Monoclonal antibody affinity purification of a 78 kDa membrane protein of *Leishmania donovani* of Indian origin and its role in host–parasite interaction

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Monoclonal antibodies were raised against pathogenic promastigotes of *Leishmania donovani* of Indian origin. Among these, one was used for immuno-affinity purification of a 78 kDa membrane protein present in both the amastigote and promastigote forms of the parasite. Results of immunoblot experiments with the anti-78 kDa antibody revealed that the protein was present only in parasites belonging to the *L. donovani* complex. The expression of the protein was observed to be the same during different phases of growth of the promastigotes. Therefore, the 78 kDa protein is neither stage-specific nor differentially regulated. Surface iodination and subcellular fractionation of the promastigotes indicated that the protein was localized on the cell surface. The 78 kDa protein was found to inhibit the binding of promastigotes to macrophages significantly, suggesting that it may play a role in the process of infection. Thus, here we report the purification of a surface protein of *L. donovani* of Indian origin, which may play an important role in the process of infection.

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1. Introduction

Protozoan parasites of the genus Leishmania cause a spectrum of diseases, varying from self-healing cutaneous leishmaniasis to potentially fatal visceral leishmaniasis (VL) or kala-azar (KA). KA is caused primarily by parasites belonging to *Leishmania donovani* complex, namely, *L. donovani*, *L. infantum* and *L. chagasi*. In the alimentary tract of sand fly vectors, *Leishmania* exist as motile and flagellated promastigotes. These are transmitted by the vector sandflies into the vertebrate host during their blood meal. The promastigotes are ingested by macrophages of the host, where they are transformed into non-motile amastigotes. Transformation of promastigote to amastigote is an important prerequisite for the establishment of infection. Although the externally exposed molecules of these protozoa have been implicated as determinants in the host–parasite cellular interaction (Chang 1983; Dwyer and Gottlieb 1983), there have been few reports on the isolation of these proteins for detailed study (Chang 1983; Jaffe and Zalis 1988).

Purification of the plasma membrane molecules can be achieved by using leishmania surface-specific monoclonal antibodies, which have already found excellent

Keywords. Affinity-purification; host-parasite interaction; *Leishmania*; monoclonal antibody

Abbreviations used: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; DOC, deoxycholic acid; EGTA, ethyleneglycol-bis-(beta-amino ethyl ether) N,N,N,N-tetra acetic acid; EDTA, ethylenediaminetetraacetate; FACS, fluorescence activated cell sorter; HRP, horse radish peroxidase; KA, kala-azar; NBT, nitrobluetetrazolium; PBS, phosphate buffer saline; PMSF, phenyl methyl sulfonyl fluoride; PKDL, post kala-azar dermal leishmaniasis; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; VL, visceral leishmaniasis.

applications so far for the separation of *Leishmania* species and for the identification of these membrane molecules (Jaffe *et al* 1984; Canto-lara *et al* 1999). Here, we report the purification of a 78 kDa surface antigen from *L. donovani* of Indian origin by affinity chromatography and its role in host parasite interaction.

2. Materials and methods

2.1 *Parasite culture*

Parasites used in this study were, *L. donovani* isolates AG83 (MHOM/IN/83/AG83), GE1 (MHOM/IN/89/GE1), GE2 (MHOM/IN/89/GE2), RS (MHOM/IN/94/RS), CK (MHOM/IN/95/CK), DD8 (MHOM/IN/80/DD8), D1700 (MHOM/SD/68/SI), *L. chagasi* isolate (MHOM/BR/00/EDMAEL), *L. infantum* isolate (Spanish isolate), *L. major* isolate (MHOM/SU/73/5ASKH), *L. tropica* (MHOM/SU/73/K27), *L. mexicana* (MHOM/BZ/82/BEL21), *L. amazonensis* (MHOM/BR/77/LTB0016). AG83, GE1, GE2 were isolated from kala-azar patients responsive to Stibanate (sodium antimony gluconate) therapy, whereas, CK was isolated from bone marrow aspirates of a kala-azar patient unresponsive to treatment with stibanate. RS was isolated from a post kala-azar dermal leishmaniasis patient. Promastigotes of these different strains of *Leishmania* were routinely maintained at 22°–25°C in medium-199 with 20·0 mM HEPES (pH 7·4) and 10% heat-inactivated fetal bovine serum (Inovar Biological Inc., USA). Amastigotes were isolated from spleens of infected hamsters following the method described by Hart *et al* (1981).

