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Metacyclogenesis II: Species-specific Antigenic Characterization and Metabolic Differences between Promastigotes of *Leishmania* spp.

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ABSTRACT

Inter and intra-specific developmentally regulated antigenic polymorphisms and some metabolic differences between promastigotes of Leishmania donovani and L. major have been characterised. There was an increase in optical density values from logarithmic to stationary phase antigen of both species justifying the increased infectivity of stationary phase promastigotes. Immunoblot analysis showed few antigens as species and stage-specific, viz., 105 KDa unique to L. donovani logarithmic phase, 140 KDa to logarithmic phase of L. major and 115 KDa to L. major stationary phase promastigotes, whereas antigen of 50 KDa was only present in logarithmic phase of both L. donovani and L. major strain. Bands at 20, 95 and 120 KDa were present both in logarithmic and stationary phase L. donovani antigen when they reacted with homologous antibody. On the other hand, no common shared antigens were detected in the case of homologous reaction of two phases of L. major strain. Fewer peptides on western blots of stationary phase than of logarithmic phase promastigotes confirmed the concept that the former phase may be less immunogenic but much more infective to its vertebrate host. Of the amino acids and sugars tested, proline was found to have the maximum role in the transformation to metacyclic form. The role of the other sugars were negligible.

INTRODUCTION

The developmental cycle of Leishmania spp. is comprised of an intracellular amastigote form in the vertebrate host and an extracellular promastigote form in the sandfly vector. Perhaps the greatest recent advance in studies of Leishmania in sandflies and cultures is the confirmation of the production of infective or 'metacyclic' promastigotes in the stationary phase of growth (Sacks and Perkins 1984). Within the promastigote population, a dividing non-infective promastigote population gives rise to infective or metacyclic promastigotes in the stationary phase of growth (Giannini 1974, Doran and Herman 1981, Franke et al. 1985, Sacks et al. 1985). In this respect,

leishmanias are similar to Trypanosoma cruzi, another trypanosomatid protozoan pathogenic to human beings (Brenner 1973). Thus, Leishmania promastigotes and T. cruzi epimastigotes multiply in the digestive tract of Phlebotominae and Triatominae insects, respectively. After few days, T. cruzi epimastigotes and Leishmania promastigotes differentiate into a mammal-infective metacyclic stage, acquiring the capacity to resist complement-mediated lysis. Transformation of main insect vector (promastigotes, epimastigotes etc.) stages of Leishmania spp. and T. cruzi to the vertebrate infective (metacyclic) stage is a transition of fundamental importance for understanding higher cell development (Krassner et al. 1993). Recently, Bates and Tetley (1993) have successfully induced metacyclogenesis in L. mexicana promastigotes at pH 5.5 in Schneider's Drosophila medium supplemented with 20% fetal calf serum. It is a remarkable advancement in understanding some unanswered questions regarding its pathogenesis. Metacyclics appear to be already adapted to life in vertebrate hosts. Considerable heterogeneity at ultrastructural, biochemical, genetic and biological level accompanies the development of promastigotes both in the sandfly midgut and in axenic culture (Sacks 1989). In vitro development of infective or metacyclic promastigotes is associated with changes in membrane carbohydrates (Doran and Herman 1981, Sacks et al. 1985, Grogl et al. 1987, Bandyopadhyay et al. 1991), increased resistance to lysis by normal human serum (Franke et al. 1985, Howard et al. 1987, Puentes et al. 1988, Bandyopadhyay et al. 1991) altered motility (Sacks et al. 1985) and enzyme activities (Mallinson and Coombs 1986, 1989). Molecular determinants of metacyclic promastigotes of Leishmania spp. have also been explored (Kweider et al. 1987, 1989, Sacks 1989). A comparison of logarithmic (log) and stationary phase promastigotes showed differences in polypeptide composition (Bandyopadhyay et al. 1991) and antigenicity (Grogl et al. 1987). Developmentally regulated glycoconjugates which can be detected by lectin PNA (Sacks et al. 1985) or by stage-specific monoclonal antibodies (Sacks and Da Silva 1987) were also reported. Very recently, lipophosphoglycan (LPG) and glycoprotein protease (GP63) the two major glycoconjugates displayed both inter- and intra-specific developmentally regulated polymorphisms (Davies et al. 1990, Sacks et al. 1990). It was shown that LPG structure is modified during the differentiation of L. major promastigotes from a less infectious form in log growth phase to a highly infectious 'metacyclic' form during stationary growth phase (Sacks et al. 1990, McConville et al. 1992).

