Kala-Azar in India: A Brief Account, Leishmania - Macrophage Interactions and Antileishmanial Chemotherapy

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ABSTRACT Human leishmaniasis is not a single disease; rather, it is a collection of diseases caused by different species of Leishmania, each of which has its own potential to cause a characteristic set of symptoms in man. In 1977, the World Health Organization identified Leishmaniasis, African Trypanosomiasis, Schistosomiasis, Malaria, Filariasis and Chagas' disease as the six major tropical diseases of immediate concern.

Macrophage is the central cell type in the reticuloendothelial system of mammals. They play a part not only in the immunological reactions but also in the initial recognition of foreign material and in the induction of immune responses. Leishmania donovani, the causative agent of human visceral leishmaniasis or kala-azar, reside and proliferate solely in the phagolysosomal vacuoles of macrophages of the infected hosts. Though the macrophage is one of the most strong cell types in its scavenging as well as immunological potencies, the Leishmania amastigotes can harbour and multiply in them. The types of human leishmaniasis, life cycle and diagnosis of Leishmania donovani have been described here explaining how Leishmania gets entered and resist against the toxic products of macrophages and how far the macrophages can develop immunological response against it.

The mainstay of leishmanial therapy are pentavalent antimonial compounds. In cases of treatment failure, second-line drugs are used. In some cases, even these agents fail to eradicate the parasite, so there is a pressing need for a new therapy for this group of diseases. In this review, most of the drugs used in kala-azar have been accounted in terms of their nature, possible mode of action and side effects; further, the concept and profound possibility of the use of targeted drug-delivery system in this disease have been discussed.

INTRODUCTION

Human leishmaniasis is caused by at least 14 different species and subspecies of the genus Leishmania. Leishmania is a flagellated dimorphic protozoan belonging to the family Trypanosomatidae. Most forms of Leishmania are zoonotic; humans are infected secondarily. Animal reservoir hosts include wild cats, dogs, jackals, foxes, sloths, hyraxes and rodents. The disease is transmitted by over 50 species of sandflies of the genus Phlebotomus (for old world forms) and Luizomyia (new world forms). Infected sandflies inject promastigotes (flagellated form) into the mammalian hosts when taking a blood meal. The parasites rapidly invade macrophages, the centrally functional cell in mammalian reticuloendothelial system, within which they transform into amastigotes (nonflagellated form). In susceptible individuals, the parasites multiply leading to the rupture of macrophages; the released amastigotes are able to infect other macrophages. The cycle is completed when the parasites are taken into the gut of uninfected sandflies when taking a blood meal. The amastigotes transform into promastigotes in the hind gut and then migrate to the proboscis; the promastigotes are now ready for transmission to other mammalian hosts. Promastigotes (sandfly stage) can be grown in large quantity in cell-free media in vitro. This has greatly facilitated the analysis of leishmanial antigens and immunological study in experimental models (Cox, 1989).

Most parasites reside in safety in their various hosts by circumventing the host defence apparatus that is potentially capable of destroying them. Leishmania donovani parasitizes the macrophage or mononuclear phagocyte system of its vertebrate host. Leishmania parasites exist in two forms: flagellated promastigote form which is spindle shaped, contains one flagellum and resides in the gut of the phlebotomine sandfly vector; the other form is the aflagellated amastigote form which contains no flagellum, ovoid in shape
and resides in the macrophage of man. Promastigotes and amastigotes differ in their temperature requirements; the former one grows at 22°C whereas the amastigote requires 37°C. Promastigotes enter the host cells just after the bite of sandfly vector. They then shed their flagella, change their shape and transform into amastigotes. The latter form then multiply within the macrophages and are released into the blood by macrophage lysis. Rapidly they then gain entry into the adjacent host cells for propagation of infection. The transformation into amastigotes initiate the disease processes. Given the role of macrophages in the eradication of invading microbes coupled with their ability to kill Leishmania if provided with correct stimulus, the parasite must have evolved under strong selective pressure strategies to ensure survival (Chang et al., 1985).

It is quite unfortunate that all serious efforts to develop a highly effective chemotherapy for visceral leishmaniasis have been proved unsatisfactory so far. A water-soluble pentavalent antimonial drug called urea stibamine was first introduced by Dr. U.N. Brahmachari which saved thousands of kala-azar victims in India in the nineteen twenties (Sanyal, 1985). To reduce toxic side-effects, urea stibamine was later replaced by other more effective antimonial compounds like antimony gluconate and glucantime. Although these are presently used as first line of defence, they elicit side effects like vomiting, bradycardia, abdominal pain, hepatic dysfunction and acute renal insufficiency. When treatment with antimonials does not prove satisfactory, aromatic diamidines are used for therapy. These also elicit serious side effects and are considered to be second line drugs. Recent clinical reports show an increasing number of cases that are becoming refractory to treatment with antimonials and also to diamidines. In these cases, amphotericin B, a polyene antibiotic is administered intravenously with continuous monitoring under hospital conditions. It has been reported to have profound antileishmanial activity but also have serious limitations. A number of other drugs of high potency and known mode of action are under experimental trial in different laboratories to find more suitable candidates against this dreaded disease (Marinkelle, 1980).