2.2 *Monoclonal antibodies*

L. donovani (MHOM/IN/83/AG83) promastigotes (2×10^8) were suspended in 200 µl of lysis buffer (20·0 mM Tris-HCl, pH 7·4, 40·0 mM NaCl, 5·0 mM EDTA, 5·0 mM iodoacetamide, 2·0 mM PMSF and 1·0 µg/ml Leupeptin) and rapidly freeze-thawed for three cycles. The lysate was adjusted to 1·0 mg/ml protein with PBS (0·1 M NaCl in 50·0 mM Na-phosphate buffer, pH 7·4). BALB/c mice (female, 6–8 weeks) were immunized with parasite proteins by four consecutive subcutaneous injections (a total of 1·0 mg) at two week intervals, the first emulsified with complete and the rests with incomplete Freund's adjuvant. B-cells derived from immunized mice were then fused with myeloma cell line (SP2/0) following standard protocols. Hybrid clones secreting parasite-specific antibodies were selected by ELISA (Jaffe *et al* 1984) using microtitre plates coated with parasite proteins (100 ng/well). Parasite specific IgG was purified from ascitic fluid by affinity chromatography using protein A-sepharose 4B (Harlow and Lane 1988).

2.3 *Affinity purification*

Affinity purified IgG was coupled with CNBr-activated sepharose 4B beads following the instructions provided by the manufacturer (Pharmacia, Sweden), extent of coupling was ~ 90–95%. Promastigotes (1×10^8/ml) were suspended in a buffer containing 0·5% DOC, 10·0 mM EDTA, 2·0 mM EGTA, 25·0 mM iodoacetamide and 5·0 mM PMSF in 20·0 mM Tris-HCl, pH 8·2. The suspension was incubated at 4°C for 20–25 min with occasional stirring and centrifuged at 10,000 *g* for 10 min. The supernatant (5 ml, diluted to 1·0 mg/ml protein with the same buffer) was applied to IgG-sepharose 4B column (1·0 ml bed volume) pre-equilibriated with the same buffer. After thorough washing with 20·0 ml of the same buffer, bound proteins were eluted with 100·0 mM glycine, pH 2·5, and 0·5 ml of the eluted fractions were collected in equal volumes of 1·0 M Tris pH 8·0 at 4°C.

2.4 *Protein estimation and gel electrophoresis*

Protein contents of cell lysates and column eluted fractions were estimated by the method of Peterson (1983) and Layne (1957) respectively. In both cases, crystalline bovine serum albumin was used as standard. Parasite proteins were analysed by SDS-PAGE (Laemmli 1970). Western blotting was carried out following the procedure described by Burnette (1981). Immunoreactive proteins were visualized by incubation with protein A-conjugated alkaline phosphatase, followed by treatment with NBT and BCIP (Blake *et al* 1984).

2.5 *Surface iodination and immunoprecipitation of promastigote antigens*

Promastigotes (5×10^6 cells/ml) were surface iodinated as described by Bouvier *et al* (1987). Radio-labelled parasite antigens were then immunoprecipitated using the anti-78 kDa antibody (Schnur *et al* 1990).

2.6 *Isolation of pellicular and flagellar membrane*

A pellicular membrane was prepared following the method of Dwyer (1980), with some modifications as described by Gottlieb and Dwyer (1981). Flagellar fraction was prepared following the procedure of Pereira *et al* (1977).

