Chapter I

Introduction

Section I

*Leishmania* and leishmaniasis

The leishmanias comprise a group of unicellular hemoflagellate protozoan parasites of vertebrates, which are transmitted from host to host by the bite of sandfly vectors. They are closely related to trypanosomes and are grouped under Kinetoplastida, which also includes *Crithidia, Leptomonas* and other species. *Leishmania* has a digenetic life cycle consisting of an extracellular flagellated motile form called Promastigotes, in the digestive tract of the insect vector and an intracellular, sessile form, or Amastigote, in the mononuclear phagocytes of vertebrate host. A wide variety of *Leishmania* species, infect reptiles and mammals, causing a spectrum of diseases in humans collectively known as leishmaniasis (1). According to a WHO/TDR report (2001), there are some twelve species of *Leishmania* which cause leishmaniasis.

Historical background

The first record of leishmaniasis is found on tablet inscriptions in Assyria (modern Iraq) in the 2-3rd century BC (2). It prevailed in 1st century AD as evidenced by representations of skin lesions and facial deformation on pre-Inca pottery from Peru and Ecuador. There are detailed descriptions of oriental sore by Arab physicians in the 10th century, and there are later records from various places in the middle east including Baghdad and Jericho. Leishmaniasis was prevalent in India as 'kala-azar', long before the parasitic nature of the organism was known. The history of kala-azar in India started from Assam, where several epidemics occurred, the severest one being in 1875. Though Cunningham (1885) first saw the parasites in the histological sections of 'Oriental sore' in India (3) and Borovsky (1898) called it protozoa, yet Rogers (1897) and Ross (1899) considered kala-azar to be a severe form of malaria. The real cause of the disease was established when Leishman, in May 1903, reported the
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finding of a parasite in the necropsy material of the splenic smear of a soldier who died in England from a fever contracted at Dumdum, Kolkata, known as the ‘Dumdum fever’ (kala-azar) (4). He correctly related it to the trypanosomes. Donovan in July 1903, found a similar organism in the biopsy material from the spleen of a patient suffering from kala-azar in Madras (5). Ross, in 1903, named the parasite as *Leishmania donovani*. Rogers (1904) succeeded in cultivating the organism and obtaining the flagellate forms. Luhe (1906) isolated the *Leishmania tropica* and Nicolle (1908) introduced the name *Leishmania infantum* for the parasites of Mediterranean kala-azar on account of the disease being strictly confined to infants and young children. According to the Indian kala-azar commission (1931-34), the sandfly, *Phlebotomus argentipes* is the probable transmitter. In 1921 the experimental proof of the transmission to humans by sand flies belonging to the genus *Phlebotomus* was demonstrated by the Sergent brothers, Edouard and Etienne. The actual mode of infection, however, was not demonstrated until 1941.

In the New World, cutaneous and mucocutaneous leishmaniasis causing disfiguring conditions have been recognized in sculptures since the 5th century and in the writings of the Spanish missionaries in the 16th century. In 1911 Gaspar Vianna found that the parasites in South America differed from those in Africa and India and created a new species, *Leishmania braziliensis*.

Geographical distribution

Leishmaniasis belongs to a globally widespread group of parasitic diseases. It is endemic in tropical regions and sub-tropical regions of America, Africa, Indian subcontinent, sub-tropics of Southeast Asia and the Mediterranean basin and Southern Europe [Fig –1]. In India leishmaniasis is endemic in the states of Bihar, Uttar Pradesh and West Bengal. Leishmaniasis currently affects some 12 million people in 88 countries on all continents except Australia. About 350 million people are exposed to the risk of infection and about two million new cases and 11000 deaths are estimated to occur annually (6). The World Health Organization (WHO) recognizes it as one of the six major communicable diseases. Almost 90% of visceral leishmaniasis cases occur in Bangladesh, Brazil, India and Sudan, while cutaneous leishmaniasis is prevalent in Afghanistan, Brazil, Saudi Arabia and Syria. Most of the cases of mucocutaneous leishmaniasis occur in Bolivia, Brazil and Peru.
In recent past, major epidemics of visceral leishmaniasis have occurred in Eastern India and Bangladesh (7, 8) and among refugees in Sudan. A ‘viscerotropic’ form of the disease was identified in American military personnel who served in the Persian Gulf War (9). Recently leishmaniasis has also emerged as an opportunistic disease in HIV patients (10) in southwestern Europe and Africa and in those who have had organ transplants (11).

Leishmaniasis in India

‘Kala-azar’ or visceral leishmaniasis has periodically appeared as an epidemic through out India, particularly in Eastern India. The first recorded epidemic dates back to 1824 in Jessore district of Bengal (now in Bangladesh), which spread to Nadia district in 1832, Hooghly district in 1857 and subsequently in Burdwan district (now in West Bengal, India) in 1862 and to the Garo hills of Assam in 1863. It was a full-blown epidemic in Assam between 1890 and 1900. After the post DDT era, incidence of kala-azar declined considerably along with other vector transmitted diseases like malaria due to elimination of the vector by pesticides. However resurgence of leishmaniasis was observed worldwide after the withdrawal of insecticide. In 1991, almost 2.5 lakh people were affected with leishmaniasis all over India, killing about 76000 people. Now the disease has remained confined to North-Bihar and Malda, Murshidabad and West Dinajpur of West Bengal.

Clinical types of leishmaniasis

Clinically leishmaniasis can be divided into four types:

(A). Visceral Leishmaniasis (VL) or Kala-azar or Dum-Dum Fever: Kala-azar is the most commonly used term to describe visceral leishmaniasis due to its characteristic symptoms which are darkening of the skin of the hands, feet, face and the abdomen. It is a systemic disease of the reticuloendothelial system, particularly the bone marrow, the spleen and the liver. Intermittent fever, hepatosplenomegaly, hypergammaglobulinemia, anemia, darkening of skin and severe deficiency of cell-mediated immunity are the main symptoms.
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Visceral leishmaniasis is caused by the parasites *Leishmania donovani donovani*, *Leishmania donovani infantum* and *Leishmania donovani archibaldi* in the old world and by *Leishmania donovani chagasi* in the new world.

A dermal form of kala-azar, called Post kala-azar Dermal Leishmaniasis (PKDL), restricted to Eastern India and Africa, occurs in a subset of patients following remission of the initial VL infection (12). Reports of PKDL have also been documented from in China and Iraq(x). It is proposed that PKDL represented the temperature dependent mutation of the originally visceralizing kala-azar strain (13).

(B). Cutaneous Leishmaniasis (CL) or Delhi Boil or Oriental Sore: This is a self-healing, self-limiting paralysed cutaneous lesion, developed at an exposed site of the body where the organisms are introduced by the bite of sand fly vector. Skin ulcers take more than a year to heal. Most cases of cutaneous leishmaniasis heal without treatment, leaving the person immune to further infection. However Diffuse Cutaneous Leishmaniasis (DCL) (14) never heals spontaneously and tends to a relapse after treatment. It produces disseminated and chronic skin lesions resembling those of leprotamous leprosy and is difficult to treat. Cutaneous leishmaniasis is usually divided into (1) Old World leishmaniasis caused primarily by *L. tropica*, *L. major*, *L. aethiopica*, and (2) New World leishmaniasis caused primarily by *L. mexicana* or *L. braziliensis*. Diffuse cutaneous leishmaniasis is caused primarily by *L. aethiopica* or *L. mexicana*.