**TYPES OF LEISHMANIASIS**

The leishmaniasis are divided into the following three categories:

**Cutaneous leishmaniasis (CL):** According to the geographic occurrence, CL can generally be classified into old world cutaneous leishmaniasis (OWCL) or new world cutaneous leishmaniasis (NWCL). OWCL, which is caused by L. major or L. tropica is found mainly in the Middle East and Africa and is variously known as 'Oriental Sore', 'Bagdad Boil', 'Delhi Sore' or 'Aleppo Button'. NWCL is also known as 'Chiclero's Ulcer' or 'Bay Sore' (in Belize, Peru and Venezuela), 'Pian Bois' (in Brazil, Columbia, French Guyana and Surinam) and 'Uta' (in Peru).

These are caused by L. mexicana, L. amazonensis and L. panamensis species (WHO Report, 1984). Another form is diffused cutaneous leishmaniasis (DCL) which occurs both in the old and the new world. CL is often self healing.

**Mucocutaneous leishmaniasis (MCL):** MCL or espundia caused by L. braziliensis is due to metastasis of organisms to mucosal sites from a primary cutaneous lesion established much earlier. Metastatic spread may occur to the oronasal and pharyngeal mucosa, causing highly disfiguring leprosy-like tissue destruction and swelling.

**Visceral leishmaniasis (VL):** VL or kala-azar (black-disease) caused by L. donovani is also known as 'Dum dum fever' or 'Ponos'. The common features are fever, malaise, weight loss, coughing and diarrhoea accompanied by anemia, skin darkening,
hepatosplenomegaly and often enlargement of lymph nodes. The disease is usually fatal if remains untreated. About 5% of the untreated kala-azar patients develop post kala-azar dermal leishmaniasis (PKDL) within a year of time (Haldar et al., 1989). PKDL is characterised by the appearance of depigmented patches of skin which progress to diffuse nodular lesions over the body. It is generally non-healing but does not lead to death. PKDL patients have been suspected to be a possible reservoir for the parasite in its life cycle.

In fact, all these diseases share relatively few properties except that the causative organisms belong to the same genus, that the vector is always a sandfly and that in human, the parasite harbours and multiplies in the phagolysosomal vesicles of macrophages.

**DIAGNOSIS OF LEISHMANIASIS**

Diagnosis of active leishmaniasis is primarily based on demonstration of the parasite in tissue biopsies. A skin test using a killed whole parasite preparation (leishmanin) is used as a presumptive test but is not fully specific. Serological tests such as complement fixation tests are not specific or sensitive and the more sophisticated immunofluorescence assay and counter-current electrophoresis require special equipment and are not suitable for field use. Some promising new test systems are currently being evaluated which include use of monoclonal antibodies (MAB), enzymelinked immunosorbent assay (ELISA), indium-based slide precipitation and polymerase chain reaction (PCR). (Jaffe et al., 1985, Mael and Belin, 1982).

**KALA-AZAR IN INDIA : A BRIEF RECORD AND THE ECOLOGICAL PERSPECTIVE**

Kala-azar entered into the history of epidemics in India from its appearance in the lower Gangetic plains in West Bengal, in Hooghly district in 1857 and in Burdwan district in 1862 (Burdwan fever). The other report was of a form of febrile disorder (Jwar Vikar) found in Jessore (district) (Bangladesh) that took a heavy toll of about 75,000 lives in three years. In 1863, kala-azar appeared in the Garo hills in Assam and between 1890 and 1900 a great epidemic took place in the valley. Similar epidemic was also observed in Purnea district (Bihar) during the last few years of nineteenth century (Kala-dukh). Later on, a severe epidemic ranged in the eastern part of the entire subcontinent from 1917 to 1929 with devastating consequences. In 1937, approximately 100,000 cases were reported from Bihar alone, 85% of which from north Bihar. Considering the heavy loss of human lives in eastern India in several decades a 'Kala-azar commission' was set up which submitted its historic reports in 1926 and 1932 outlining the dynamics of transmission of the parasite through the sandfly vector (Kala-azar Commission Reports, 1926, 1932). Places at an altitude of less than 700 meters heavy annual rainfall, mean humidity above 70%, alluval soil, abundant vegetation and rural setting were some of the ecological factors often found to be related with epidemics in India. All these conditions still prevail in the Assam valley, West Bengal, Bihar, Tamil Nadu and Bangladesh (Sanyal, 1985).

Before 1922, there was no specific treatment for visceral leishmaniasis. Dr. U.N. Brahmachari first introduced a soluble form of urea stibamine, a pentavalent antimonials that worked as a wonder drug against visceral leishmaniasis. Afterwards this drug has been replaced by less toxic synthetic compounds; however, the pentavalent antimonials are still the anchor-sheet for antileishmanial therapy.