2.7 *Promastigote–macrophage interaction*

Anti-78 kDa Fab fragments were prepared as described by Coulter and Harris (1983). Binding of promastogotes to macrophages were carried out following the method of Chang and Chang (1986), except that thioglycolate activated peritoneal macrophages (Reiner and Malemud 1985) were obtained from BALB/c mice instead of cultured mouse J774 macrophages.

3. Results

The hybridoma growth efficiency for the fusion of immune B cells and myeloma cells was 50%, of which only 10% of the hybridomas secreted *Leishmania*-specific antibodies (data not shown). However after secondary screening, only five stable hybrid clones secreting parasite-specific antibodies were obtained (figure 1). Since the antibodies secreted by all the hybrid clones reacted with the same 78 kDa parasite protein (data not shown), only one of the five hybrid clones was used for subsequent studies. Immunoblot experiments using the monoclonal antibody indicated the presence of the 78 kDa protein in the parasites belonging to *L. donovani* complex, namely *L. chagasi* and *L. infantum* and absence in *L. major*, *L. tropica*, *L. amazonensis* and *L. mexicana* (figure 2). Thus these results suggest that the monoclonal antibody was *L. donovani* complex-specific. The protein was also found to be present in the different isolates of *L. donovani* including GE1, GE2, freshly transformed i.e. virulent and *in vitro* adapted i.e. avirulent promastigotes of AG83, RS, CK, DD8 and D1700 (figure 3). Furthermore, it was observed that the protein was present in both amastigote

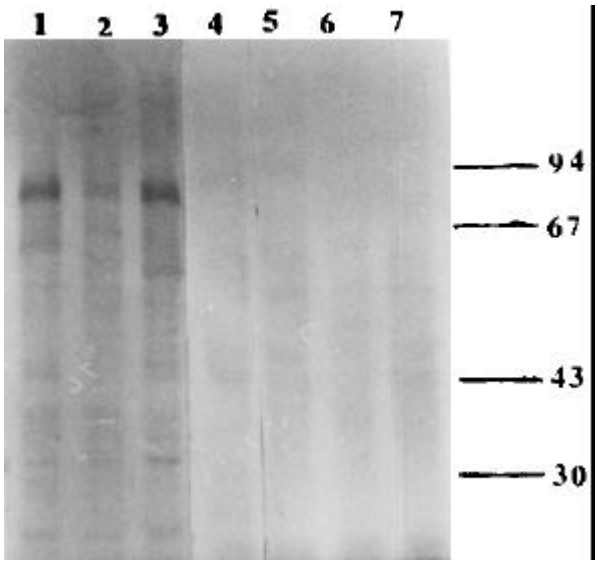

Figure 2. Immunoblot analysis of different species of *Leishmania* using the monoclonal antibody. Proteins (20 µg) were separated by SDS-PAGE and after transfer, proteins bands were visualized by immunoblotting using 1 : 50 dilution of the monoclonal antibody. Lanes 1–7 represent promastigotes of *L. donovani* (AG83), *L. chagasi*, *L. infantum*, *L. major*, *L. tropica*, *L. amazonensis* and *L. mexicana* respectively.

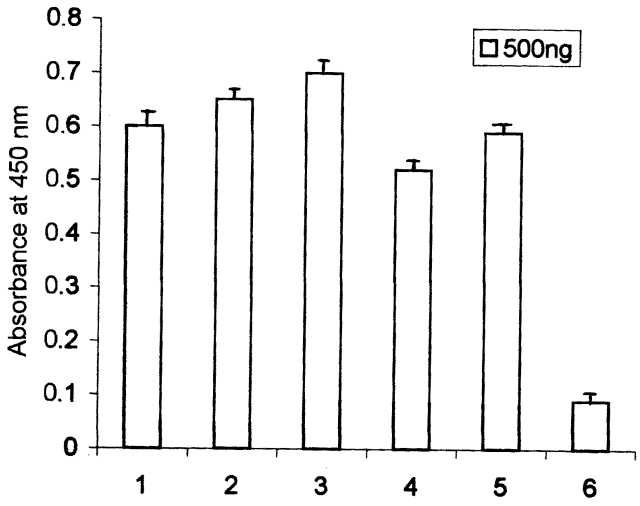

Figure 1. Antibody titres of culture supernatants of different hybridomas. Wells of ELISA plates were coated with 500 ng/50 µl of promastigote lysate and ELISA was performed using 100 µl of culture supernatants (1–5) and culture media alone (6). Results are mean ± SD of experiments performed in duplicate.