(C) Mucocutaneous Leishmaniasis (MCL) or Espundia: Mucocutaneous leishmaniasis is also initiated by the same manner as in oriental sore but some patients develop metastatic lesions with various degrees of disfiguring and sometimes fatal tissue damages, localized particularly in the nasal and buccal cavities. Mucocutaneous leishmaniasis causes severe suffering because of social and economical trauma associated with the disease. Mucocutaneous leishmaniasis is caused primarily by *L. braziliensis*.

(D) Diffuse cutaneous leishmaniasis: This form produces widespread skin lesions which resemble leprosy and is particularly difficult to treat.
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A

Fig. 1. Worldwide distribution (shown in red) of cutaneous (A) and visceral (B) leishmaniasis. (Source: http://www.who.int/leishmaniasis/leishmaniasis/maps/en/).

B

Epidemiology of kala-azar

Kala-azar, although a tropical disease, shows its particular prevalence either during or after the rains. Certain climatic factors such as temperature, humidity and rainfall influence the incidence of the disease in endemic areas in India. The disease has been found to be limited to alluvial points with a high humidity and rarely occurs in altitudes of 2000 feet and above. It has been further observed that kala-azar is essentially a disease of rural districts rather than of towns. The factors which are
necessary for the spread of the disease are: (i) a primary source of infection (ii) conditions favoring transmission like climatic conditions as well as the conditions favoring the development and infection in the transmitting agent (sand-fly) and (iii) a susceptible population. In sporadic and epidemic cases both sexes are equally affected, although in certain areas higher incidence may be noticed among the males. In the endemic areas adolescents are generally affected.

**Diagnosis of leishmaniasis**

Preliminary diagnosis is based on the symptoms and clinical signs of the different types of leishmaniasis. However these alone are not enough to differentiate this disease from other similar conditions such as malaria, relapsing fever, liver abscess and trypanosomiasis.

The various laboratory procedures that may be carried out for the diagnosis of kala-azar may be divided into direct and indirect evidences.

Direct evidence mainly involves demonstration of amastigotes in the bone marrow, blood, lymph gland or splenic aspirates by (i) microscopical examination of a stained film, (ii) cultural examination, and (iii) inoculation into hamsters.

The indirect tests include monitoring the changes in blood cell count and the serological tests. Serological tests for visceral leishmaniasis include Montenegro test (15), Direct Agglutination Test (DAT) (16), indirect-fluorescent antibody test (IFAT) (17), enzyme linked immunosorbent assay (ELISA) (18), counter-current electrophoresis (CCIE) (19) etc. Antigen detection in the circulation or urine of the host indicates active stage of the disease. Recently, specific molecular probes have been developed which hybridize to specific regions of the parasites' kinetoplast DNA (kDNA), ribosomal RNA (rRNA), mini exon derived RNA (medRNA), and genomic repeats. The use of these probes in diagnosis is more advantageous as a large number of samples can be analyzed quickly without compromising efficiency (20). The probes are also more specific since they are made to target regions which are unique to a particular *Leishmania* species, complex or isolate (21).

The latest development in the molecular diagnostic technology is based on PCR amplification methods involving parasite specific targets: kDNA minicircles (22, 23) and the med RNA genes (24). An improved 'PCR-solution hybridization enzyme linked assay' (PCR-SHELA) was developed and has been used to diagnose infection.
of *L. donovani* in patients in India, Kenya and Brazil with 90% sensitivity and 100% specificity (25).

**Therapeutic strategies**

**Chemotherapeutic strategies:** Chemotherapy remains the only practical means of combating leishmaniasis. Traditional drugs include pentavalent antimonials, aromatic diamidines and Amphotericin B. The two pentavalent antimonial compounds, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) were first introduced in the 1940’s and have since been used as first-line chemotherapeutic agents against all forms of leishmaniasis including visceral leishmaniasis. Urea stibamine developed by U. N. Brahmachari (26) along with sodium stibogluconate continues to be an effective anti-leishmaniasis drug in India. It is not completely understood how these drugs act against the parasite; they may disrupt its energy production or trypanothione metabolism. Pentavalent antimony [Sb(V)] has been found to inhibit glucose catabolism and ATP formation via the glycolytic pathway and fatty acid β-oxidation in *Leishmania mexicana* (27). Furthermore, Sb(V) can form complexes with various carbohydrates. Trivalent antimonite [Sb(III)] appears to interact with key sulfhydryl groups of leishmanial proteins, thereby probably causing enzyme inhibition (28). The drugs are the safest currently available since they are rapidly excreted by the kidneys and there is virtually no accumulation in the body. However due to wide-spread antimony resistant cases of Indian PKDL and kala-azar, treatment over four months is recommended. This is expensive and potentially toxic (29).

Aromatic diamidines have been considered the second line of defense against this disease. Electron microscopy revealed that treatment of *Leishmania* with pentamidine leads to the disintegration of the network of intercatenated circular DNA molecules that comprise the mitochondrial genome, termed the kinetoplast and also the mitochondrion (30). It is actively concentrated by parasite mitochondria where it is capable of inhibiting mitochondrial topoisomerase II (31). The two preparations which have been used are pentamidine isethionate and pentamidine dimethane sulphonate. Both of these compounds are very effective in the treatment of kala-azar but because of their toxicity and potential side effects they are used as drugs of second choice.
Amphotericin B, a polyene microlide antibiotic, used in case of patients unresponsive to antimonials and pentamidines has also been found to be effective (32). It acts on the cell membrane sterols and phospholipids of *Leishmania* spp. (33). Several studies confirmed that the leishmanicidal activity of amphotericin causes the formation of pores in the membrane that alter the permeability of the membrane to ions which in turn promote its disorganization and depolarization (34,35). Recent research has been oriented towards reducing its toxicity. Lipid associated Amphotericin B (LAAMB) enables the targeting of the drug to particular sites of infection, thereby reducing its toxic effects. The three commercial preparations of LAAMB currently under trial are AmBisome (Vestar, USA), Amphocil (Liposome technology inc, USA) and Amphotericin B lipid complex (Bristol Meyers Squibb, USA). Therapy with aminosidine (paromomycin) and methyl benzethonium chloride has been successful for the treatment of old world cutaneous leishmaniasis (36). Aminosidine is an antibiotic of the aminoglycoside family. (37). The drug acts on RNA synthesis and modifies membrane polar lipids thereby affecting membrane fluidity and altering membrane permeability (38). Also under clinical evaluation are imidazoles and triazoles and purine analogues (allopurinol). The latter works on the principle that *leishmania* are unable to synthesize purines *de novo* and, therefore rely upon the uptake and salvage of preformed purines for survival. So allopurinol is hydrolyzed to allopurinol riboside, an analogue of inosine. This nucleoside is incorporated instead of ATP into *leishmanial* RNA where it interferes with protein synthesis. Miltefosine, an anticancer agent, has shown promising results in treatment of patients in India (39) and with HIV/VL co-infection (40) and also in case of new world cutaneous leishmaniasis (41). The cure rate of miltefosine in phase III clinical trials is 95%. Recently, piperine has also been used for treatment of visceral leishmaniasis (42).

**Immunotherapy:** Immunotherapy is used to improve antileishmanial drug action. One of the methods employed is the use of interferon gamma (IFN-gamma) to treat visceral leishmaniasis. The cell mediated immune system fails to recognize the leishmanial antigens. IFN-gamma enhances the intracellular killing of the leishmanial parasites and reduces the dose of antimony required for treatment.
Elimination of sandfly vectors and reservoir hosts: In endemic areas such as Bangladesh and India sandfly control is an on-going process with mass spraying of chemicals such as DDT, Malathion, Fenitrothion, Propoxur and Diazinon.