Within a decade after 1950 under the National Malaria Eradication Program extensive spraying of DDT (Dichloro Diphenyl
Trichloroethylene) wiped sandfly population along with mosquito; as a result, the number of kala-azar cases started declining and came to a point of almost disappearance. Unfortunately, this dormant epidemic disease has again appeared in North Bihar after 1975, an epidemiological survey in Bihar revealed that about 700,000 cases of kala-azar occurred with 4000 deaths. The disease in now endemic in most districts of Bihar and some districts of West Bengal. Recently it has been reported that 2.5 lakh cases of visceral leishmaniasis have been identified in Bihar in 1991-1992 which clearly indicate the urgent need for control of the disease in India (Thakur, 1992).

**LIFE CYCLE OF LEISHMANIA**

*Leishmania* has two stages in its life cycle, the amastigote form occurring in man (or in other hosts) and the promastigote form occurring in sandfly. The amastigote form resides in reticuloendothelial system and multiplies by binary fission. The host cell is thereby enlarged and eventually ruptures. The liberated parasites invade fresh cells and the cycle is thus repeated. In this way the entire reticuloendothelial system becomes progressively infected. In the blood stream, some of the amastigotes are phagocytised by the neutrophils and monocytes. The blood sucking sandfly draws these free amastigotes as well as those within the monocytes during its blood meal. In the hindgut of the sandfly, amastigotes transform into promastigotes.

**Amastigote (aflagellated) Form:** *Leishmania* amastigote is a minute round or ovoid body, 2 to 4 μm in diameter. It has a rounded nucleus, usually eccentric in position, and a kinetoplast which appears as minute dot beside the nucleus. The kinetoplast has a mitochondrial structure containing DNA. A filament called axoneme representing the root of the flagellum extends from the kinetoplast to the body margin.

**Promastigote (flagellated) Form:** Elongated spindle-shaped, these are about 14 to 20 μm long and 1.5 to 3.5 μm broad. The round or oval nucleus is situated centrally and the oval parabasal body lies transversely near the anterior end. In front of the parabasal body is placed the light staining eosinophilic vacuole, over which runs the root of the flagellum, which is longer than the body.

**Transformation Events From Amastigote to Promastigote:** (Chang et al. 1985)

a) In *L. donovani*, respiration is increased.

b) Cytochromes are produced concurrently with the mitochondrial extension.

c) Cell volume is increased ten-fold and length of body is increased four to five fold.

d) A prominent locomotory flagellum is produced.

e) 30 - 80% amastigotes are transformed into promastigote; the time taken to complete the process may vary from 20-96 hours. The whole process is independent of cell division.

f) Mitochondrial amplification is demonstrated by outgrowth of branches from the kinetoplast capsule during transformation.

g) The transformation is accompanied by a slight but significant decrease in relative mitochondrial volume.

h) An increase in polyamine levels is marked.

i) Cyclic AMP may play a part in the control of the differentiation process.

j) The differentiation process may be blocked by a number of anti biotics and also by spleen cell extracts.

k) A marked increase in tubulin biosynthesis has been reported in *L. mexicana*.

**STAGES OF LEISHMANIA INFECTION TO MACROPHAGES**

a) Attachment of promastigote to macrophage surface.

b) Transformation in amastigote.
c) Neutralization of macrophage microbicidal machinery.
d) Multiplication of amastigotes within phagolysosomes of macrophages.
e) Bursting of host macrophage and infection to adjacent macrophages.
Macrophages have different roles to *Leishmania* infection:
a) Function as host-cell to the parasite.
b) Present parasite antigen to the immune system.
c) Act as effector cells responsible for parasite killing in the healing stages of infection.

**PHAGOCYTOSIS OF LEISHMANIA BY MACROPHAGE**

Serum from many animal species displays potent lytic activity against *Leishmania* promastigotes due to complement activation by the alternate pathway. It may therefore be speculated that survival of the parasite within animal hosts depends on its activity to become intracellular. Interestingly, no specific organelle that might facilitate cell invasion has been detected at the ultrastructural level. Leishmaniasis appears to rely entirely on the phagocytic activity of the macrophages to reach a safe intracellular location. A scanning electron microscopic study of the phagocytosis of *L. donovani* by hamster macrophages *in vitro* revealed no preferential orientation (tail first, head first) of the parasite during internalization.

Attachment to the macrophage membrane is a prerequisite to phagocytosis. Possible ligands on the parasite include:

a) A 60 to 65 kilodalton glycoprotein (gp 63) – the promastigote surface protease (Russell and Wilhelm, 1986).
b) A complex lipophosphoglycan (LPG) (Hamman et al., 1986).
c) Fucose and mannose containing glycoconjugates.
d) Third component of complement (C3) attaches to gp 63 or LPG of promastigote (Russell, 1987).