Ramamoorthy et al. (1992) showed that the increase in the surface expression of gp63 during the in vitro development of L. chagasi promastigotes from a less (log phase) to a more infective form (stationary phase) correlated with expression of RNA transcripts derived from different gp63 genes. While cDNA clones specific for either log or stationary growth phases were found to differ from each other only at the 3'-untranslated ends. More recently Steinkraus and Langer (1992) sequenced a gp63 cDNA isolated from a stationary phase promastigotes library of L. guyanensis and that gene encoded a sequence of 20 amino acid residues shorter than the predicted form of the L. mexicana gp63 cl and contained several potential cleavage sites for the addition of a GPI anchor.

In previous communication we have shown stage and species specific comparative study of L. donovani and L. major with respect to lysis by normal human serum, enzyme activity, carbohydrate and protein profiles (Bandyopadhyay et al. 1991, Bhattacharya et al. 1993). In this communication we carried out further antigenic characterization and some metabolic differences between log and stationary phase promastigotes of L. donovani and L. major.

MATERIALS AND METHODS

Leishmania donovani (MHOM/IN/78/UR6, Chakraborty and Das 1988) and Leishmania major (MHOM/IN/71/LRC/L408), maintained in RPMI-1640 (GIBCO, Grand Island, New York) supplemented with 10% (v/v) heat inactivated fetal calf serum (GIBCO), penicillin (200 U/ml) and streptomycin (200 µg/ml) were used in this study.

Soluble antigens were prepared (Leon et al. 1986) from log and stationary phase cultures (Bandyopadhyay et al. 1991).

Antibody against log phase promastigotes of both species of Leishmania were raised after Ray and Ghose (1985).

Modified Ouchterlony gel diffusion precipitin test (GDP) and counter-current immunoelectrophoresis test (CIEP) were done according to Aiket et al. (1979).

Enzyme-linked immunosorbent assay (ELISA) was carried out after Badaro et al. (1986). Stock log and stationary phase antigens of both species were diluted to a concentration of 30 µg/ml with 0.05 M carbonate/bicarbonate buffer, pH 9.6. After overnight incubation with 0.1 ml diluted antigen at 4°C and repeated washing using PBS (pH 7.4) with 0.05% Tween 20, 0.1 ml serially diluted (upto 1:256,000) homologous or heterologous sera were added to each well. After incubation for 90 min at 37°C, the plates were washed with PBS-Tween 20. One hundred microlitre conjugate [Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Mo, USA)] diluted 1:1,000 times with dilution buffer was added and the plates incubated for 90 min at 37°C. After incubation the plates were washed for 15 min, dried and 0.1 ml O-phenylene diamine enzyme substrate solution was added (Voller et al. 1979). The resulting reaction was stopped by the addition of 5N H_2SO_4 after 20 min at 37°C. The absorbance was read at 490 nm on a Multiskan plus P ELISA reader (Flow Laboratories Ltd., Irvine, Scotland).

SDS-PAGE analysis was performed with log and stationary phase antigens, as previously described (Bandyopadhyay et al. 1991).

Gel for immunoblots were transfered to nitrocellulose as described by Towbin et al. (1979). The nitrocellulose sheets with log and stationary phase antigens of both species of Leishmania were placed in blocking buffer (3% BSA in Tris buffer saline, TBS) for 12 h at 4°C on a rocking platform. Strips were washed three times with TBS-Tween 20 for a total period of 30 min. To detect antigens, blots were incubated for 90 min at 37°C on a shaker (Luckham-R-100 TW, England) in appropriate antibody solution. The dilution for L. donovani antibody was 1:100 and for L. major was 1:50 in 5% BSA in TBS. The membranes were washed three times for 30 min and incubated for 90 min in HRP conjugated goat anti-rabbit IgG at 1:4,000 dilution in TBS. After washing as before the blots were incubated with 0.05% 3,3 diaminobenzidine tetra hydrochloride (Sigma) and 0.01% hydrogen peroxide (Merck AG, Darmstadt, FRG) until the colour developed. The reaction was stopped by washing in deionized water.