Drug resistance: Drug resistance is a growing problem during the chemotherapeutic treatment of leishmaniasis. The common strategy of resistance involves amplification and over expression of either a gene encoding the target enzyme of the particular drug or a gene that encodes a protein involved in drug transport, thus affecting its concentration in the cell. In many instances, resistance of Leishmania species is due to over-expressed efflux pumps belonging to the super family of ABC (ATP-binding cassette) transporters. Three different classes of ABC transporters have been described in Leishmania parasites. The first group is homologous to the human multidrug resistance protein (MRP) subfamily of transporters and is linked with drug resistance to antimony and other compounds. The metalloid-thiol transporter Pgp A is implicated in antimony resistance. The gene, pgpA was identified as part of an extrachromosomal DNA, the H-circle, amplified in L. tarentolae (ltgpA) and L. major (ImpgpA). Transfection experiments have shown the involvement of pgpA in arsenite and antimony resistance. Another gene that is amplified in antimonite tolerant Leishmania cells is gsh1. It encodes γ-glutamylcysteine synthetase, an enzyme involved in the rate limiting step in glutathione biosynthesis. This suggests that PgpA transports metalloid-glutathione conjugates. Resistance to miltefosine has been observed in cultures of L. tropica, also involving a Pgp-like transporter.

The second class of ABC proteins identified in Leishmania parasites are transporters with a higher similarity to mammalian P-glycoproteins that confer a multidrug resistance (MDR) phenotype similar to that observed in cancer cells. The first Leishmania mdr1-like gene was described in the early 1990’s in a vinblastine - resistant L. donovani line (Idmdr1). Later, homologous genes were also described in other drug resistant lines. The parasites were stepwise-selected with the indicated drug and presented an MDR phenotype, with a cross-resistance to nonrelated hydrophobic drugs such as puromycin, daunomycin, vinblastine, adriamycin and doxorubicine. The third class of ABC transporters shows high homology with members of the mammalian ABCA subfamily. However, they have not yet been implicated in drug resistance in Leishmania.

Other mechanisms, unrelated to drug efflux, might also be involved in Leishmania resistance to antimonials. The antimony active against leishmaniasis is the trivalent
form; a change in the reductase activity of the parasite could lead to drug resistance. The mechanism of resistance to paromomycin in *L. donovani* has been related to decreased drug uptake, probably as a consequence of altered membrane composition. Resistance to allopurinol is related to differences in the affinity of enzymes of the purine salvage pathway of the parasites.

Resistance to drugs with no therapeutic importance in leishmaniasis is well documented. Resistance to methotrexate occurs due to mutations in the gene for its transporter (43). Stepwise selection of parasites by methotrexate cause amplification of the DHFR/TS gene (44) and a member of the MDR-related gene family (45) as extra chromosomal circles, termed as R-circles and H-circles respectively. Similarly *Leishmania* selected for resistance to tunicamycin possess amplified extra chromosomal circles containing the glycosyl transferase coding region (46). However, selection with mycophenolic acid and &-difluoromethylornithine resulted in amplification of inosine monophosphate-dehydrogenase (47) and the ornithine decarboxylase gene (48) respectively as linear extra chromosomal elements.

People are also trying to investigate any relationship between sterol methylation and amphotericin B resistance (49). In case of methotrexate resistance, recently roles of methionine adenosyltransferase and s-adenosylmethionine have been implicated (50). Resistance to pentamidine has been observed in *Leishmania*. A decrease in the mitochondrial membrane potential (MMP) in pentamidine resistant *Leishmania*, indicates that MMP is diminished in the drug resistant line. So, the drug does not accumulate rapidly into the mitochondrion, but remains free in the cytosol. Efflux pumps appear to be operative in removing cytosolic or membrane associated pentamidine from the cell.

**Classification of Leishmania**

The classification of Leishmania is assigned based mainly on their morphological, serological and epidemiological characteristics and selectivity for both vertebrate and invertebrate host. Recently isozyme variant types, antigenicity and buoyant density of kinetoplastid DNA are used for proper classification. The unique characteristics of mini circle DNA of Leishmania has permitted selection of 12 complex species, subspecies, strain and even isolates specific DNA probe (51). The sub-kingdom protozoa (gk,protos=first,zoo=animal) comprises all unicellular animals. Based upon the locomotor organ (pseudopodia, flagella or cilia), it has been divided into several
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Phylum sarcomastigophora (gk, sarx=flesh, mastix=whip, phoreus= bearer) contains those groups of organisms that contain either fleshy pseudopodia or a whip like flagella as their locomotor organ. Organisms containing flagella only, are placed under sub-phylum mastigophora. Mastigophores that do not possess chlorophyll bearing chromatophores but contain glycogen as reserve food, are placed under the class zoomastigophora (gk., zoo=animal ). Unique mitochondria or kinetoplast bearing organisms are placed under order kinetoplastida. The organisms which always appears moving under the microscope because of the constant undulation of their undulating membrane, are placed under the sub-order Trypanosomatida (gk., trypan=anger, soma=body ).

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Systemic position of Leishmania (Levine et.al. 1980).

Kingdom – Protista (Haeckel, 1866)
Subkingdom – Protozoa (Goldfuss, 1817)
Phylum – Sarcomastigophora (Honigberg & Balamuth, 1963)
Subphylum – Mastigophora (Diesing, 1866) 13
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Class – Zoomastigophora (Calkins, 1909)
Suborder – Trypanosomatina (Kent, 1880)
Family – Trypanosomatidae (Doflein, 1901, emend, Grobben, 1905)
Genus – Leishmania (Ross, 1903)

Lainson and Shaw (52) have divided Leishmania into two subgenera, Viannia and Leishmania, based on their development in sand fly. The species belonging to Viannia subgenus develop in the hindgut before migrating anteriorly to midgut and foregut (peripylaria). Species in Leishmania subgenus occupy only the midgut and foregut of the vector (suprapylaria) and have lost the primitive hindgut development (Table 1).

Evolution of Leishmania

A specialized densely stained body, made up of mitochondrial DNA and known as kinetoplast, is present in all kinetoplastids. Kinetoplastids may be free living, monogenetic (single host) or digenetic (alternating between invertebrate and vertebrate or plant host). Based on their morphology and parasitic life style, digenetic Endotrypanaum, Leishmania, and Trypanosoma are thought to have evolved from monogenetic Leptomonas. Recently, based on nuclear small subunit ribosomal RNA (SSU rRNA) and large subunit ribosomalRNA (LSU rRNA) sequences, multiple origins of digenetic parasites have been proposed. The genus Leishmania forms a closely related group with Crithidia and has some distant relationship with Trypanosoma brucei and Trypanosoma cruzi. Trypanosoma brucei represents the most ancient divergence (Fig. 2). There are over 20 different known species of Leishmania and over a dozen are associated with various forms of leishmaniasis [Fig.2].
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Insect vector of leishmaniasis

Insects are of great medical importance as they serve as vectors or intermediate hosts for a variety of diseases. Various types of leishmaniasis are transmitted by the female sand flies. The spread of leishmaniasis actually follows the distribution of these phlebotomine vectors in different parts of the globe. There are ~30 species of sand flies that can become infected during the blood meal from a host. Sand flies of the genus *Lutzomyia* in Americans, and *Phlebotomus* elsewhere transmit leishmaniasis (53). However in the case of the *L. braziliensis* complex, *Psychodopygus* spp. is also implicated as vector. *Phlebotomus papatasi* is an established vector for *Leishmania tropica* and *Leishmania major* in the old world causing cutaneous leishmaniasis. In India, *Phlebotomous argentipus* is the vector for *Leishmania donavani*. In these vectors *Leishmania donavani* parasites were experimentally shown by PCR amplification of the 180bp mini exon derived (med) RNA (54). Recently a role of sand flies belonging to the genus *Sergentomyia* in kala-azar transmission in India has been suggested (55). However, vector control has become difficult with *Phlebotomus papatasi* becoming resistant to DDT and Dieldrin.