Possible receptors on macrophages responsible for *Leishmania* internalization are:

a) Complement receptor 3 (CR3), through fixation to iC3B, to the Arg-Gly-Asp sequence on gp 63 or to externally exposed sugars (Russell and Wright, 1988).
b) The fibronectin receptor.
c) Carbohydrate-recognizing receptors, e.g. the mannose - fucose receptor.
d) A receptor for advanced glycosylation endproducts.

Differences reported in different experimental systems as to the nature of the main surface interactions between *Leishmania* and macrophages relate to:

a) Type of macrophage.
b) Species of *Leishmania*.
c) Developmental stage of *Leishmania*.
d) Presence or absence of serum during assays *in vitro*.

**INTRA-MACROPHAGE EVENTS AFTER INTERNALIZATION**

**Phagolysosome Formation:** After internalization of the parasite, the parasitophorous vacuole undergoes fusion with secondary lysosomes where the promastigotes transform into amastigote form. Amastigotes not only harbour in this phagolysosomes but also multiply. Promastigotes are more susceptible to intracellular destruction and their survival in phagolysosomes is dependent on their capacity to be rapidly transformed into amastigotes. Their location within phagolysosomes renders the parasites potentially susceptible to the toxic activity of the lysosomotropic agents, such as certain amino-acid esters (Rabinovitch et al., 1987). These are trapped in host cells by protonation; they then presumably accumulate in the lysosomal compartment of the parasite itself where their hydrolysis would occur, leading to osmotic
swelling of the organelle and to rapid parasite disruption.

Nutritional Requirements of Intracellular Amastigotes: Intracellular survival implies that:

a) the nutritional requirements of the parasite is satisfied,
b) its metabolism has become adapted to the conditions prevailing within the host cell, and

c) measures have been taken to avoid the harmful effects of lysosomal hydrolases and of toxic oxygen metabolites produced by the respiratory burst during phagocytosis (Mukkada et al., 1985).

Intracellular leishmanias have access to the extracellular environment via the vacuolar apparatus of host cell. Leishmania is unable to synthesize purine and depends on the availability of preformed basis of host macrophage in order to synthesize its own nucleic acids. By autoradiography, it has been demonstrated that L. donovani can synthesize DNA from RNA precursors derived from host macrophages in vitro.

Nutrient uptake by the parasite appears to be mediated by different plasma membrane transport systems. Incorporation of glucose and amino acids is carrier-mediated in several Leishmania spp. and the membrane glucose transporter of L. donovani has been characterised. An extremely oriented acid phosphatase and the gp 63 surface protease may also help the parasite to obtain essential nutrients from its surroundings.

In the acidic environment (pH 4.5 to 5.5) of host cell phagolysosomes, amastigotes proliferate and multiply. Presence of the parasite seems to have little impact on lysosomal pH because it remains acidic. Moreover, the parasite membrane contains a proton-translocating ATPase which probably helps to maintain pH homeostasis in the parasite and contributes to lysosomal acidification. This proton gradient drives the active transport of nutrients required for parasite growth (Antoine et al., 1990).

METABOLIC CHANGES FROM PROMASTIGOTE TO AMASTIGOTE AFTER TRANSFORMATION

a) The respiration rate and glucose catabolism are strongly decreased in amastigotes, correlating with the lower activity of several glycolytic enzymes.
b) Concomitantly, nonesterified fatty acids become a predominant energy source.
c) The temperature shock from vector to host body (22°C to 37°C) triggers the synthesis of several heat-shock proteins (HSP), preceding the morphological and metabolic changes characteristic of the amastigote stage. This heat-shock also increases infectivity of the microorganisms (Mukkada et al., 1985).

RESISTANCE APPARATUS OF AMASTIGOTES AGAINST TOXIC MACROPHAGE PRODUCTS

Phagocytosis of Leishmania promastigotes induces macrophages to produce a strong metabolic burst and the production of active derivatives of oxygen. Resistance to such toxic agents must be a prerequisite to intracellular survival of parasites. Amastigote properties that weaken respiratory burst of macrophages may be summarised as below:

a) Amastigotes contain scavenging enzymes such as superoxide dismutase and catalase which lead to a weaker respiratory burst than that induced by ingestion of promastigotes, which contain little catalase (Lewis and Peters, 1977).
b) Trypanothione, a novel reducing agent that may help to detoxify oxygen metabolites, is found in Leishmania.
c) The acid protease on the cell surface has been shown to reduce the respiratory burst of the neutrophils.
d) Presence of amastigotes within macrophages depresses their response to macrophage activating factors. One acid phos-
phatase of *Leishmania* blocks superoxide anion production. Further, leishmanial lipophosphoglycan can inhibit protein kinase C, a modulator of oxidative metabolism in macrophages (Lewis and Peters, 1977).

