Identification of amastigote-specific antigens of *Leishmania donovani* using kala-azar patient sera*

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Received 8 February 2000; revised 9 March 2001

Antigenic characterization of the soluble fraction of axenic amastigotes of *Leishmania donovani* (strain Dd8, causative agent of Indian kala-azar) and their comparison with promastigotes is reported. The axenic amastigotes were assessed for their immunological status employing anti-A2 monoclonal antibody which is extremely specific for *L. donovani* amastigotes. SDS-PAGE of 35[\(\text{S}\)]methionine labeled proteins of the two parasite stages exhibited few stage specific and some conserved antigens in both the stages. An increased synthesis of heat shock proteins was observed in axenic amastigotes. Western blot experiments employing sera of kala azar positive patients identified immunodominant antigens of 116, 83, 26 and 12 kDa in axenic amastigotes which were not present in promastigotes. These amastigote stage specific antigens may have immense potential in immunodiagnosis and prophylaxis of kala-azar.

Species of genus *Leishmania* are biologically diverse group of trypanosomatid flagellates causing a wide spectrum of diseases in man. The disease manifests itself in three clinical forms (cutaneous, mucocutaneous and visceral) depending upon the species of *Leishmania* involved. Of these visceral leishmaniasis or kala-azar, caused by *L. donovani* is the most severe form and usually fatal if left untreated.

*Leishmania* are dimorphic protozoan parasites with an extracellular flagellated promastigote stage in the sand fly vector and an intracellular stage occurring within mammalian macrophages. The morphological differences marking the transformation from promastigote to amastigote are accompanied by several biochemical and molecular modifications of both quantitative and qualitative nature.

*Leishmania* promastigotes have been the subject of numerous biochemical and immunological studies as they can be cultured axenically with relative ease. In contrast, little is known about the antigenic nature of amastigotes due to methodological difficulties in obtaining sufficient amounts of organisms free from host contaminants. Methods have been developed to purify amastigotes from infected host tissues. However, the resultant material is mostly contaminated with small percentage of host debris which may alter results. Since the amastigote stage is responsible for causing severe pathologies in the vertebrate host, vaccines and chemotherapeutic agents need to be essentially developed against this stage of the parasite.

Initially the comparative antigenic profile of intracellular/lesion amastigotes and promastigotes was studied by radio-iodination in several species. Handman and Curtis reported two polypeptides (94 and 43kDa) to be amastigote specific by surface iodination. Sadick and Raff found only one amastigote component (95kDa) to be intensely iodinated in *L. tropica*. In another study in *L. major* two amastigote specific antigens and five antigens expressed predominantly in amastigotes were detected in biosynthetically labelled preparations. However, detailed immunochemical studies on amastigotes were initiated only after the development of axenic cultivation of this stage in a few leishmania species, viz. *L. pifanoi*. Eperon and McMahon-Pratt raised monoclonal antibodies against membrane proteins of axenic amastigotes of *L. panamensis* and *L. braziliensis* and identified a 12 kDa component and three bands within a heterogeneous component of >50 kDa which were specific for amastigotes of these two species. Another study revealed common antigenic molecules in axenic and intracellular amastigotes using monoclonal antibodies (P-2/A-2, A-2, P-4 and P-8) raised against *L. pifanoi* axenic amastigotes. Hodgkinson et al. demonstrated two antigens (36 and 27 kDa) in axenic amastigotes of *Lamazomensis* using monoclonal antibodies. Regarding *L. donovani*, there are several reports on antigenic makeup of promastigotes. However, there are only two reports on amastigote specific antigens of *L. donovani*.

*Communication No. 5789*

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Gupta et al.\textsuperscript{16,17} have been successful in developing a long term culture of amastigotes of \textit{L. donovani} (strain Dd8, causative agent of Indian kala-azar) and have established their resemblance with intracellular amastigotes at ultrastructural and membrane levels. As amastigote stage is the developmental form found in the infected mammalian host, molecules upregulated or selectively expressed in this stage represent potential antigenic vaccine targets. Therefore the present investigation has been undertaken to detect immunodominant antigens specifically expressed in amastigote stage of \textit{L. donovani}.

