Review

Intermediary metabolism in *Leishmania mexicana*

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Introduction

Leishmaniasis (oriental sore; kala azar) is a trypanosomatid protozoan infection caused by several species of the genus *Leishmania*. The world-wide incidence of the disease is hard to determine but has been estimated to be as high as 12 million cases.[1] All continents are affected with the exception of Australia. The clinical manifestations of the disease are protean ranging from a self-healing skin lesion, through various mucocutaneous forms, to disseminated visceral leishmaniasis with hepatosplenomegaly, weight loss, fever, leukopenia, and death.

The parasite exists as a mobile flagellated promastigote form and as a smaller non-flagellated intracellular form, the amastigote. The reservoir for the parasite is extensive and includes rodents, squirrels, cats, porcupines, opossums, foxes, wolves, and dogs. Infection is acquired from the reservoir via ingestion of a blood meal by the sandfly vector (subgenus *Phlebotomus*). Promastigotes grow rapidly in the gut of the sandfly and, as in culture, the optimum temperature range is 22–26° C. Promastigotes can be transferred to man by the bite of the infected fly, allowing emergence of the amastigote form which parasitizes the phagolysosome of the macrophage. The epidemiology of the disease is complex due to the great variations in the reservoir, the vector, the species of *Leishmania*, and the immunological status of the host.

Much of the literature concerning Leishmaniasis has been based on studies carried out in multiple different *Leishmania* species, and frequently data from different strains have been juxtaposed and extrapolated in attempts to synthesize a unified understanding of the parasite. The pitfalls of this process are obvious. In contrast, this monograph will focus exclusively on the *Leishmania mexicana* group, and specific aspects of its intermediary metabolism will be reviewed. Limited discussion will, whenever possible, be augmented by references to detailed studies which often include other *Leishmania* species and are referenced after each sub-heading in the text. The majority of publications apply to the extracellular promastigote form of the parasite; this will also apply in the following discussion unless the intracellular amastigote form is indicated specifically.

Growth requirements in vitro

Early studies of *Leishmania* were hampered by the difficulties of in vitro cultivation of the parasites. The pioneering studies of Trager[2] laid the groundwork for the development of agar, semi-solid, and liquid media, which were supplemented with blood or fetal calf serum (FCS) to satisfy the requirement of hemoflagellates for exogenous heme and other substances. The recognition of this requirement for heme led to the observations that both promastigotes and amastigotes of *Leishmania mexicana* rely on exogenous heme due to a partial deficiency of heme biosynthetic enzymes[3] and, more recently, the demonstration of a plasmalemma heme binding site on promastigotes.[4] This is an excellent example of how requirements for in vitro cultivation can form the basis for subsequent elucidation of inherent physiological and biochemical characteristics of the parasite. This absolute requirement for exogenous heme may offer a potential site for chemotherapy. Interestingly, it has been reported that both *Trypanosoma brucei* and *Leishmania donovani* are destroyed when exposed to hematoporphyrin and that the effect is augmented by menadione, suggesting a possible cidal role for hydrogen peroxide.[5,6] It is evident that the composition of the medium in which parasites are grown may influence subsequent analysis of the biochemical pathways under study. For example, there is evidence that the efficiency of transformation of *L. mexicana* may be affected by exogenous factors such as non-esterified fatty acids (amastigote → promastigote[7]) or oxygen tension (promastigote → amastigote[8]) in the medium.

Media which have been used commonly in the studies cited in this review include Medium 199, Grace's insect, and Schneider's Drosophila (all with 30% FCS),[9] a semi-defined medium containing liver infusion broth and hemin[10] and, most commonly, modified

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Eagle's minimal essential medium with 10% FCS.[11] Despite the report of propagation of amastigotes in a cell-free medium,[12] the most common method for cultivation of amastigotes is that of Chang[13] which utilizes the cultured mouse macrophage line J774G8.