*Leishmania* amastigotes may inhibit lysosomal hydrolyases of macrophages by shedding polyanionic substances capable of complexing the positively charged hydrolyases or binding calcium ions (Sett et al., 1993). GP 63 or the ecto-acid protease may be involved in parasite destruction.

**LIGAND-RECEPTOR INTERACTION AND INTRACELLULAR SURVIVAL OF *LEISHMANIA***

Whether the nature of the ligand-receptor interaction that precedes phagocytosis has some role in the successful infection of macrophages by *Leishmania* is not fully understood. Infectivity of cultured promastigotes is strongly dependent on the growth phase in vitro (logarithmic vs. stationary) of *Leishmania* (Sett et al., 1992), and it correlates with the increased expression of a surface glycoprotein presumed to be gp 63 and of a high molecular weight glycolipid structurally related to the LPG. Interestingly, intracellular parasite survival was inhibited when promastigotes were coated with a monoclonal antibody of gp 63 (Chan, 1989). Further, one strain of *Leishmania major* lacking surface LPG was found to be unable to infect hamsters or mice and is rapidly killed by mouse macrophages in vitro; treatment of this strain with LPG from virulent strain restored their capacity to infect macrophages (Handman et al., 1986). It is noteworthy that, in addition to its postulated capacity to dampen macrophage respiratory burst, LPG also appears to function as an acceptor of C3 cleavage products. This observation is consistent with the finding that C3 deposition on the parasite surface promotes intracellular survival, presumably favouring parasite interaction with macrophage CR3 (C3bi), a receptor known to trigger phagocytosis with little stimulation of respiratory burst activity.

Serum-independent attachment of *Leishmania* to macrophages involves mannose-fucose receptor that binds specifically with ligands having terminal L-fucose or D-mannose. Binding follows saturation kinetics, requires calcium and is inhibited by these sugars or their polymers. Large quantities of C3 fragments of the complement system are known to be deposited on parasite surface which serve as opsonins for binding to complement receptors (C3bi) on macrophages. Interestingly, the protease activity of gp 63 has been implicated in cleaving C3 into C3b and other C3 products. GP 63 probably also interacts in direct binding through its mannose moieties. Because of their surface abundance, excretory nature and definite role in parasitization, both LPG and gp 63 are being systematically explored as possible candidates for vaccine development (Handman et al., 1987).

**MACROPHAGES AND THE IMMUNE RESPONSE IN LEISHMANIASIS***

Macrophages are involved in both the inductive and the effector phases of the immune response, and leishmanial infection appears to interfere with such activities (Sett et al., 1993). Macrophages from patients with *L. braziliensis* or *L. mexicana amazonensis* infection, or mouse macrophages infected with *L. donovani* in vitro, display a lesser capacity to produce interleukin 1 in response to bacterial stimulants relative to control cells. Similarly, T cell mitogenic stimulation and interleukin 2 production by spleen cells from *L. major* infected susceptible mice are depressed by a macrophage mediated mechanism perhaps as a result of excessive prostaglandin synthesis. Of particular interest is the observation that in vitro infection of mac-
rophages form BALB/c mice with *L. donovani* suppressed the expression of both class I and class II major histocompatibility complex and give products as induced by interferon γ (Murray et al., 1983). Such a phenomenon would be expected to contribute to both the deficient immune response to parasite antigen observed in certain forms of infection of humans and animals and a reduced capacity to function as effector cells for intracellular parasite killing.

Regarding the parasite-antigen presentation by infected macrophages, it has been found that soluble antigenic material could be recovered from the supernatants of short-term cultures of organs from infected hamsters. Similarly, the demonstrations of immune complexes containing anti-*Leishmania* antibodies in the sera of patients with visceral infection and of parasite antigen in their urine are the indications that *Leishmania* antigen is released from infected cells (Handman et al., 1981).

Membrane-bound *Leishmania* antigen has also been demonstrated on infected macrophages in vitro. Immunochemical analyses revealed that the antigen to be of 51 kilodalton molecular weight constituting of three subunits of 26, 11 and 10 kilodalton. The capacity of macrophages to display such molecules would be expected to have a critical role in both the induction and effector phases of the immune response. It has been postulated that depending on the nature of the molecules or fragments thereof that are presented by parasitized macrophages, different subsets of T cells might be stimulated.

**NATURE, POSSIBLE MODE OF ACTION AND SIDE EFFECT OF THE MAJOR ANTILEISHMANIAL DRUGS**

a) Urea stibamine:
*Nature*: A pentavalent antimonial; a product of p-aminophenyl stibonic acid and urea.
*Mode of Action*: Unknown.