**Materials and Methods**

Acrylamide, sodium dodecyl sulphate (SDS), bisacrylamide, HBSS, Tween20, molecular weight markers, antihuman IgG, alkaline phosphatase conjugate, amidoblock, BCIP/NBT tablets, TPCK, PMSF, aprotonin, leupeptin were procured from Sigma Chemical Co. St. Louis, USA. Nitrocellulose paper was obtained from Bio-Rad Laboratories, Richmond CA, USA and \textsuperscript{35}S methionine from Bhabha Atomic Research Centre, Mumbai, India. All other chemicals used were of analytical grade. Blood samples were collected from kala-azar patients from endemic areas of Muzaffarpur, Bihar, India.

**Parasite—\textit{Leishmania donovani} (MHOM/IN/80/Dd8)** originally obtained in the form of promastigotes from Prof. P.C.C. Garnham, London and maintained at CDRI in golden hamsters (\textit{Mesocricetus auratus}) was used. Promastigotes were maintained at 26°±1°C under in vitro conditions and axenic amastigotes were generated and maintained in NNN biphasic medium at 34°±1°C\textsuperscript{16}.

**Preparation of antigen samples—**Promastigotes and axenic amastigotes (5x10\textsuperscript{8} cells) were harvested at 4000 rpm for 20 min. at 4°C twice with PBS, pH 7.2. The pellets were suspended in 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA, 2 mM phenylmethyl sulphonyl fluoride (PMSF), 0.3 μM aprotonin, leupeptin and 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and immediately sonicated for 5x30 sec. at 4°C. The resulting lysates were spun at 20,000 rpm for 60 min at 4°C. The supernatants were treated with 2xSDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 2% w/v SDS, 20% v/v glycerol, 0.1% w/v bromophenol blue) and boiled for 5 min. Samples were recentrifuged for 10 min at 4°C and the resulting supernatants were subjected to SDS-PAGE and western blot analysis.

**Metabolic labeling—**Promastigotes and axenic amastigotes (5x10\textsuperscript{8} cells) were washed with methionine free Hank's balanced salt solution (HBSS) and incubated in HBSS containing 0.1% glucose, 50 mCi/ml of \textsuperscript{35}S methionine for 2 hr at 26°C and 34°C respectively. Labeled cells were then washed with HBSS and lysed in SDS-PAGE sample buffer containing 1 mM iodoacetate and 2 mM PMSF by 6-8 cycles of repeated freezing and thawing in liquid nitrogen. Extracts of labeled cells were analyzed on SDS-PAGE.

**SDS-PAGE**—SDS-PAGE was carried out under denaturing conditions in presence of SDS according to the method of Lammeli\textsuperscript{18}. A gradient separating gel (7-12% or 10-15%) and stacking gel (5%) were prepared from 30% acrylamide in 1.5 M Tris-HCl buffer system, pH 8.6 and 6.8 respectively. Antigen samples (in the form of equal amount of protein/counts) were loaded and electrophoresed (Bio-Rad Labs, Richmond CA, USA) was carried out with 2.5 mA/lane from cathode to anode till the bromophenol blue marker reached the bottom of the gel. Molecular weight markers were also run in parallel. After electrophoresis, gels were placed in 50% methanol for 3 hr., then silver stained according to the Morrissey\textsuperscript{19} in order to visualize the protein bands.

Metabolically labeled samples were also electrophoresed on 7-12% gradient gel in the similar way and then gels were fixed in 25% isopropanol, 10% acetic acid solution and impregnated in AMPLIFY (Amersham) for 30 min. Gels were subsequently dried and exposed onto X ray films (Kodak X-Omat AR) with an intensifying screen. These were developed after an exposure time of 3-5 days at -70°C.

**Immunoblotting (Western Blotting)**—The polypeptides resolved after SDS-PAGE (10-15% gradient) were electrophoretically transferred from gels onto nitrocellulose paper (0.22 μM) according to the method of Towbin et al.\textsuperscript{20}. Transfer was made using a Transblot cell (Bio-Rad Labs, Richmond, CA, USA) at 4°C for 3 hr at 4-5 volts in a transfer buffer (20 mM Tris base, 150 mM glycine and 20% v/v methanol, pH 8.3). Nonspecific sites of antigens were blocked by placing the NCP strips in blocking buffer (5% BSA in PBS, pH 7.2) overnight at room temperature, control strips were washed twice with PBS-Tween 20 and were then stained with 0.1% amidoblack in order to monitor complete transfer of protein bands from gel onto NCP, whereas the other NCP strips were probed with (a) anti-A2 monoclonal antibody C9 (which was kindly provided by Dr. W.W. Zhang, Canada) (b) pooled sera.
of kala-azar patients for 4 hr. After washing with PBS Tween 20, strips were incubated with antihuman IgG conjugated with alkaline phosphatase (dilution 1:4000) for 2 hr at room temperature with gentle shaking. Further, after subsequent washings with PBS-Tween 20 and then twice with PBS, the blots were developed using the chromogenic substrate 5-bromo-4chloro-3 indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