Morphologically, sand flies are small (<5mm in length), slender with piercing mouthparts and long antennae. The body is hairy, sand or buff colored with wing veins parallel to each other [Fig.3]. They are weak fliers and tend to remain close to...
the ground near their breeding sites. Eggs are laid in dark, humid burrows, cracks or crevices or under leaves. Egg laying starts after 30-36 hours of a blood meal. A female lays 30-35 eggs in 6-12 days. There are four larval instars, lasting a total of 4-6 weeks. Pupa requires ~10 days for development into adult. They feed on plant sap with only the adult female being hematophagus.

**Fig.3:** A1, 2. Sandflies of the genus Lutzomyia. B1, 2. Sandflies of the genus Phlebotomus

**Morphology of Leishmania**

**A. Promastigote or Leptomonad stage:** Promastigote (gk., pro= in front, mastix= whip) is a pear or spindle shaped free surviving form measuring between 5-20 μm in length and 1-4 μm in width [Fig. 4A]. Each cell has a single flagellum emerging from the basal body within the parasite, extending 15-30 μm (56). It contains a centrally located nucleus and a single well developed terminal/ sub-terminal tubular mitochondria. Promastigotes divide by simple binary fission with the mitochondria dividing in phase with nuclear division. They exist as large rosettes attached to each other by flagella, oriented towards the center of the mass, in *in vitro* cultures and smears of gut of infected sand flies [Fig 4C].
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Within the midgut, two morphologically distinct types of promastigotes are seen (57). The long thin nectomonad promastigotes (body length > 12 µm) are free swimming or attached by interdigitation of the flagellum to the ciliated epithelium of the midgut; while the short, broad haptomonad (body length 5-12 µm) is bound by hemidesmosome attachments of the flagellum to the cuticular intima of the stomodeal valve (58).

The infective metacyclics are small (10 µm) having slender body with a comparatively long flagellum. They are non-dividing but highly active cells and are unattached forms found in proboscis of sand flies.

Another form, distinct from the promastigote configuration, is found in the sand-fly pharynx. They are round to oval shaped (5-10 µm x 4-6 µm) with a very deep flagellar pocket. The kinetoplast lies by the side or posterior to the nucleus rather than anterior to it (57). This form, called paramastigote, gives rise to the metacyclic promastigotes.

![Fig. 6: A representation of the life cycle of Leishmania in mammalian host and sandfly vector. (www.dpd.cdc.gov/.../Leishmania_LifeCycle.gif)](https://www.dpd.cdc.gov/.../Leishmania_LifeCycle.gif)

B. Amastigote or Leishman-Donovan body: Amastigotes (gk., a= no, mastix= whip) are small (2x4 µm), non-motile, uninuclear spheroid or ovoid organisms found in the macrophages of the infected host [Fig.4B]. Each cell contains a large,
eccentrically located nucleus and a distinct rod shaped kinetoplast associated with the flageller rudiment that does not extend beyond cell margin. The cytoplasm appears vacuolated and contains tiny vacuoles and granules. Amastigotes of *L. braziliensis* contain refractile bodies (59).

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**Fig. 5: Amastigotes of *Leishmania.* A. Electron micrograph of *Leishmania tropica* in the cytoplasm of a macrophage. The amastigotes contain a round nucleus with characteristic chromatin condensation, mitochondria, a blepharoplast, a kinetoplast without forming flagella, and a row of microtubules just beneath the plasma membrane.

B. Stamp cytology preparation shows round-shaped amastigotes (marked by arrow), phagocytized by macrophages (Giemsa). The nucleus and kinetoplast (paranucleus) can be recognized. (Source: pathy.fujita-hu.ac.jp).

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**Life cycle of *Leishmania***

*Leishmania* spp. has a dimorphic life cycle (60), which involves a regular alternation of hosts between the phlebotomine vector and the vertebrate host [Fig.5]. While the *L. donovani* is adapted to transmission from man to fly to man, others have specific zoonotic reservoirs. *Leishmania* exist as a promastigote in the sand fly gut, and as amastigotes in vertebrate macrophages. Multiplication of each form is by binary fission (61). Till date a sexual phase of *Leishmania* has not been found.
Introduction

Development in the sand fly

When a female sand fly bites, the amastigotes present in the skin (dermis) or in the peripheral blood phagocytes are taken into the abdominal midgut of the sand fly with the blood meal from an infected host. The parasite initially resides within the peritrophic membrane secreted by the midgut cells. These amastigotes measuring 6-10 μm (62) are released from the macrophages and undergo growth phase within 24 hrs. They divide once before differentiating into the promastigote stage, characterized by a dense coat of glycocalyx (63). The procycls (proliferative promastigotes) may be found in different morphotypes. The nectomonads are large promastigotes that proliferate within the sac-like quitinous peritrophic matrix. With the disruption of the anterior part of this matrix by promastigote chitinolytic enzymes (~2 days), the parasites migrate and attach to the microvilli of the midgut epithelial cells, besides specific binding of surface molecules (LPG); and then divide rapidly. There the proliferating nectomonads may give rise to either infective metacyclics or to shorter and broader haptomonads. After 4-7 days, the parasites cease dividing and differentiate through a series of flagellated intermediate stages (64) into infective metacyclic promastigotes. They migrate through the stomodeal valve, from pharynx to the foregut and esophagus where they are suspended in the sand fly saliva, which promotes survival and development of the promastigotes till inoculation during the next blood meal (65).

Development in the mammalian host

The infected fly gains access to the dermal capillaries when it bites the vertebrate host and the parasites are regurgitated into the bite wound. As they ingest their blood meal, the sand flies continuously salivate which serves the parasite an important adjunct to establish a successful infection (66, 67), possibly by inhibiting oxidative metabolic processes and antigen presentation by macrophages (68, 69). Most extra cellular promastigotes are killed by complement factors before entry into host cells and those who escape, rapidly adhere to the residential or recruited cells of monocyte/macrophage lineage including Langerhans cells (70, 71). Attachment to red blood cells protects promastigotes from complement mediated lysis and from there they are transferred to leukocytes.
Parasites bind to the cell surface mostly through complement receptors type-I (CR1) and type-III (CR3) (72). After the complement mediated adhesion, the promastigotes are internalized by conventional “zipper like” interactions (73) or “coiling phagocytosis” (74). Within minutes of phagocytosis, promastigotes are located in phagosomes, which undergo maturation to form parasitophorous vacuole (PV). Inside the parasitophorous vacuole, the promastigotes transform into amastigotes within 2-5 days. They multiply rapidly and are eventually released by lysis of the host cell followed by invasion of neighbouring macrophages, resulting in pathogenicity.