**Side Effects**: Cardiotoxicity and glucosuria.
*Remarks*: It has been observed that in the treatment of *Leishmania donovani* infected Chinese hamster, urea stibamine is the most potent drug amongst many other antimonials. However, the use of this drug was discontinued due to the ambiguity of its chemical nature, stability and toxicity (Sanyal, 1985).

b) Sodium stibogluconate (Pentostam):
*Nature*: A pentavalent antimonial.
*Mode of Action*: Possibly by inhibition of glucose catabolism via glycolytic enzymes and of B-oxidation of fatty acids.
*Side Effects*: Cardiotoxicity and renal toxicity.
*Remarks*: Pentostam is recommended as a first line antileishmanial drug for its high efficacy (Ballou et al., 1987).

c) Mechline antimoniate (Glucantime):
*Nature*: A pentavalent antimonial.
*Mode of Action*: Unknown, but supposed to be similar to sodium stibogluconate.
*Side Effects*: Cardiotoxicity and renal toxicity.
*Remarks*: Glucantime is recommended as a first line antileishmanial drug, specially in the case of cutaneous and mucocutaneous infections (Belazzou and Neal, 1986).

d) Pentamidine isothionate:
*Mode of Action*: Not very clear, but studies have indicated that kinetoplast DNA of *Leishmania* spp. is probably the susceptible site of action of this drug.
*Side Effects*: Many and frequent; these include hypotension, nausea, vomiting, hypoglycemia and renal insufficiency.
*Remarks*: Despite its use in various forms of leishmaniasis, the drug is highly toxic with several side effects, and therefore, it is recommended as a second line antileishmanial drug (Berman, 1982).

e) Amphotericin B:
*Nature*: A polypeptide antibiotic.
*Mode of Action*: Amphotericin B is highly antileishmanial. Recent ultrastructural studies
of amphotericin B treated *Leishmania* infected human monocyte derived macrophages showed that the drug directly acts on the parasite. Amphotericin B probably interacts with phospholipid in the cell membrane of *Leishmania*.

**Side Effects**: Immediate effects of intravenous administration of this drug are nausea, vomiting, chills, fever, anemia and renal insufficiency.

**Remarks**: It is considered as a drug of second choice and is used in the treatment of antimony resistant cases (Berman, 1982).

e) *Allopurinol*:

*Nature*: Allopurinol [4-hydroxyprazolo (3,4-d) pyrimidine] is a synthetic pyrazolopyrimidine. Pyrazolopyrimidines are structural analogues of purines in which there is a change in the position of nitrogen which alters their metabolic fate.

*Mode of Action*: Allopurinol has been found to be effective against *L. donovani*, *L. mexicana* and *L. braziliensis*. Its activity can be reversed by adenine, inosine, hypoxanthine and by the compounds that represent the available adenine metabolic pool in most organisms. These findings have led to the hypothesis that allopurinol acts to interrupt purine metabolism in *Leishmania* spp (Kager et al., 1981).

**Side Effects**: Allopurinol has little significant side effects.

**Remarks**: Since allopurinol has considerable advantage over the antimonials and since early in vivo work had been encouraging, allopurinol was tested clinically against visceral leishmaniasis. Some success was reported from India and Kenya. Allopurinol riboside is many fold more effective against *L. braziliensis* and *L. donovani* than the free base. The riboside is expected to be on limited clinical trial in near future.

f) *Formycin B*:

*Nature*: Formycin B [prazolo (3,4-d) pyrimidine C-nucleoside] is another prazolo-pyrimidine drug. It is a structural analogue of inosine.

*Mode of Action*: Metabolism and mechanism of action of this drug were shown to be qualitatively similar to those of allopurinol riboside.

**Side Effects**: Formycin B has little significant side effect.

**Remarks**: Efficacy of this drug by oral administration was found to be 4 to 8 times greater than that of the standard antimonial drug glucantime when administered intramuscular. It caused 85 to 92% reduction in the number of hepatic *L. donovani* amastigotes of infected hamster after oral administration at the dose of 13 mg/kg per day for 4 days (Rees, 1982).

g) *WR 6026*:

*Nature*: WR 6026 is a compound that belongs to the class 8-aminoquinoline.

*Mode of Action*: The high antileishmanial property of WR 6026 is possibly due to the in vivo occurrence of some active metabolite of WR 6026.

**Side Effects**: This drug catalyzes hemoglobin oxidation and a moderate amount of reversible methemoglobinemia has been demonstrated in dog.

**Remarks**: WR 6026 has shown real promise as a potential chemotherapeutic agent against *Leishmania*. When *L. donovani* infected hamsters were orally treated with WR 6026, the compound was shown to be 400-700 times more active than the antimonials. WR 6026 is under toxicity studies and if no untoward side effect is revealed, it can emerge as the first oral antileishmanial drug (Berman and Lee, 1984).

h) *Ketoconazole*:

*Nature*: Ketoconazole is an antifungal imidazole.

*Mode of Action*: Both leishmanial and fungal membranes contain ergosterol instead of cholesterol which is present in mammalian cell membrane. Antifungal azoles exploit this difference by preferential inhibition of ergosterol synthesis. Ketoconazole was shown to
act by blocking the cytochrome p 450-dependent C-14 demethylation of lanosterol (Goad et al., 1984).

