pentomidine isothionate has been extensively used against Sudanese kala-azar. It is given intramuscularly at a dose of 4 mg/kg diluted in water on successive or alternate days. Side effects include hypotension, nausea and vomiting. It is nevertheless linked with possible subsequent polyneuritis and the development of diabetes. Hypoglycaemia has been reported and may be severe as to cause death. Reversible renal insufficient occurs as well (Bryceson et al., 1985).

1.15.3 Other Antileishmanial Drugs

[A] Berberine Chloride

This is a derivative of the plant alkaloid berberine and has had some success when used to infiltrate lesion due to *L. tropica* (oriental sore). It is very effective against post kala-azar dermal leishmaniasis.

[B] Cycloguanil Pamoate (Cycloguanil Embonate)

Camolar is a "Single shot" intramuscularly administered drug with a repository action. Its efficacy against leishmaniasis was observed by chance in initial trials on malaria prophylaxis in the Americans. Interestingly although, good results have been obtained against American cutaneous
leishmaniasis. The drug seems uneffective against oriental sore due to *L. aethiopica* in Ethiopia.

1.15.4 Antibiotics

1) Amphotericin B (Fungizone)

This antibiotic is derived from the soil fungus *Streptomyces nodosus*, first discovered in Venezuela and it is a potent antileishmanial agent (Sampaio et al., 1970). However, its use is largely restricted to the treatment of advanced cases of mucocutaneous leishmaniasis that have not responded to the more conventional drugs, since it is highly toxic and very expensive (Thakur et al., 1993).

A test dose (1 mg) of the drug should be used to determine patient response to this polyene. If there is no reaction then the drug may be started at 0.1 mg/kg of the body weight per day and enclosed by 0.1 mg/kg per day to a daily doe of 0.3 – 0.7 mg/kg dose of 1-1.5 gm. The immediate side effects are nausea, vomiting, chills, and fever associated with infusion of the drug. Later anemia may occur along with progressive renal insufficiency. The renal insufficiency usually disappears upon discontinuation of the drug but can be a serious problem during treatment.

[B] Monomycin

Russian workers (Arustcumyan 1967; Akovyan and Mukhamedov, 1968) have reported on the efficacy of this antibiotic after injection into skin lesions due to *L. major*,
but there seems to have been no work on this substance for other forms of leishmaniasis.

[C] Rifampicin

This antibiotic has recently been used to treat cutaneous leishmaniasis due to *L. tropica* in Kuwait. Results have not been clear cut and the drug is at present very expensive.

1.15.5 Other Potential Agents for Visceral Leishmaniasis

There are several approaches which are still in the pre-clinical evaluation. It may be quite appropriate because only these modalities are reasonably likely to supplant Sb as primary therapy for kala-azar. WR 6026 is the primaquine analogue with high activity against visceral disease in some animal models. WR 6026 would be the first effective orally administrable agent for kala-azar.

Liposomes are artificial lipid particles that are predominantly phagocytized by macrophages of the visceral reticuloendothelial system. The administration of drug containing liposomes delivers large quantities of drug to the macrophage in which amastigotes reside. In fact, a disease due to an obligate intra macrophage microorganism such as leishmaniasis would seem to be ideally suited for liposomal therapy. At least several formulations of liposomes contain antimony (Berman, 1985) and one containing amphotericin B (Berman et al., 1986) is being considered for human trials.
The exciting potential of the liposomal approach has to be tempered by the reality of developing an agent that requires new technology that meets US Food & Drug Administration requirements and that will be marketed primarily in the developing world.

1.15.6 New Oral Agents

There are published reports on the use of allopurinol in visceral disease and of ketoconnazole in cutaneous disease. In Indian kala-azar allopurinol treatment for 14-31 days (11-36 mg/kg per day) produced cures in eight out of sixteen patients on the basis of negative bone marrow cultures after therapy, but only three (19%) of the sixteen cases could be shown not to relapse (Jha, 1983). In a different study, five patients in whom Sb therapy had failed were cured with allopurinol plus the same daily dose of Pentostam that had failed.

One adverse reaction to allopurinol in these studies has been rash. A major theoretical concern about high dose allopurinol therapy is metabolism of the drug via xanthine oxidase to oxypurinol and generation of oxypurinol renal stones. The ribonucleoside of allopurinol has been administered at a dosage of 1,5000 mg (25 mg/kg) without apparent toxicity (Research Triangle Park, 1984). The upper limit on dose may be based on cleavage of the ribonucleoside
to allopurinol by gastrointestinal bacteria and on the toxicity of allopurinol and secondarily generated oxypurinol.

The first reported clinical use of ketoconazole was Nicaraguan cutaneous leishmaniasis, and several studies have investigated this oral agent for American cutaneous disease. Treatment with 200 mg twice a day for 3 months resulted in cure of all of six Nicaraguan patients (Urcuyo, 1982). The data on the efficacy of ketoconazole for cutaneous leishmaniasis are encouraging in part. In the ongoing Panamanian study, this oral agent is as active as the positive control drug (Pentostam) and patients are being randomized between the two groups.

Ketoconazole does not seem to be very active against *L. braziliensis* disease in other regions of the New World. As in the case of allopurinal ribonucleoside, biochemical considerations suggest that keto conazole is likely to be active and the drug should be tested in randomized trials with positive and negative control on regions endemic for cutaneous leishmaniasis.