**Morphological transformation**

**Amastigotes to Promastigotes**

The peritrophic membrane of the insect gut is the site of this transformation and it is carried out in 12-24 hrs. (75). It is triggered by oxygen as the amastigotes are predominantly anaerobic (76), while promastigotes are more oxidative (77). A shift in
ambient temperature from 22°C to 35°C is reported to hasten the process of transformation (78). During transformation amastigotes first lengthen and the flagellar vacuole lying near the flagellar pocket increases in size. Within 20 hrs, a stumpy flagellar rudiment is protruded and the body assumes an elongated promastigote form followed by flagellar vacuole contraction (63). A change in the surface coat occurs leading to an increasingly dense coat of glycocalyx composed of a variety of glycoconjugates bound to the parasite surface by glycosphingolipids (GPI) anchor (79). These glycoconjugates comprise mostly stage specific lipophosphoglycans (LPG), proteophosphoglycans (PPG) and low molecular weight glycosphingolipid phospholipids (GIPLS). This transformation is also accompanied by an increase in respiratory rate, synthesis and accumulation of cytochrome C, an increase in cellular and mitochondrial volume, synthesis of flagellum and intraflagellar bodies, acquisition of antigens and changes in the respiratory stimulation by L-proline (80).

A sequential development from a non-infective promastigote to a non-dividing infective ‘metacyclic promastigotes’ form in insect vector and in vitro culture is also demonstrated in Leishmania spp. and progressive number of this form appears when the cell population reaches the stationary phase of their growth cycle (81). In vitro development of metacyclics is associated with changes in the membrane carbohydrates (82), increased expression of gp63 (GPI anchored Zn- stimulated metalloproteinase), increased resistance to lysis by normal human serum (83), altered mobility (84), enzyme activities and antigenicity (82). Modification of LPG during metacyclogenesis results in loss of binding to the 65 kDa LPG binding protein in the midgut microvilli allowing the infectious metacyclics to move forward to the foregut. Increased level of gp63 is instrumental in degrading host molecules such as immunoglobulins and complement. Metacyclics also express enhanced membrane bound and secreted acid phosphatase and cysteine proteinase activities (101).

Promastigotes to Amastigotes

Results from in vitro studies reveal that this transformation takes place at 37°C and 25°C (85) and requires amino acids and glucose (86). There is also an increased activation of enzyme such as phosphoenol pyruvate, malate dehydrogenase (87) and adenosine kinase (88) whereas there is dramatic increase in tubulin biosynthesis (89), polyamine and CAMP levels (90) and transporters of glucose (91) and nucleotides.
However, LPG (92) and gp63 levels are down regulated (93). The low pH of the parasitophorous vacuole is overcome by the action of a parasite membrane H\(^+\) - translocating ATPase which maintains the leishmanial cytoplasmic pH at neutrality. The proton electrochemical gradient created across parasite membrane drives the active transport of glucose and proline, essential to parasite survival.

**Ultrastructure of Leishmania**

Ultrastructure of *Leishmania* was revealed mainly by Electron Microscopy [Fig-6].

**A. Plasma membrane:** *Leishmania* at all stages presents a typical trilaminar unit membrane 2-4 nm wide with underlying sub-pellicular microtubules. The surface membrane of the kinetoplastids has been divided into three morphologically distinct domains each with a highly specialized membrane with distinctive functions and unique protein and lipid compositions (94).

(i) The pellicular membrane: It surrounds the body of the cell and is attached to a corset of subpellicular microtubules that lies in cortical area free of ribosomes (95). This membrane contains many permeases that mediate the uptake of nutrients and is densely covered with proteins or a glycolipid coat that protect the parasite against the host immune response.

(ii) The Flagellar Membrane: It is distinct in protein and lipid composition and has a high sterol / phospholipid ratio.

(iii) The Flagellar Pocket: It is present as a deep invagination of a cell membrane at the base of the flagella which is surrounded at its opening by a desmosome like thickening called ‘junctional complex’ (96). It is devoid of microtubules and responsible for uptake of nutrients via receptor mediated endocytosis or pinocytosis, for secretion of proteins and for integration of membrane proteins into cell surface. In amastigotes the flagellum is confined to this pocket.

**B. Flagella and Basal body complex:** The flagellum consists of nine pairs of peripheral axonemal microtubules encircling a central pair which arises from the first basal plate and can be found distal to the basal body. A large lattice like structure, the paraflagellar rod (PFR), runs along side the axoneme. The proximal region of the PFR is linked to the axonemal doublets 4 through 7 (97). Two main protein components of
Introduction

the PFR termed PFR-1 and PFR-2 (98). The two central microtubules (99) and PFR (100) are absent from amastigotes.

The basal body complex lies close to the kinetoplast and responsible for nucleation of the flagellar axoneme microtubules. It is the only recognizable microtubule-organizing centre (MTOC) in kinetoplastids. The central axonemal doublet terminates on the first basal plate while the outer doublets terminate close to the kinetoplast.

C. Mitochondria-Kinetoplast Complex: *Leishmaniae* present a single branched mitochondrion which may extend the whole length of the cell with a large DNA content in a region located in the vicinity of the flagellum basal body, termed kinetoplast. Isolated k-DNA network observed by electron microscopy is a planar structure, elliptically shaped. In vivo the network is probably compacted into a disk (101). The inner mitochondrial membrane is in voluted to form plate like cristae (102).

D. Nuclear Structure: Like all eukaryotes, the nucleus of *Leishmania* is surrounded by two nuclear membranes, 7nm thick. It has pores of 30-100 nm and the centrally located nucleus is of 0.6 –1.0 μm in diameter. During cell division the nuclear membrane remains intact, while the nucleolus disperses. The chromatin is arranged peripherally with no apparent chromosome condensation during cell cycle. It is organized in 16-23 chromosomes or chromosome sized molecules (101).

E. Cytoplasmic Structure: Both promastigotes and amastigotes contain typical eukaryotic organelles such as Endoplasmic Reticulum (both smooth and rough), Golgi apparatus, lysosome-endosome and ribosomes, while the ER is present the nucleus, the Golgi apparatus and the lysosomes are found near the flagellar pocket. A specialized transitional ER (tER) lies directly opposite to Golgi apparatus and constitutes the major site of protein and lipid export to Golgi apparatus (103). An acidified vacuole, acidocalcisome, is found at the aflagellate end of the promastigotes, which acts as the cellular reserve for calcium, phosphate, sodium, magnesium and in some cases zinc (103). Acidocalcisomes are thought to be involved in pH homeostasis and osmoregulation in parasites. The cytoplasm of amastigotes appears vacuolated. A single large caveola, present posteriorly in amastigotes may be involved in endocytic activity (104). The amastigote forms belonging to the *L. mexicana* complex display
unique lysosome like membrane bounded organelles presenting spongy like matrix which may occupy as much as 15% of the cell volume, termed megasomes. Megasomes are cysteine proteinase rich organelles associated with accumulation and degradation of endocytosed macromolecules. *Leishmania* also contains glycosomes, microbodies and peroxisomes that play an important role in oxidative metabolism (95). Peroxisomes, Glyoxisomes and glycosomes are microbodies belonging to a single organelle family, grouped under the generic name 'peroxisomes', that is represented in virtually all eukaryotic cells. Glycosomes are specialized peroxisomes present in trypanosomes and related protozoa. They are globular organelles with a mean diameter of 0.2-0.3μm surrounded by a single membrane unit. Cells may present between 10 and 100 glycosomes which take part in processes like glycolytic ATP generation, β-oxidation of fatty acids, pyrimidine biosynthesis and purine salvage. They contain the major part of the glycolytic pathway. Surprisingly, the hallmark enzyme catalase is absent from the organelles of *Leishmania* (101). Inclusions of lipid bodies (0.5μm across) are found in both forms of the parasite.