Side Effects: Ketoconazole has little significant side effects.

Remarks: This drug virtually eliminated all organisms in in vitro experiments at concentration of 10 to 15 μg (micron) per ml and was shown to be effective against the visceral form in rodent models.

i) Methotrexate:

Nature: Methotrexate or amethopterin (4-amino-10-methyl-folic acid) is a structural analogue of folic acid.

Mode of Action: Methotrexate is a strong inhibitor of dihydrofolate reductase which catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. Formation of thymidylate acid takes place during DNA synthesis by the action of thymidylate synthetase which converts dUMP to dTMP by methyl-group transfer, which is donated by tetrahydrofolate. As the blocking of dihydrofolate reductase results the cessation of DNA synthesis, methotrexate has used as the chemotherapeutic agent for a number of diseases. It is most commonly used and thoroughly studied as an antineoplastic agent; it has also been reported to have high leishmanicidal activity by several workers (Scott et al., 1987).

Side Effects: Methotrexate is a cytotoxic drug. Its general toxic effects are stomatitis, diarrhoea, hemorrhagic enteritis, hepatic dysfunction, myelosuppression, renal dysfunction, etc.

Remarks: The extent of inhibition of dihydrofolate reductase activities in different Leishmania promastigotes by methotrexate differs with the species of the parasite. Considering the wide application of this drug and the potential cytotoxicity together with the observed antileishmanial activity, it would be an attractive proposition to use this drug for selective delivery to a diseased site like Leishmania infected phagolysosomal compartment of macrophages through some targeted specific carrier molecules.

j) Doxorubicin:

Nature: Doxorubicin or adriamycin is an anthracycline antibiotic isolated from Streptomyces peucetius. It is a daunomycin derivative with alteration in amino sugar.

Mode of Action: Doxorubicin is used in recent years as the 'wonder drug' in cancer chemotherapy which forms a complex with nuclear DNA by intercalating between base pairs, thus causing steric hindrance to DNA replication and DNA-dependent RNA synthesis; recently it has been reported to inhibit DNA topoisomerase II, a DNA untwisting enzyme. Besides the vital point that the DNA turnover rate of L. donovani amastigotes is much higher than its host cell, there are two additional reasons in favour of using doxorubicin as an antileishmanial agent:

a) Doxorubicin accumulates in the cytoplasmic inclusions especially in macrophages unlike its intercalation with the nuclear DNA in other mammalian cells.

b) Doxorubicin also acts as a macrophage-activating agent, the degree of activation being comparable to that of interferon γ.

Side Effects: Like many other strong anticancer drugs, doxorubicin may produce many toxic side effects. It is highly cytotoxic and found to be mutagenic and carcinogenic in high doses. The general toxic effects are hematologic and cardiac, e.g., leucopenia, thrombocytopenia, anemia, sinus tachycardia, arrhythmia, etc.

Remarks: Doxorubicin has been recently reported by us to be highly leishmanicidal; almost 95% inhibition of splenic amastigotes of L. donovani was found when a total dosage of 625 μg doxorubicin per kg body weight per day was administered intravenously in four consecutive doses in mice, which is far less than the toxic dose (Sett et al., 1992). Though it may be included as a second line antileishmanial drug after a thorough pharmacological and toxicological evaluation, it would be a very attractive candidate for se-
lective drug targeting in leishmaniasis for both of its high leishmanicidal and cytotoxic effects.

MACROPHAGE DIRECTED DRUG DELIVERY: THE PROBABLE ANTHEISHMANIAL THERAPEUTICS IN THE NEXT DECADE

One of the most recent trends in pharmaceutics is to deliver active drug moieties specifically to their desired site of action. The idea of selective targeting was first conceived by Paul Ehrlich who proposed the idea of "magic bullet", i.e., drugs or toxins when bound to a molecule with special affinity for a specific organ or tissue will be able to selectively destroy target cells without injuring other unrelated host tissue or cells. Needless to say, such accurate targeting presupposes extensive knowledge at the molecular level of cell biology, mechanism of endocytosis, vehicle development etc. that is only becoming available in recent years.