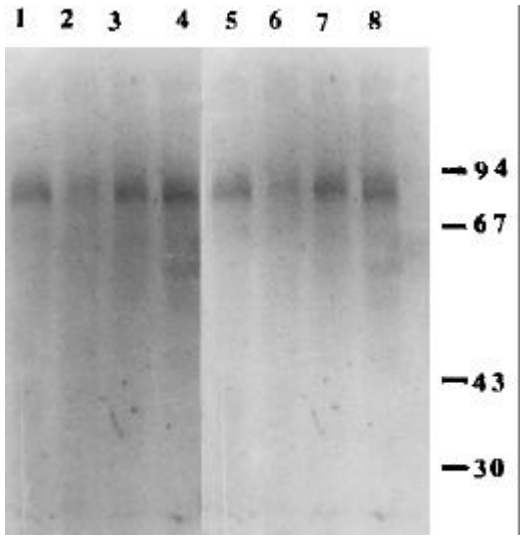

Figure 3. Western blot analysis of different isolates of *L. donovani*. All conditions are same as figure 1. Lanes 1–8 represent GE1, GE2, freshly transformed and *in vitro* adapted AG83, RS, CK, DD8 and D1700 respectively.

and promastigote forms of the parasite (figure 4) and also the amount of this protein remained the same during the different phases of growth of the promastigotes (figure 5). The 78 kDa protein was purified by immunoaffinity chromatography using the monoclonal antibody (figure 6). Surface iodinated *L. donovani* promastigotes were immunoprecipitated using the monoclonal antibody and analysed by autoradiography (figure 7).

Results indicated the presence of a distinct band of molecular mass of 78 kDa. Analysis of subcellular fractions by SDS-PAGE and immunoblotting using the monoclonal antibody also indicated the presence of the 78 kDa protein in the purified pellicular membrane fraction (figure 8). Thus these results suggested that the 78 kDa protein was exclusively present on the cell surface.

To study the role of 78 kDa protein in leishmania-macrophage interaction, the effect of time on the optimum attachment of promastigotes to murine macrophages was analysed. A monolayer of murine macrophages was infected with [^{3}H]leucine labelled promastigotes at a ratio of 1 : 10. Attachment of promastigotes to macrophages was completed within 45–60 min of incubation at 37°C (data not shown). Pre-incubation of the promastigotes with different concentrations of anti-78 kDa (Fab) fragments indicated about 50% inhibition of attachment when 0·5 mg/ml of the antibody was used (figure 9). However, in the absence of any antibody or with an irrelevant antibody (Fab fragments of normal rabbit IgG), attachment of the promastigotes to the

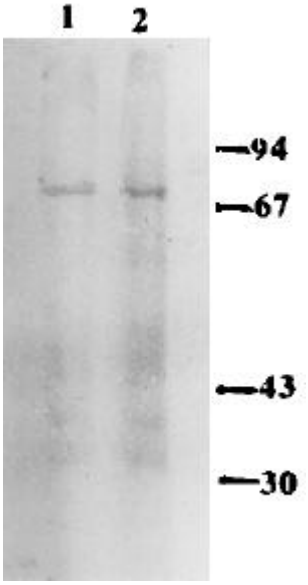

Figure 4. Analysis of stage specific expression of the 78 kDa protein by Western blot. Lanes 1 and 2 represent promastigote and amastigote lysates of AG83. Other conditions are same as in figure 1.