**F. Cytoskeleton:** The parasite cells are shaped by a layer of parallel subpellicular microtubules spaced about 44nm apart, running longitudinally underneath the plasma membrane at a distance of ~ 8.5 nm all over the cell body. They terminate near the flagellar pocket that presents only four associated microtubules thought to take part in vesicle transport. Endocytosis and exocytosis take place in the flagellar pocket reservoir of both parasite forms (101).
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Fig. 7: A. Transmission electron micrograph of an amastigote with a kinetoplast characteristic of Leishmania spp. (arrow). (Scale bar = 1 μm.) (Source: www.mja.com.au) B. Transmission electron micrograph showing promastigotes adjacent to the microvillar surface in the midgut region of the sand fly. (Source: www.liv.ac.uk) C. The catenated network of DNA within the sand fly. (Source: www.ebi.ac.uk/parasites) D. Transmission electron micrograph of Leishmania kinetoplast. (Source: www.ebi.ac.uk/parasites)

Biochemistry of Leishmania

Sialic acid moieties contribute to the net negative charge of promastigotes and amastigotes (105). Several GPI anchored molecules are abundantly found on parasite membrane and the common surface ligands include mannose/glucose and galactose moieties. LPG, PPG, glycoproteins like gp63, GIPL and N-glycans (106,107) that are anchored to the membrane by GPI glycolipid (108) are the major glycoconjugates. The main backbone of the LPG is conserved as PO₄[Gal(β1-4)man] in all the species but a stage specific abundance of those molecules with difference in oligosaccharide branching unit is seen. Leishmania promastigotes also synthesize high levels of free GPI that are 10 fold more abundant (~5×10⁷ copies per cell) than LPG.

Three different phosphomonoesterase activities viz., a non specific acid phosphatase (AC Pase) and 5' and 3' nucleotidases (N Tases) are distinguished on the plasma membrane of L. donavani promastigotes (109). Transporters for hexoses (110), amino
Introduction

acids (111), folate (112) and purines (113) apart from H\(^+\)-ATPases (114), Ca\(^{2+}\) – ATPase (115), Mg\(^{2+}\) stimulated ATPase and adenylate cyclase (116) are also present on promastigote surface of several species. Protein targeting to membrane by acylation bypassing the classical pathway has also been reported (117). *Leishmania* preferentially catabolizes amino acids except in stationary phase of growth (118). Principal pathways of carbohydrate metabolism like Embden–Mayerhof pathway, hexose monophosphate shunt, Kreb’s cycle exist (119), while the electron transport chain in mitochondria has an unusual feature of high concentration of cytochrome-\(b\) as compared to cytochromes-c and a+a\(_3\) (120). In amastigotes the activities of enzymes involved in β-oxidation of fatty acids are higher suggesting fatty acids to be principal energy substrate (121). *Leishmania* being purine auxotroph relies solely on the salvage pathway for the synthesis of purines (122), while unusually, XPRTases are present in both stages of the parasite (123), adenosine deaminase is absent in promastigotes (124). The pyrimidine metabolism involves both *de novo* and salvage pathway with the last two enzymes of UMP biosynthesis being located in glycosomes (125).

To date, energy metabolism has been studied more extensively in the related kinetoplastid *T. brucei* compared with other trypanosomatids. So, conclusions drawn from experiments with these cells might be applicable *Leishmania* also. Whereas the classical model of procyclic metabolism proposes a functional TCA cycle and a key role for the electron transport chain in ATP generation, this view began to shift during the 1990s. Van weelden *et al.* (126) showed by targeted gene deletion that an aconitase deficient cell line is not ATP deficient and seems to grow normally in vitro. They conclude that a complete TCA cycle is not used by the parasite for the oxidation of glucose derived products because most, if not all, of the acetyl-CoA produced from glucose is converted into excreted acetate. Also, it has been shown that procyclic cells grown in glucose depleted medium are > 1000 times more sensitive to oligomycin than those grown in glucose rich medium. These data support the view that in the presence of glucose, procyclic cells do not depend on oxidative phosphorylation for ATP production, but in the absence of glucose, when cells switch to amino-acid metabolism, oxidative phosphorylation becomes essential. In the presence of glucose, the production of ATP is likely to occur primarily by substrate-level phosphorylation (126,127). Whether these results can be extrapolated to be applicable in case of *Leishmania* remains to be seen.
Introduction

Section II

Molecular biology of Leishmania

Leishmania genome

The genome is largely diploid with 60-65% single copy genes (128) and a total haploid genome content of ~35 Mb (129). The average DNA content per cell is 0.116 pg in L. donovani and 0.266 pg in L. braziliensis (128) of which ~15% is kDNA. The parasite has a strong preference for G or C in the 'wobble' position and has an average GC content of 64%. In case of L. braziliensis, the GC content was found to be lower (57%) (130). A novel modified base J (α-D-glucosyl hydroxymethyluracil) is found at the telomeres and transcriptionally silent regions (131) where it replaces a fraction of thymine in DNA. A conserved hexameric 5'-TTAGGG-3' telomeric repeat constitutes the telomere (132) protruding as 3'-G overhangs that eventually form t-loop structures (133). One interesting feature of the Leishmania genome is the occurrence of frequent intra-chromosomal amplification and/or deletion events, occurring generally at the subtelomeric regions.

L. major 'Friedlin' strain (MHOM/IL/81/Friedlin) had been selected as the reference organism for the mapping and the sequencing of the Leishmania genome, the project undertaken by WHO under the Leishmania Genome Network (LGN). This effort led by the Tri-Trypanosomatidae 'Tritryp' genome consortium yielded the publication of the three prominent kinetoplastid parasite genome sequences (Trypanosoma brucei, T. cruzi and L. major). The 36 chromosomes of the 32.8-megabase haploid genome have been sequenced and analysis of the sequence by several algorithms predict 911 RNA genes, 39 pseudogenes, and 8272 protein-coding genes, of which 36% can be ascribed a putative function. Most of the smaller (<10 members) gene families appear to have risen from tandem gene duplication, whereas most members of larger (>10 members) families have multiple loci containing single genes and/or tandem arrays; many of the latter contain Leishmania specific genes. The extremity of each L. major chromosome contains the repeat previously reported. Six telomeres contain 0.7 to 25 kb of the subtelomeric repeat sequences between the TAS region and the first gene. Five additional groups of telomeres share varying amounts of sequence, including two...
cases (chromosome 17 and chromosome 10) in which the ends of a single chromosome contain the same subtelomeric sequence (134,135).

Fig. 8: Estimated haploid genome sizes in megabases (Mb) and the estimated number of genes per haploid genome. The Venn diagram depicts the distribution of clusters of orthologous genes among and between the three species: Trypanosoma brucei, Trypanosoma cruzi and Leishmania major. The numbers in parentheses indicate the number of TIGRFAM and Pfam protein domains identified in each group. Taken from El-Sayed et al. (2005) Science 309, 404-409.
### Table 2: Summary of the L. major genome

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Taken from Ivens et al. Science (2005) 309, 404-409.