The effect of L-proline, L-alanine, L-glutamine, L-leucine, L-isoleucine, glucose, sucrose, fructose and mannose were used as substrate on metacyclogenesis. The above substrates (10 mM) were added to artificial triatomine urine medium (TAU) (Contreras et al. 1985) one at time. It is useful because differentiation is not accompanied by self-replication and interpretation of results is therefore simplified. Controls were run simultaneously without any substrate. L-leucine and L-isoleucine, known inhibitors of proline metabolism were added one by one along with L-proline. The percentage of metacyclic stages formed and the total number of cells were determined with a Neubauer hemocytometer on second, third and fourth days of culture. Control cultures contained no metabolites.

RESULTS AND DISCUSSION

The trypanosomatids appeared relatively early in the evolutionary history of the eukaryotes, and then gradually developed their parasitic mode of life. In general, they have two forms in the life cycle : one in the gut of insects and other in humans (or certain mammals). It is very reasonable that a large number of metabolic, immunogenic and morphogenetic alterations occur during the transition from the insect to the mammalian host (Blum 1993). The idea of 'metacyclogenesis' that is the transformation of non-infective form to infective ones had previously been developed in Trypanosoma spp. (Vickerman 1985). So, metacyclogenesis occurs in response to a variety of changes in environmental stimuli, and is accompanied by dramatic changes in gene expression, protein synthesis and morphology.

The results of GDP and CIEP tests failed to provide any distinct differences between log and stationary phase antigens of both species of Leishmania. The band pattern was identical in both log and stationary phase antigen when reacted with homologous and heterologous antibody (Bandyopadhyay 1992).

There was a clear increase in the optical density (O.D.) valaue from log to stationary phase in case of both L. donovani and L. major. But this increase was not found to be statistically significant. Another interesting feature was the increase in O.D value of L. major with heterologous antibody than with homologous antibody. This feature was not observed in case of L. donovani (Fig. 1).

Though GDP and CIEP were not sensitive enough to reveal any stage-specific differences, ELISA was sufficiently sensitive to distinguish stage-specific antigen, despite, some cross-reactivity among the two stages. Badaro et al. (1986) have shown the highest mean absorbance of kala-azar patient's sera

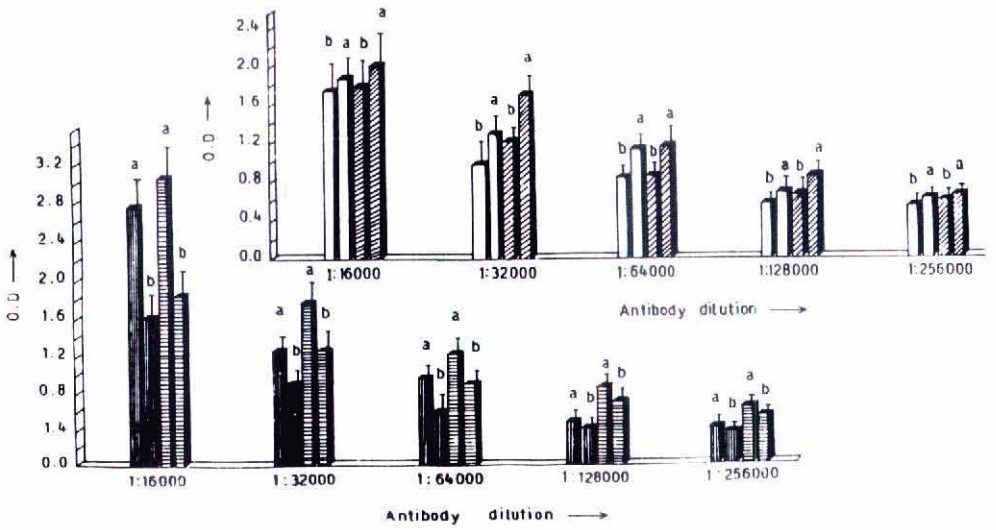

Fig. 1. ELISA results of log and stationary phase antigens of L. donovani and L. major against homologous and heterologous antibody, L. donovani log antigen is represented by ▥, L. donovani stationary antigen by ▤, L. major log antigen by □, L. major stationary antigen by ▨. L. donovani log antibody and L. major log antibody is represented by **a** and **b** respectively.

with L. mexicana amazonensis. Our results also demonstrated similar type of absorbance profile. This could be explained by the fact that immunodominant components of L. donovani membrane are also the major shared antigen (Handman and Hocking 1982). Increase in absorbance value from log to stationary phase of both L. donovani and L. major with sera from immunized rabbits in the present study justify increased infectivity of stationary phase promastigotes. El Amin et al. (1987) have also found that the older forms of L. donovani were highly reactive with patient's sera. Our results of high infectivity between both log and stationary phase L. major antigen and L. donovani antibody support the idea of Rosen et al. (1986), Bogdan et al. (1990) that the L. major derived antigen rather than L. donovani equivalent, was used because sera of kala-azar patients respond best to L. major antigen.