The surface membrane[14,15]

Despite the critical roles of the surface membrane in the acquisition of substrates and biosynthetic precursors, exocytosis, protection from the relatively inhospitable environments of the sandfly gut and the phagolysosome, and immunoprotection, relatively little is known of the biochemistry and physiology of *L. mexicana* plasmalemma. *L. mexicana* promastigotes grown in lipid-free medium were shown to contain total lipids of 3% of their dry weight with roughly equal proportions of neutral and polar lipids; interestingly, growth in MEM + 10% FCS shifted this ratio to 30:70 in favor of polar lipids.[16] The major sterol was found to be ergosta-5,7,24 (28)-trien-3β-ol with appreciable concentrations of lanosterol.[17] Inhibition of the cytochrome P450 isozyme which demethylates lanosterol is thought to be the basis of the inhibitory action of ketoconazole on the growth of *L. mexicana*[18,19] and amastigotes,[20] probably, at least in part, by influencing the fluidity of parasite biomembranes. Uptake of cholesterol and its incorporation into promastigotes,[18] and uptake of palmitic, stearic, and oleic acids into amastigotes[21] have been reported, but details concerning the transport processes are lacking.

Studies utilizing colloidal iron hydroxide and cationized ferritin revealed that *L. mexicana* promastigotes are more electronegative than amastigotes, but in contrast to other *Leishmania* species, surface charge did not correlate with infectivity of promastigotes.[22] Removal of sialic acid from the membrane by neuraminidase treatment decreased the electrophoretic mobility of promastigotes but not amastigotes; the rapid puromycin-inhibitable recovery of sialic acid residues on membranes after removal by neuraminidase is evidence that *L. mexicana* synthesizes sialic acid rather than acquires it passively from the medium.[22] Acid phosphatase also has been detected cytochemically in the plasmalemma, Golgi, endoplasmic reticulum, close to the flagellar pocket and in what appear to be lysosomes of both infective and non-infective *L. mexicana* promastigotes. However, none were found in amastigote plasmalemma.[23] Acid phosphatase has been suggested to play a role in protection of the amastigote in other *Leishmania* species, but the findings do not support such a role for *L. mexicana* in which its functions remain obscure.

Plasmalemma of *L. mexicana* promastigotes express a wheat-germ agglutinin-like ligand that binds to an N-acetyl glucosamine moiety on macrophage plasmalemma and facilitates uptake of the parasite into macrophages.[24] Further characterization of *L. mexicana* cell surface carbohydrates utilizing 28 purified lectins revealed differences in the carbohydrate composition between promastigotes and amastigotes, and between infective and non-infective forms.[25] Curiously, there was specificity in binding to promastigotes versus amastigotes even among lectins which bound to the same carbohydrate composition (e.g., D-GalNAc); evidently, this area and its potential contribution to selective uptake deserve further study. Utilizing a Boyden chamber, it also has been shown that *L. mexicana* promastigotes migrate up a concentration gradient of sugars, albumin, hemoglobin, and an unidentified factor generated by the parasite itself.[26] Such chemotactic factors might influence migration of the promastigote within the sandfly gut or entry into the mammalian host from the proboscis.

Early studies (1969) on post 10,000 g extracts of *L. mexicana* promastigotes revealed a total protein of 61 μg/mg dry weight and 11 antigens were demonstrated by immunoelectrophoresis.[27] Subsequently, a major surface glycoprotein (M_r 65,000) was isolated from nine *Leishmania* species, including *L. mexicana*; upon electrophoresis in SDS-page under non-reducing conditions, there was a shift to M_r 50,000.[28] The glycoprotein was found in both amastigotes and promastigotes, and it was suggested that changes in glycosylation of this protein might account for changes in immunogenicity. In a subsequent report,[29] the presence of N-linked glycoproteins was found to correlate with protective immunity to *L. mexicana* in BALB/c mice. The major surface glycoprotein (M_r 65,000) identified in *Leishmania* species (termed p63) later was shown to be a protease which was released following digestion with phospholipase C.[30] This protease is abundant on the surface of *Leishmania* species (5×10^5 mol/cell in L. major), and recent studies have shown that it binds to complement receptor type 3.[31] Another *L. mexicana* membrane N-linked glycoprotein (63KDa) has been purified by affinity chromatography and shown to inhibit the binding of promastigotes to J774 macrophages.[32]