**Leishmania chromosomes**

Old world *Leishmania* (*L. donovani* and *L. major* groups) have 36 chromosome pairs (0.28 to 2.8 Mb), whereas New World species have 34 or 35, with chromosomes 8+29 and 20+36 fused in the *L. mexicana* group and 20+34 in the *L. braziliensis* group.(134)

A typical *Leishmania* chromosome contains a central core of conserved single or low copy number sequences with long stretches of sub-telomeric and telomeric repeats (136). The variation in the repeat sequences of the sub-telomeric regions is responsible for the size polymorphism between homologous chromosomes of different *Leishmania* species (137,138).
The chromosomes are characterized by their unique arrangement of directional gene clusters (DGCs), previously described in *L. major*. The full extent of this organization is now evident. The *L. major* genome is organized into 133 clusters of tens to hundreds of protein-coding genes, with unrelated predicted functions, on the same DNA strand. The clusters can span up to 1259 kb and are separated by 0.9 to 14 kb divergent or convergent strand-switch regions, which show an unusual base composition. Experimental evidence suggests that polycistronic transcription by RNA polymerase II initiates bidirectionally within the divergent strand switch regions and terminates within the convergent strand-switch regions, which often contain tRNA, rRNA, and/or snRNA genes. The intergenic regions (IRs) in *Leishmania*, separate the polycistronic nuclear gene clusters and also play critical roles in post-transcriptional events. The gene organization reveals that some genes exist in multiple copies of tandem repeats within the same or different chromosomes and other genes map to a single locus. While genes coding for heat shock proteins, hsp 70, hsp 83, hsp 100 (139,140,141), surface glycoprotein gp63 (142), tubulins (143), cpb cysteine protease (144), mini exon derived RNA (145) belong to the first category, the genes coding for DHFR (146), LPG biosynthesis enzymes, glucose transporters (147) etc. belong to the second group. The ribosomal RNA (rRNA) genes are arranged in head to tail tandem repeats of ~160 units on a single chromosome over a stretch of 10kbp. Each repeat unit encodes the typical eukaryotic 28S, 18S and 5.8S rRNA species and the 28S rRNA genes are further divided into two larger 28s\textsubscript{a} and 28s\textsubscript{â} rRNAs and four small RNAs of 220,180,140 and 70 nucleotides that are highly conserved among species. At most chromosome ends (55 of 72 for *L. major*), transcription proceeds towards the telomere, and in 12 cases, the DGC closest to the telomere is very short (one to three genes).

**Kinetoplast DNA**

The kinetoplast DNA (kDNA) exists as a massive network of thousands of topologically linked double stranded circular DNA molecules of two types, that are catenated with each other. The larger types of DNAs are called ‘maxicircles’ and the smaller ones are called ‘minicircles’. kDNA replication occurs coincident with that of the nucleus (148) with both maxicircles and minicircles replicating simultaneously (149).
Maxicircles: Maxicircles are 20-50kb in size with 25-100 copies in a network. They resemble conventional mitochondrial DNA as they encode rRNA and a small number of proteins mainly involved in mitochondrial energy transduction. Interestingly there is a total absence of any tRNA encoding genes in the maxicircle as well as in the minicircle DNA (150). The structural genes include those encoding three subunits of cytochrome oxidase (CO I-III), three subunits of NADP dehydrogenase (ND1-3), Apocytochrome b (cyb) and four unidentified open reading frames (MURF 1-4) (fig.9). Five of the thirteen maxicircles transcripts are ‘cryptogenes’, which are post-transcriptionally edited to various extents by either insertion or deletion of uridines to restore the proper open reading frames (151,152). Genes for mitochondrial 12S and 9S ribosomal RNAs (153) and guide RNAs (154) are also present. A unique non-coding region, the variable region, initiates maxicircle replication, which proceeds unidirectionally as a θ structure. The function of the divergent region, which consists of tandem AT-rich repeats of different complexities, is unknown.

Minicircles: Minicircles are 0.5-2.7 kb in length and 10,000 copies are present in mitochondria (155,156). The sequence heterogeneity, lack of protein coding open reading frames (ORFs) and rapid rate of evolution implied a role other than conventional protein coding. A common feature of the minicircles is the sequence region of 100-300bp, of which a 13bp sequence, 5’-GGGGTTGGTGTAA-3’, termed as universal minicircle sequence (UMS) (157), which is conserved and thought to be the minicircle origin of replication. An UMS- binding protein has also been identified (158). The sequence, outside that conserved region, called variable region, are found to encode guide RNAs (gRNAs) that controls the specificity of the maxicircle mRNA editing (159). Unusually the minicircles are topologically relaxed (160) and stand...
perpendicular to the plane of the network. During replication the minicircles are released from the network by Topoisomerase II (161) and migrate to one of the two antipodal protein complexes where they replicate as ë structures (162) and are attached to the periphery of the network.

![Organisation of a Leishmania minicircle](Source: www.ebi.ac.uk/parasites/kDNA/Source.html)

**Transcription in Leishmania**

The three classes of eukaryotic nuclear RNA polymerase (I, II and III) have been identified in the kinetoplastids, along with a mitochondrial RNA polymerase that generates mRNAs from the maxicircle DNA. However the TriTryp RNAP I, II and III components differ appreciably from those in other eukaryotes (134).

Transcription of kinetoplastid rRNA genes is performed by an RNA polymerase I that is resistant to α-amanitin. The general structure of the rRNA gene promoter resembles that of other eukaryotes in having a bipartite core structure in the -70 to -10 region and having an upstream core element. Mapping of the rRNA gene promoter elements in *L. donovani* has allowed the development of a tetracycline-inducible system for *Leishmania*. Transcription termination downstream of the *L. infantum* rRNA gene has been associated with tandem repeats that contain Chi-like elements. The variant surface glycoprotein and procyclin gene of African trypanosomes have been also found to be transcribed by RNA polymerase I (163).
Transcription of housekeeping genes such as tubulin and actin is undertaken by an RNA polymerase II that is sensitive to α-amanitin. The genes are organized and transcribed as polycistrons, followed by rapid trans splicing and polyadenylation of the pre-mRNA. In trans splicing, the same spliced leader, which is derived from the 5' terminus of the small nuclear SL RNA, is fused to the 5' end of each mRNA. A specific RNA polymerase II initiation site has not been determined for protein coding gene transcription. The TriTryps harbor a single TBP (TATA binding protein) homologue termed TBP-related factor 4 (TRF4). Analysis of the completed genomes from these organisms revealed no orthologs of the general transcription factors TFIIA, TFIIB, TFIIE and TFIIF.

RNA polymerase II is also utilized for the synthesis of SL RNA and, in contrast to the protein coding genes, SL RNA genes are transcribed monocistronically from a concrete transcription initiation site. SL RNA is encoded in ~100 tandemly arranged SL RNAs per haploid genome, whose transcription represents 6% of the total cell RNA synthesis. The SL RNA promoter is conserved within trypanosomatids and consists of a bipartite upstream sequence element (-60 and -30) and an initiator element (-5). Downstream of the intron, all SL RNA genes possess a run of 5-31 thymidines that has been proposed to serve as a termination element. The resulting primary SL RNA transcript thus contains a poly-U tail of heterogeneous length (134,163).

Small nucleolar RNAs are also transcribed by an α-amanitin sensitive RNA polymerase.

The kinetoplastid RNA polymerase III shows an intermediate level of resistance to α-amanitin and recognizes the three classes of promoters for higher eukaryotes. The first consensus eukaryotic promoter sequence to be identified in kinetoplastids was the RNA polymerase class I promoter (box A and box C elements) within the 5SrrRNA gene. The class II promoter (box A and box B elements) was found conserved within tRNA genes. The proteins that bind to the tRNA gene promoter elements have also been reported. The class III promoter (distal and proximal sequence elements) has not been found in kinetoplastids. On the other hand, there is a dependence of all U-rich (U1-U6) snRNA (and other small RNA) gene transcription on the class II promoter of an upstream tRNA gene that is oriented in the opposite direction (163).