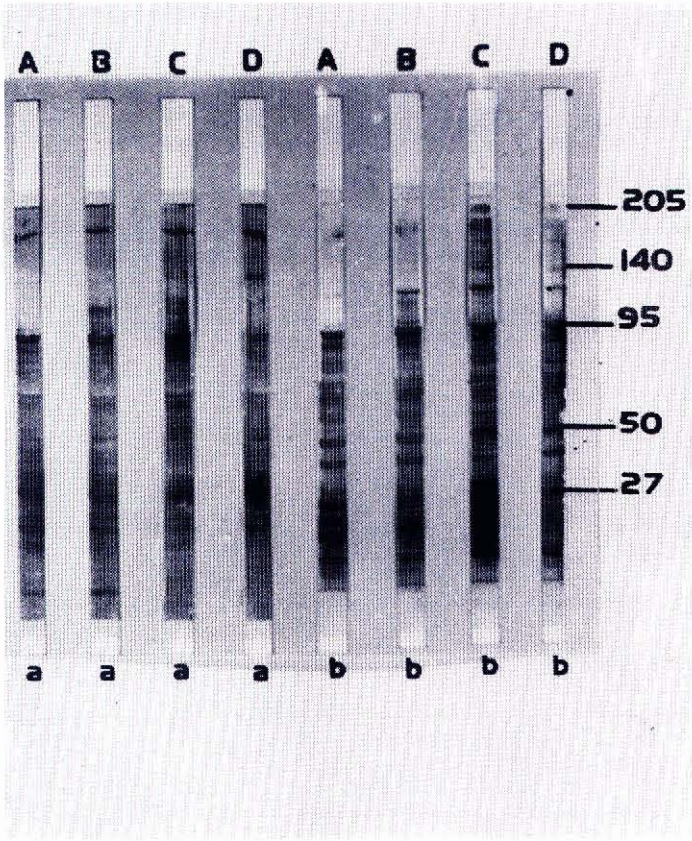

Fig. 2. Western blot analysis of log and stationary phase antigens of L. donovani and L. major. L. donovani stationary antigen (A), L. donovani log antigen (B), L. major stationary antigen (C) and L. major log antigen (D) were reacted with L. major log antibody (a) and L. donovani log antibody (b) respectively. Molecular weights in kilodaltons (KDa) are indicated at the side of the lane.

Results from the antigenic analysis (immunoblot) of log and stationary phase promastigotes of L. donovani and L. major strains identified by anti-promastigotes antisera are shown in Fig. 2. A comparative western blot analysis was performed to determine if there are commonly shared antigens among the two morphologic stages of L. donovani and L. major promastigotes. Results showed that bands at 20 KDa, 95 KDa and 120 KDa were present both in log and stationary phase when L. donovani antigen reacted with homologous antibody, whereas 20 KDa and 120 KDa were not detected by heterologous antisera. On the other hand, in the homologous reaction of two phases of L. major strain, no common shared antigens were detected. Only a band at 120 KDa was observed when both log and stationary phase antigen of L. major reacted with L. donovani antiserum. There were few antigens recommended as species and stage-specific, viz., 105 KDa unique to L. donovani log phase, 140 KDa to log phase of L. major and 115 KDa to L. major stationary phase promastigotes, whereas antigen of 50 KDa was only present in log phase of both

L. donovani and L. major strain. Immunoblotting showed that antipromastigote antisera raised in rabbit detected fewer antigens of stationary phase promastigotes of both species than log phase promastigotes, which are in well agreement with the observation of Grogl et al. (1987). Fewer antigens that are regarded as unique species and stage-specific were identified. The antigen of 50 KDa is the only stage-specific marker present in log phase of both L. donovani and L. major. The identification of antigens raised among the two stages of L. donovani and L. major is also important. Fewer peptides on western blots of stationary phase than of log phase promastigotes strengthened the observation of Grogl et al. (1987) that promastigotes from stationary phase cultures may be less immunogenic to the vertebrate host, whereas this stage is much more infective than log phase (Giannini 1974, Sacks et al. 1985). The expression of several polypeptides exclusively in the infective stage (stationary) is of special interest.