The mechanism and sites of uptake of substrates and biosynthetic precursors by *L. mexicana* organisms remain poorly defined. The plasma membrane of *Leishmania* is invaginated anteriorly to form a reservoir which gives rise to the flagellum. The membrane lining the reservoir is the only known site for endocytosis and secretion.[33] Although carrier-mediated transport of sugars, amino acids, and purines has been demonstrated in other *Leishmania* species,[14] the only receptors studied in *L. mexicana* have been the heme receptor[4] and the adenine receptor.[34] The latter was shown to display specific binding which resulted in increases in adenylate cyclase activity and cAMP concentrations: The functional correlate was a decrease in the rate of amastigote to promastigote transformation.

Carbohydrate metabolism[35-38]

Carbohydrate metabolism and energy production are the most thoroughly characterized aspects of *L. mexicana* metabolism. Martin et al.[39] have demonstrated the presence in *L. mexicana* of all the enzymes of the

Embden-Meyerhof pathway, the hexose monophosphate shunt, and the tricarboxylic acid (TCA) cycle. In contrast to other species of *Leishmania*, neither lactate dehydrogenase nor lactate have been identified in the Embden-Meyerhof pathway in *L. mexicana*; instead, NAD may be regenerated utilizing dihydroxyacetone phosphate as a hydrogen acceptor, which is reduced to glycerol phosphate and hydrolyzed by glycerophosphate phosphatase to yield glycerol and inorganic phosphate. Phosphoglucose isomerase is highly active whereas hexokinase is relatively inactive.[35,39] Hexokinase, phosphofructokinase, and pyruvate kinase are all irreversible in *L. mexicana* and may be sites of allosteric control.[40] It is unknown whether fructose 2,6-biphosphate or adenosine monophosphate are allosteric regulators of pyruvate kinase in *L. mexicana* as they are in other species of *Leishmania*. Apparently, pyruvic acid may be carboxylated to oxaloacetic acid by pyruvic carboxylase or decarboxylated to acetyl CoA by pyruvate dehydrogenase.[35] Subcellular fractionation has been used to demonstrate that hexokinase, phosphofructokinase, fructose-1,6-diphosphatase, glycerol-3-phosphate dehydrogenase, and glycerokinase are found in small organelle fractions (P_2) which have been termed glycosomes.[41] In contrast, pyruvate kinase and glucose-6-phosphate dehydrogenase are mainly cytosolic.[41] Why glycosomes appear to be limited to the kinetoplastid flagellates is unknown. The relative flux of glucose through the Embden-Meyerhof pathway and the hexose monophosphate shunt remains unknown.

Early evidence to support the existence of the TCA cycle in *L. mexicana* came from studies that demonstrated utilization and metabolites of various sugars. Although all the enzymes of the TCA cycle now have been demonstrated in *L. mexicana*,[39] the activities of citrate synthase, α-ketogluterate dehydrogenase, and succinate dehydrogenase are very low whereas that of pyruvate carboxylase is high. It has been suggested that pyruvic acid entry into the TCA cycle is slow relative to its conversion to oxaloacetate and acetyl CoA and that the conversion of pyruvate to succinic acid is via malic and fumaric acids.[35] Carbohydrate utilization in *L. mexicana* has been shown to occur mainly at the end of the log phase and at the beginning of the stationary phase.[42] Phosphoenol-pyruvate carboxykinase was highly active during glucose consumption but decreased after glucose depletion, suggesting that its primary function is glycolytic rather than gluconeogenic.[42] Isocitrate lyase and malate synthase have been identified in *L. mexicana* and serve to cleave isocitrate into succinate and glyoxylate. Glyoxylate may react with acetyl CoA to yield malate, which can partake in gluconeogenesis via oxaloacetate. Evidence that the glyoxylate cycle may be operative in *L. mexicana* has been provided,[40,43] and its contribution to gluconeogenesis has been suggested.[43]