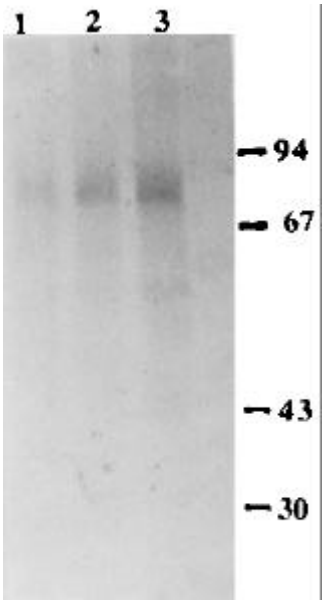

Figure 5. Expression of 78 kDa protein during *in vitro* growth of AG83 promastigotes. All conditions are same as in figure 1. Lanes 1–3 represent promastiogote lysate prepared from cells harvested after 120, 72 and 24 h of growth.

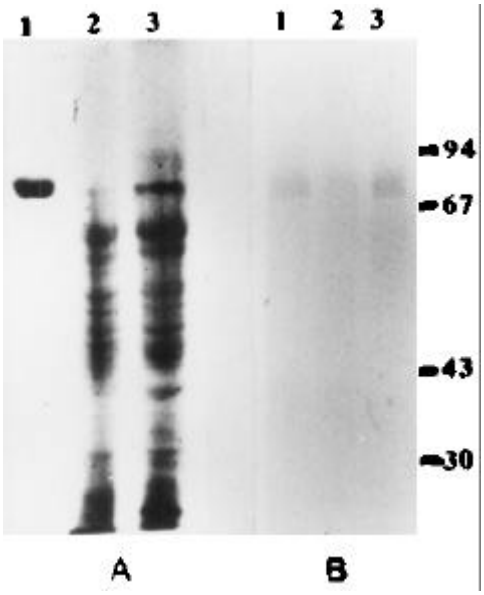

Figure 6. Analysis of affinity purified 78 kDa protein. (**A**) Proteins eluted from the column were precipitated with 80% chilled acetone and were analysed by SDS-PAGE and protein bands are visualized by silver staining. (**B**) Proteins (10 µg) were separated by SDS-PAGE, transferred onto nitrocellulose membrane and immunoblotted using 1 : 100 dilution of purified anti-78 kDa IgG. Lanes 1–3 represents eluted protein, flow through and parasite lysate respectively.

macrophages was almost 100%. Similarly when macrophages were preincubated with different amounts of the purified 78 kDa antigen, it was observed that 1 μg of protein inhibited the binding of promastigotes to the macrophages by more than 60% (figure 10). Hence these studies suggested that the 78 kDa protein may mediate attachment of promastigotes to host macrophages.

KA serum and normal serum were collected from different endemic parts of India. These sera were pooled and used as the primary antibody in the Western blot analysis using promastigote lysate and the purified 78 kDa protein (figure 11). Several proteins were recognized by KA serum in the promastigote lysate, whereas normal sera failed to do so. When purified 78 kDa protein was used, the immunoblot with KA serum produced a distinct protein band of molecular mass 78 kDa. This band was absent when the immunoblot was done with normal serum. Thus the antibody directed against this protein was found to be present in the pooled KA sera.

4. Discussion

The major accomplishment of the present study is the purification of a surface membrane antigen from *L. donovani* promastigotes of Indian origin by monoclonal antibody affinity binding. The purified antigen appears to play a role in host–parasite interaction. In this present study, highly pathogenic *L. donovani* promastigotes were used to immunize mice to develop monoclonals. Stable hybrid clones secreting anti-78 kDa antibodies were

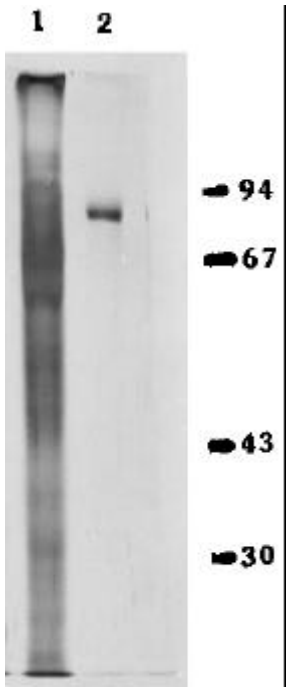

Figure 7. Cellular localization of the 78 kDa protein. Lane 1; [^{125}I]-labelled promastigotes protein (2×10^6). Lane 2: Labelled promastigotes immunoprocipitated with 1 : 50 dilution of the monoclonal antibody and analysed by SDS-PAGE.