In both L. donovani and L. major L-proline was primarily responsible for producing metacyclogenesis, however, L-alanine and L-glutamate too had some effect on the transformation from log to stationary (metacyclic) phase (Table 1). When L-leucine and L-isoleucine were added to with proline, the percentage of metacyclics in TAU was reduced (Table 2).

Table 1. Role of L-alanine, L-glutamate and L-proline in metacyclogenesis.

Species	Substrate	No. of Promastigotes X 10^6			% Metacyclics		
		2nd day	3rd day	4th day	2nd day	3rd day	4th day
L. donovani	L-alanine	8.61±0.06	14.00±0.04	6.81±0.02	3.60%	5.14%	4.90%
L. major		6.24±0.04	8.02±0.02	4.73±0.03	0.32%	3.68%	0.97%
L. donovani	L-glutamate	9.80±0.012	5.75±0.018	1.07±0.004	0.54%	2.22%	4.80%
L. major		7.33±0.021	3.24±0.016	0.01±0.003	-	1.46%	1.89%
L. donovani	L-proline	12.69±0.09	10.30±0.005	3.20±0.005	1.09%	18.00%	27.00%
L. major		9.31±0.02	7.53±0.03	1.99±0.012	0.40%	6.00%	14.22%

Table 2. Role of L-leucine and L-isoleucine as inhibitors of proline metabolism.

Species	Substrate	No. of Promastigotes X 10^6			% Metacyclics		
		2nd day	3rd day	4th day	2nd day	3rd day	4th day
L. donovani	L-Proline +	5.60±0.012	7.32±0.062	1.80±0.009	0.70%	1.01%	2.77%
L. major	L-leucine	3.67±0.012	5.84±0.013	0.98±0.01	-	0.98%	1.09%
L. donovani	L-Proline +	4.62±0.057	4.01±0.012	2.67±0.004	-	0.90%	-
L. major	L-isoleucine	3.11±0.014	3.69±0.008	2.03±0.016	0.01%	-	-

In the control experiments no promastigotes could be observed after the second day. D-glucose, which is an important constituent of most media had very little role in metacyclogenesis. In case of L. major no promastigotes

were observed in the medium (in which glucose had been added) after the third day. The percentage of metacyclics produced was also negligible (upto 3% in the third day). The other sugars, i.e. sucrose, fructose and mannose had no role in producing metacyclic. It is established that the parasitic protozoa belonging to the kinetoplastids can use both sugars and amino acids as carbon and energy sources. Recently Ter Kuile (1993) suggested that in T. brucei and L. donovani, glucose is a primary nutrient, but in the insect stage, amino acids can also be used as carbon and energy source. Our results also showed that L-proline has a distinctive role in producing metacyclic promastigotes, but higher in L. donovani (27% on 3rd day) than L. major (14%). Glutamate and alanine can also stimulate metacyclogenesis but at much lower rate because these two are intermediates and/or end products in proline metabolism (Krassner and Flory 1972). L-proline can be considered as an essential substrate for producing metacyclic form as in case of Trypanosoma spp. (Contreras et al. 1985). This assumption is further strengthened by the observation that when L-leucine or L-isoleucine were added to TAU along with L-proline, metacyclogenesis was reduced. Both leucine and isoleucine are known to be inhibitors of proline metabolism. Glucose too, was found to have very little effect both in case of L. donovani and L. major, besides fructose, mannose and sucrose. While TAU containing glucose supported the growth of L. donovani upto the fourth day, L. major promastigotes died within the third day. Ter Kuile and Opperdoes (1993) have pointed out that L. donovani can consume all available culture medium glucose before the end of log phase growth. On the other hand, L. tropica exhibit delayed glucose utilization in culture (Mukkada et al. 1974) and do not utilize glucose to any extent until late in the log or early in the stationary growth phase. From the very limited information regarding leishmanial metacyclogenesis, it is conceivable that some biochemical and antigenic changes occur to switch on the phase transformation process and establish some preadaptations to survive in the immunologically hostile environment of the host. In order to get more information on the antigenic changes occurring during this transformation process we are at present carrying out the studies on antigenic characterization against anti-promastigote stationary antisera raised in rabbits. A clear understanding of all the aspects of metacyclogenesis will help in development of strategies for control of the infective form of the parasites that infect more than 400,000 peoples every year.

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