Results

Figure 1 is the western blot employing anti-A2 monoclonal antibody C9. There was no antigen recognition in promastigotes (lane P) but in axenic amastigotes (lane A) a 26 kDa band was visible.

Figure 2 is the autoradiograph of $^{35}$S-labeled proteins synthesized by axenic amastigotes and promastigotes. There was a reduction in the total cellular protein synthesis in the axenic amastigotes as compared to promastigotes. Prominent bands were observed at 14, 16, 18, 25, 35, 48, 52, 55, 60, 68, 70 and 80 kDa in promastigotes while the axenic amastigotes had major bands at 12, 14, 22, 28, 30, 44, 48, 60, 70 and 80 kDa. There was a comparative increase in the active synthesis of polypeptides at 80, 70, 60, 44 and 14 kDa in axenic amastigotes. The comparative antigenic profile of different parasite stages as determined by SDS-PAGE and silver staining is shown in Fig. 3. Separation of proteins of the soluble fraction in promastigotes revealed 20-25 bands, of which 15 were present below 66 kDa. The main bands were present at

![Fig. 1](image1.png)

Fig. 1— Autoradiograph of *L. donovani* promastigotes (lane P) and axenic amastigotes (lane A) metabolically labelled with $^{35}$S methionine. Numbers represent molecular weight standards in kDa: β-galactosidase (116), phosphorylase b (97), bovine serum albumin (66), ovalbumin (45) and carbonic anhydrase (29).

![Fig. 2](image2.png)

Fig. 2—SDS-PAGE patterns of protein profile of *L. donovani* promastigotes (lane P), axenic amastigotes (lane A). Numbers represent molecular weight standards in kDa.

![Fig. 3](image3.png)

Fig. 3—Immunoblot of *L. donovani* promastigotes (lane P) and axenic amastigotes (lane A) antigens probed with sera of kala-azar positive patients. Numbers represent molecular weight standards in kDa.
Fig. 4—Immunoblot of *L. donovani* promastigotes (lane P) and axenic amastigotes (lane A) antigens probed with sera of kala-azar positive patients. Numbers represent molecular weight standards in kDa.

8,9,10,14,16,22,25,29,35,35,48,52,60,66,70, and 83 kDa (Fig. 3, lane P). Reduction in protein bands in axenic amastigotes was further confirmed by silver staining where the prominent bands were observed at 6,8,9,12,14,26,29,35,44,46,60,70,72,83 and 116 kDa (lane A). Bands of 10,16,22,25,52, and 66 kDa were found missing in axenic amastigotes.

Results of immunoblot experiments are shown in Fig. 4. The results revealed that major common/conserved antigens in promastigotes and axenic amastigotes were recognized by kala-azar patient sera at 70, 60, 35, 29, 14 and 10 kDa. Among them, 70 kDa was found to be most immunoreactive in both the parasite stages. Several of these are also actively synthesized proteins. In addition, patient sera was found to react strongly with 116,83,26 and 12 kDa antigens of axenic amastigotes. It also reacted weakly with 90 and 44 kDa antigens (lane A). However corresponding bands could not be detected in promastigotes (lane P).

**Discussion**

*Leishmania* parasites exhibit complex mixture of potentially antigenic components, out of which very few evoke protective host immunological responses. Handman et al. have suggested that amastigotes of infected macrophages rather than the promastigotes are the target of host protective immunity in vaccinated mice and mice resistant to infection with *L. major*. In the present study certain amastigote specific antigens of *L. donovani* have been identified that may be critical in host protection.