The core concept of drug delivery experiments is that the drug should be toxic for most of the cells but it should be allowed to reach its designated target. Most drugs exhibit very little site specificity. A drug generally distributes itself passively in tissue and body fluids primarily according to its physical and physicochemical properties. The first requirement for site-specific targeting is that the cell type to which the drug is to be directed is unique in the presence of the drug surface and is involved in internalization. In circulation, the ligand molecule binds specifically and with extremely high affinity to the receptor. Once the ligand and receptor are characterised, a site-directed delivery mode for the drug of interest can be developed. Most drugs are small molecules and tend to be distributed all over the system. In targeting drugs this problem is generally overcome by entrapping the drug in liposome or by covalently conjugating it to a carrier macromolecule. Both natural and synthetic macromolecules have been investigated as potential drug carriers. These include plasma proteins, glycoproteins, lectins, DNA fragments and synthetic molecules like polyvinyls and polylysines. In general, proteins are preferred carriers though in some cases adverse immunological response may lead to problems. Finally targeting is achieved by conjugating a homing device which normally happens to be the ligand that will specifically recognize the receptor of the particular cell type. Antibodies conjugated to toxins have been attempted in a number of studies for targeting to cancer cells. Alternatively sugar residues conjugated to drug have the potential as the targeting device to specific cells since mannose glycoprotein receptor on macrophages, mannose-6-phosphate receptor on fibroblasts and galactose receptor on hepatocytes have been discovered (Basu et al., 1991). Visceral leishmaniasis is a disease of macrophages. It can therefore be regarded as an ideal primary model for developing targeted delivery system for macrophages. In fact, some of the early successes of liposome research were recorded with experimental models of visceral leishmaniasis (Croft et al., 1989). In 1978, two groups simultaneously showed that pentavalent antimonials when encapsulated in liposomes without any homing device were 200 to 700 fold more effective when tested in a rodent model (Alving, 1983). The rationale behind the approach of liposome as a carrier in leishmaniasis is based on the knowledge that liposomes injected into blood stream are immediately cleared or taken up by the cells of the reticuloendothelial system, e.g., hepatic and splenic macrophages where the parasites harbour and multiply (Sett et al., 1993a). Liposomes containing amphotericin B have also been proved to be more effective than the free drug against L. donovani in rodent and monkey models. Liposome entrapped WR6026 was almost eight times more effec-
tive than the free drug against *L. donovani* in hamsters. Liposome encapsulated muramyl dipeptide and lymopokines can reduce the parasite burden from *L. donovani* infected mice suggesting their possible immunomodulatory role in activating macrophages. Because of occasional bioincompatibility, efforts have also been made to replace liposome with erythrocyte ghosts with some success. Although liposomes may have some adverse pharmacological effects, the development of this carrier of antileishmanial drugs warrants further investigation. Commercial development of a liposomal preparation containing sodium stibogluconate with more shelf-life stability is at present being seriously explored.

A few attempts have been made to selectively deliver antileishmanial drugs using macromolecule as a carrier in leishmaniasis. Mukhopadhyay et al. (1989) have shown that methotrexate coupled to maleylated bovine serum albumin is taken up efficiently through the macrophage scavenger receptor leading to selective killing of intracellular *L. mexicana amazonensis* in vivo and in vitro experimental models. Our group have efficiently demonstrated the profound potentiality of mannosylated albumin as a unique drug carrier for macrophage related disorders using visceral leishmaniasis as the model disease (Chakrabarty et al., 1991; Sett et al., 1993b). The biological phenomena relevant to drug targeting are very complex. A clear understanding of many, physiological and pathological mechanisms will allow to design truly selective drug or prodrug. It would be an interesting approach if a versatile drug vehicle would be designed which might accomodate a number of drugs for a number of related diseases.

**CONCLUSION**

Macrophages play a prominent role in the defence against a number of infectious agents. A variety of substances like endotoxins, complement components, immune complexes etc. attract them to the infected area where they remain under the influence of a migration inhibition factor released by the T cells (Lasser, 1983). After arriving the infected focus, the macrophages phagocytose the infectious agents, and once ingested, the organism may be killed by both oxygen-dependent and oxygen-independent mechanisms. Oxygen-dependent mechanisms include the production and intracellular release of reactive oxygen species, such as superoxide, hydroxyl peroxide and hydroxyl ions derived from the respiratory burst. Oxygen-independent mechanisms are mainly related to the acidification of the phagocytic vacuole after lysosomal fusion (Gabig and Babior, 1981).

Injected into humans by sandflies as extracellular promastigotes, *Leishmania* spp. bind via a surface glycoprotein gp 63 to the CR3 receptor (specific for C3bi of complement) of macrophages and are quickly phagocytosed. Although a percentage of the phagocytosed promastigotes are killed, those that survive convert to the intracellular amastigote form within 48 hours. The *Leishmania* amastigotes live and multiply within secondary (fused) phagolysosomes containing all of the enzymes and chemicals associated with such organelles. The cell membrane of *Leishmania* amastigotes contains proteins which are resistant to many proteases. Further, they contain proteins such as a complex glycolipid and lipophosphoglycan (PG) which are efficient oxygen scavengers protecting the amastigotes from the reactive oxygen and nitrogen intermediates produced by macrophages. Moreover, the amastigote membrane contains a proton translocating ATPase which probably helps to maintain intraparasite pH homeostasis and contributes to lysosomal acidification. Eventually, the amastigotes lyse the host cell and infect surrounding macrophages and thus proceed the disease towards fatality.

Leishmaniasis occurs in most part of the world with the exception of Oceania. Over-
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