Respiratory rates in *L. mexicana* decreased above 35° C and after 2 hr of incubation in diluted blood; the latter effect could be reversed after addition of fresh blood.[45] The activity of phosphogluconate dehydrogenase also decreased with increasing temperature.[46] These changes are consistent with the temperature optima of promastigote propagation and may presage metabolic changes which accompany transformation to the amastigote. Although overall respiratory rates (when corrected for cellular protein content) were similar in promastigotes and amastigotes, and respiratory inhibitors indicated similar cytochrome-containing respiratory chains,[47] substrate utilization differed between the two forms. Promastigotes utilized more glucose, while amastigotes and stationary phase promastigotes utilized more nonesterified fatty acids.[47] In both forms, asparagine, glutamine, leucine, lysine, methionine, threonine, and, to a minor extent, proline were consumed avidly; alanine and glycine were excreted.[48] Amastigotes also appear to lack pyruvate carboxylase and malic enzyme (carboxylating) but have high levels of malate dehydrogenase and phosphoenolpyruvate carboxykinase.[49] Even within the *L. mexicana* group, multiple isozyme analysis of 23 enzymes has shown considerable variation and enabled differentiation of three subgroups—mexicana, amazonensis, and peruviana.[50]

In summary, carbohydrate metabolism and energy production in *L. mexicana* follow broadly similar patterns to these seen in many other organisms. The areas which are still unexplored are those of regulation of the various competing pathways and, most important, detailed knowledge of these pathways in the intracellular amastigote form of the parasite.

Purine and pyrimidine metabolism[51]

Based largely on in vitro studies of cultivation, it has been realized that leishmanias lack the pathway for de novo purine synthesis and require exogenous purines which are utilized via the salvage pathway. Subcellular fractionation studies in *L. mexicana* showed that phosphoribosyltransferase activities were associated with glycosomes, adenine phosphoribosyltransferase, nucleosidases, and deaminases were cytosolic, and the particulate enzymes 3′- and 5′-nucleotidases were plasmalemma associated; inosine-5′-phosphate dehydrogenase was associated with endoplasmic reticulum.[52] Direct evidence for a lack of de novo purine synthesis in *L. mexicana* was provided in both promastigotes and amastigotes by the lack of incorporation of radiolabelled formate or glycine into purine nucleotides whereas labelled adenine, guanine, and hypoxanthine were readily incorporated via the salvage pathway.[53] Quantitative but not qualitative differences have been found in these pathways between promastigotes and amastigotes, both of which display higher activities of adenine and guanine deaminases than adenosine deaminase.[54]

Amastigotes concentrate radiolabelled sodium stiboglucanate (Pentostam)[55,56] and pentamidine to a greater degreee than promastigotes. Pentostam has been reported to decrease ATP and GTP synthesis,[57] possibly secondary to decreased glucose catabolism and β-oxidation of fatty acids.[58] Two other compounds of therapeutic interest are formycin B and allopurinol. Formycin B is an inosine analogue which is phosphor-

ylated in *Leishmania* species but not in mammals. The resulting formycin B monophosphate inhibits the conversion of IMP to adenylosuccinate, RNA synthesis, and mRNA translation, and may account for the anti leishmanial action of formycin B.[59-61] Allopurinol also inhibits *L. mexicana* growth in vitro probably by phosphoribosyl-transferase-catalyzed conversion to its ribonucleoside derivative and subsequent incorporation into RNA.[62,63]

In contrast to purine metabolism, *L. mexicana* is capable of de novo pyrimidine biosynthesis; the mechanism is essentially that seen in mammals except that orotate phosphoribosyltransferase and orotidine-5′-phosphate decarboxylase are particulate and associated with glycosomes and dihydroorotate dehydrogenase is cytosolic.[64,65] Activities of the former two enzymes are approximately 40 times higher in promastigotes than amastigotes, but that of the latter enzyme is approximately equal in both forms.[66,67]