In order to establish the authenticity of axenic amastigotes at the immunological level, the monoclonal antibody C9 was used. Presence of 26 kDa band in axenic amastigotes as revealed by western blot analysis employing anti A2-monoclonal antibody C9 (Fig. 1) firmly establishes the authenticity of these forms, as the A2 protein represents a unique and probably the best (till date) amastigote specific protein marker for *L. donovani*. A typical family of A2 proteins ranging from 45-100 kDa has been identified in *L. donovani* (strain 1S2D) using C9 antibody. The occurrence of a low molecular weight protein in axenic amastigotes as observed in the present study could be the outcome of change in pH range (Zhang pers. Comm.), and also strain variation. These workers suggest a heterogeneity in length of DNA sequences encoding the repeat units of A2 protein which further gives rise to different molecular A2 protein products.

The autoradiograph of 35S labeled polypeptide showed marked stage specific differences (Fig. 2). There was a significant increase in the active synthesis of polypeptides at 80,70,60 and 14 kDa in axenic amastigotes. Protein bands at 80 and 70 kDa correlate well in size with heat shock proteins (Hsps), whose presence has been well documented in *Leishmania* amastigotes as well as in promastigotes subjected to transient heat stress. Throughout their life cycle, *Leishmania* encounters stressful conditions as fluctuation in temperature, pH, nutritional depletion or exposure to oxidative products. These changes may evoke increased synthesis of Hsps. Temperature stress alone has been shown to influence the stage differentiation of some species of *Leishmania* including *L. donovani*.

Hsps are a group of molecules that serve several functions and act as molecular chaperones. Heat stress even for a short period has led to 6 and 8-fold increase in relative synthesis of Hsp 70 and Hsp 83 respectively in three *Leishmania* species; *L. donovani*, *L. mexicana* and *L. amazonensis*. The genes encoding *Leishmania* Hsps have been cloned and characterized at molecular level.

The majority of Hsp 70 proteins are constitutively expressed and some are induced by elevated temperature. Comparative SDS-PAGE analysis of antigens of two parasite stages further confirm increased synthesis of Hsps (Fig. 3).

Western blot analysis with kala-azar patient sera revealed 70kD antigen band as most immunoreactive
antigen in both stages (Fig. 4). Hsp 70 has already been shown as major immunogen in T.cruzi and L. donovani. Another common immunoreactive 14 kDa antigen is in accordance with the report of Mary et al. who have reported two antigens of great specificity at 14 and 16 kDa while studying the specificity of anti L.infantum promastigote antibodies in patients with VL (including patients with AIDS) by western blot analysis. Four distinct, strongly reactive antigens were detected at 116,83,26 and 12 kDa and two weakly reactive antigens at 90 and 44 kDa in axenic amastigotes. Surprisingly Chattopadhyay et al. reported the presence of an immunoreactive antigen of 116 kDa in membrane fraction of promastigotes of L. donovani instead of amastigotes. They identified a specific 200kDa antigen in axenic amastigotes using rabbit sera raised against these forms. 90 and 83 kDa immunogens may be the members of Hsp family. These had been shown as immunodominant antigen in leishmanial and fungal infections. The 44 and 26 kDa antigens could be cysteine proteinases in nature, which is in accordance with the recent study by Hodgkinson et al. who have shown the presence of 27,41 and 45 kDa proteins as cysteine proteinases found in axenic amastigotes of L. amazonensis (absent in promastigotes) by western blotting and immunoprecipitation. 116 and 12 kDa proteins identified in axenic amastigotes could be the stage specific immunodominant polypeptides. Thus the comparative antigenic profile of both parasite stages reveals that there are similarities as well as variations in the exposure of different antigens, thereby suggesting that specific antigenic moieties are lost/acquired during transformation and differentiation from the promastigotes to amastigotes and that their immunogenicity is also altered. Identification of amastigote specific immunodominant antigens have immense value in vaccine development against VL. Recent evidences reveal that immunization with molecules preferentially expressed in amastigotes can provide effective protection against infection as seen in CL where both CD4+ and CD8+ cells appear to be crucial to protection; further protective vaccination with defined antigens is mediated by TH1 type of immune response. Hence the immunodominant antigens of axenic amastigotes (116,83, and 12 kDa) identified in the present study may be of immense value to be exploited for immunodiagnosis or prophylaxis of kala-azar.

Acknowledgement

Financial assistance to N. Gupta and N. Bhatia from CSIR and UGC, New Delhi respectively is gratefully acknowledged. N. Goyal acknowledges financial support from the Department of Science and Technology, New Delhi (DST grant no.SP/so/D/96).

References