No consensus promoter elements for a mitochondrial RNA polymerase have been identified have been identified for minicircles or maxicircles. The minicircle derived
guide RNAs have been shown to possess 5' triphosphates and are thus primary transcripts. A nuclear encoded, single subunit mitochondrial RNA polymerase is responsible for synthesis of maxicircle mRNA. It is not known whether this RNA polymerase is responsible for the transcription of minicircle gRNAs (163).

**Stage specific gene expression**

Differential gene expression in *Leishmania* is a natural phenomenon necessary for survival and virulence as it sequentially passes through different developmental stages during its life cycle. With the advent of microarray technique, it has become quite easy to have an idea of differentially regulated genes during different stages. cDNA microarrays screened with mRNAs from procyclic, metacyclic and amastigotes of *L. major* have identified 430 unique stage specific genes (164). In another effort of proteome analysis of *L. mexicana* differentiation by two dimensional gel electrophoresis followed by cap LC-QTOF mass spectrometry, another set of 47 stage specifically expressed protein have been identified (165). While some genes have been found to be differentially regulated in amastigote stage like β-tubulin (166) and SL RNA (167), others are expressed solely at the infective stage of the parasite including A2 gene family (168), amastin (169), histone H1 (170), cpb cysteine proteases (171), p4 antigen (172) etc. Gp63 and gp46 are induced as the promastigote grows from the logarithmic phase to the more infective stationary phase (173). The possible mechanism of this stage specific gene expression is post-transcriptional, involving sequences present mostly in the 3'-UTR (174), some in the 5'-UTR (175) and in the intergenic regions between the tandemly repeated genes (176). The 3'-UTR sequence modulates transcript stability and translation efficiency either by forming a secondary structure (177) or through transacting protein factors that bind to AU-rich sequences (ARE) found in 3'-UTR (178,179).

**Splicing**

*Cis*-splicing: Intron like intervening sequences has been found recently in poly (A) polymerase gene, (180) besides the gene for tRNA^{Tyr} (GUG) (181). rRNAs are transcribed polycistronically as in other eukaryotes and are processed by the removal of internal transcribed spacer sequences by endonucleases to yield mature rRNAs.
Trans-splicing: The mRNAs of kinetoplasts are transcribed polycistronically followed by posttranscriptional processing by trans-splicing reaction to yield mature mRNAs. Only 39 nucleotides at the 5’ end of the 140 nt. long mini-exon derived RNA (med RNA) are trans-spliced to the 5’ end of the pre-mRNAs (182). About 200 copies of med-RNA genes are arranged in tandem arrays in *Leishmania* (183). Splice leader RNA has a modified cap structure (called cap 4) with a 7-methyl guanosine linked to four modified nucleotides (184). SL RNA is present in the cytoplasm of *L. tarantolae* and *T. brucei* and this cytoplasmic stage is necessary for SL RNA biogenesis and it is dependent on nuclear exporter Exportin (xpo 1) (185). It has also been found recently that the splice leader contains critical structure or sequence determinants for association with polysomes and hence with translation (186). Although, a consensus polyadenylation signal is lacking, trans-splicing and polyadenylation have been found to be coupled (187). Recently, a splicosomal snRNP core complex involved in trans-splicing has been identified (188).

Editing: RNA editing of mitochondrially encoded mRNAs is another unique characteristic of kinetoplastids, involving post-transcriptional maturation of mRNA in which U residues are inserted into, and less frequently deleted from primary mitochondrial transcripts to produce mature mRNAs (189). Editing regulates gene expression at the mRNA level helping in mRNA translation by frequently creating initiation (190) and termination codons (191) in otherwise cryptic transcripts and eliminates internal frameshifts. Most of the edited mRNAs include those codons for the mitochondrial respiratory chain. Guide RNAs (gRNAs) are the short minicircle encoded transcripts, which contains the genetic information for kRNA editing (192). The entire process is a combination of several enzymatic reactions. According to the enzyme cascade model (193), a short anchor duplex of 10-15 bp is formed first between the gRNA and its cognate mRNA leading to cleavage of the pre-mRNA at 3’ to the first unpaired nucleotide by a site specific nuclease. Next, uridylate residues were added to the pre-cleaved mRNA, at an insertional site by Terminal Uridylyl Transferase (TuTase) or a 3’-U- specific exonuclease removes uridylate residues at a deletion site. The last step is the rejoining of the mRNA generating a mature translatable mRNA.
The editosome (native mitochondrial editing complex) is a ribonucleoprotein complex (~30S-40S) with a ~20S particle as the core complex, containing the pre mRNA, gRNA and other proteins involved in editing. The 3' TuTase (194) a U-specific 3'-5' exonuclease (195), RNA ligases (196,197), gRNA binding protein gbp 21 (198), a RNA helicase (199) and editosome associated proteins TBRGG 1 (200) and REAP 1 (201) are some components of the editosome identified so far.

Genetic transformation

Transfection has been the useful tool for genetic manipulation in *Leishmania* and has enabled the study of the molecular basis of virulence, mechanism of drug resistance and gene expression and assessment of parasite specific enzymes as usual chemotherapeutic targets. DNA mediated transient and stable transformation systems by electroporation have been developed in *Leishmania* over the past decade. Vectors for transient transformation systems contain a reporter gene flanked in the 5' side by sequences required for trans-splicing and on the 3' side by polyadenylation sequences on a prokaryotic plasmid backbone. Genes of bacterial origin, such as chloramphenicol acetyltransferase (CAT) (202,203), β-galactosidase (β-gal) (204) and Luciferase (LUC) (205) have been used as reporter genes.

Stable transformation is achieved either by maintenance of a freely replicating episomal plasmid (206,207) or by insertion of a DNA fragment into chromosomes by homologous recombination (208,209). The vectors additionally require a dominant selectable marker for selection of transformants such as aminoglycosidase phosphotransferase (neo), hygromycin B phosphotransferase (hyg), N-acetylglucosamine-1-phosphate transferase (nagt), streptothricin acetyltransferase (sat) genes and the phleomycin (ble), puromycin (pac) resistance genes (210,211,212,213). Integrative transformations have been used to disrupt (214) and replace target genes (215,216) to create 'null' mutants. While 100 bp of homologous sequence is sufficient to promote site-specific integration, free ends of DNA were found to be more recombinogenic than closed circular plasmids. Vectors either completely deficient in *Leishmania* sequences (217) or with herpes simplex virus thymidine kinase gene (hsvtk) as a negative selectable marker (218) have also been reported. Recently there has been wide use of shuttle vectors (219) and cosmid vectors (220) for *Leishmania* which helps in gene ‘knock-outs’ allowing functional
analysis of *Leishmania* genes. Inducible gene expression using prokaryotic tetracycline (tet) repressor operator system has gained popularity for functional analysis of a particular indispensable gene in both *Leishmania* spp. (221) and *Trypanosoma* spp. (222).

Moreover, RNA interference (RNAi) has also been used in kinetoplastids to functionally knock out genes. Two types of episomal constructs are used for this purpose. In one case the gene is inserted between two head to head T7 RNA polymerase promoters (223,194) in a vector, which is transfected in genetically modified parasites expressing stably integrated T7 RNA polymerase gene. In another type the gene is expressed in the form of a hair-pin structure (224). Both of these methods result in *in vivo* expression of double stranded RNA that specifically degrades the target mRNA. Lack of finding of any RNAi effect in *Leishmania* has forced the investigators to study functional role of a particular gene by antisense RNA method, transfecting only the antisense version of the gene as an episome under a *Leishmania* specific promoter (225).