Polyamine metabolism

As in most organisms, polyamines appear to be involved in the growth of *L. mexicana*. Putrescine, spermidine, and spermine have been identified at highest concentrations during the logarithmic growth phase.[68] Decreased uptake of putrescine and disordered polyamine synthesis have been demonstrated following treatment of promastigotes with pentamidine isethionate, ethidium bromide, and methylglyoxal-bis (guanylhydrozone).[69] Promastigotes excrete urea, ammonia and putrescine but not spermine or spermidine; putrescine and spermidine were detected at higher concentrations in promastigotes than amastigotes, in which spermine was undetectable.[70] Evidently, polyamine metabolism may be another fruitful area of study for chemotherapeutic intervention.

Recent studies of a small spermidine-containing peptide hold considerable promise in this regard. The peptide (N^1,N^8-bis(glutathionyl)spermidine) was found to be essential in the reduction of glutathione disulphide by NADPH in *Trypanosoma brucei*.[71] Rejoicing in the trivial name of trypanothione, this peptide has been found in all species of *Leishmania*.[72] Although *Leishmania* species display little or no evidence of catalase or peroxidase hemoproteins, hydrogen peroxide metabolism has been shown to occur via trypanothione-dependent peroxidase and reductase[73] (cf. glutathione peroxidase and reductase of mammalian cells).

Trypanothione reductase is present in both promastigotes and amastigotes of *Leishmania* species and presents an excellent target for chemotherapy. Difluoromethylornithine is a drug which inhibits putrescine and spermidine synthesis and may therefore affect trypanothione contents.[74] Furthermore, the substrate binding domain of trypanothione reductase is less specific than that of glutathione reductase enabling reduction of compounds unrelated to trypanothione.[75] Thus, substituted naphthoquinone and nitrofuran derivatives undergo enzyme catalyzed redox cycling generating O_2- and H_2O_2; such compounds are trypanocidal.[76]

Protein metabolism

The quantity of protein per cell in *L. mexicana* has been calculated to be 5.3 pg for promastigotes and 1.3 pg for amastigotes,[77] yet characterization of such proteins is scant. Secretion of two proteins, free amino acids, and a protein-RNA complex have been reported in *L. mexicana*; excretion was abolished by treatment with stibophen and in contrast to *L. donovani*, there was no effect of glucose on TCA-precipitability[78] of these proteins. It has been suggested that these excreted factors may play a role in parasite protection. In this regard, multiple proteinases have been demonstrated in *L. mexicana*; most were thiol proteinases and the distribution was different in amastigotes and promastigotes[79]; antipain and leupeptin (cysteine protease inhibitors) were found to inhibit both the growth and transformation of promastigotes.[80] Five enzymes (cysteine proteinase, arylsulphatase, β-glucoronidase, DNase, and RNase) were found in much higher concentrations in amastigotes than promastigotes in lysosome-like organelles; this finding appears to be specific for the *L. mexicana* group.[81] Tubulin biosynthesis has been studied and has been shown to increase concomitantly with the amastigotes to promastigotes transformation and the morphological changes in flagellar microtubules.[82]

Conclusions

An outline of the intermediary metabolism of *L. mexicana* has been presented. As is readily apparent, there are large gaps in our understanding of the biochemistry and physiology of this organism. Overall, the general outlines of most major metabolic pathways have been described and localized, at least partially, at the subcellular level. Further characterization is urgently required, most especially concerning the sites and effects of metabolic control. The delineation of unique biochemical processes which are different from those occuring in the host remains one of our better hopes for therapeutic intervention in human leishmaniasis (see Ref.[83] for review of chemotherapy). This is especially important in view of the widespread incidence of the disease and the fact that for over 50 years the mainstay of treatment for leishmaniasis has remained the use of highly toxic pentavalent antimony compounds such as sodium stibogluconate.

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