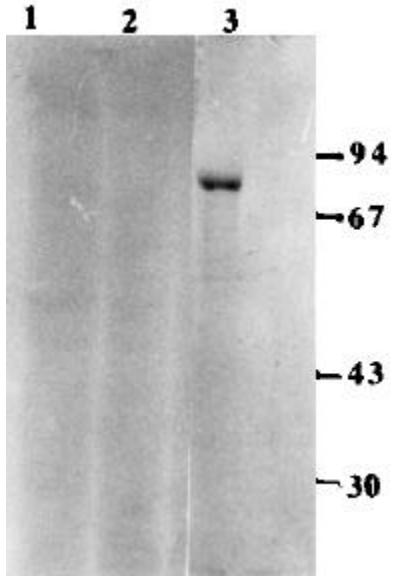

Figure 8. Subcellular localization of 78 kDa protein. Lanes 1–3 represents cytosolic, flagellar, and pellicular fractions. Other conditions are same as in figure 1.

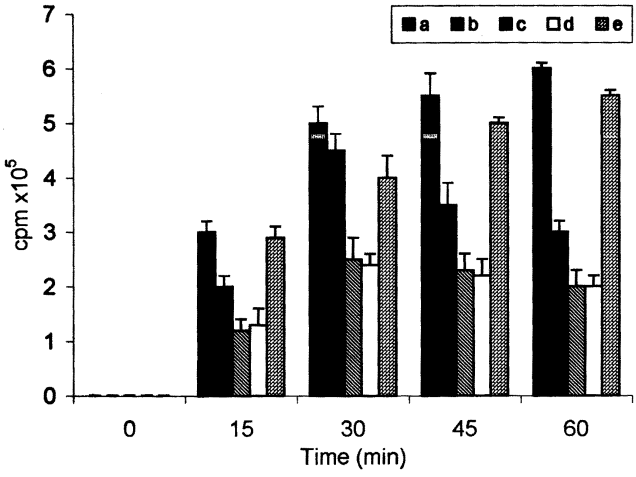

Figure 9. Effect of anti-78 kDa (Fab) fragments on the attachment of promastigotes. [^{3}H]-leucine labelled untreated (a) or promastigotes pretreated with 0·25 mg/ml (b), 0·5 mg/ml (c), 1·0 mg/ml of anti-78 kDa (Fab) (d) respectively and 0·5 mg/ml of anti-rabbit IgG (Fab) (e) were incubated with the macrophages in the ratio of 10 : 1. [^{3}H]leucine counts are taken as the extent of promastigote attachment to macrophages. Values represent mean ± SD of two experiments.

obtained (figure 1). Jaffe *et al* (1984), developed several species-specific monoclonal antibodies which were used for species classification by immunofluorescence. Results of figure 2 indicated that the 78 kDa protein was found to be *L. donovani* complex-specific. However studies with more isolates of different species are needed to determine whether this monoclonal antibody can be used for routine identification/classification of parasites by immunofluorescence. During the course of long term *in vitro* cultivation of *L. donovani* (AG83) parasites, the promastigotes were found to lose their infectivity or pathogenicity (De and Roy 1999). Since the monoclonal antibody reacted equally well (figure 3) with cell lysates prepared from parasites which are maintained in culture for a long time, the 78 kDa antigen appears to have no role in the pathogenicity of the organism.

There are many stage-specific differences between amastigotes and promastigotes. For example, gp63, the surface metalloprotease of *L. major* is down regulated in amastigotes (Schneider *et al* 1992), whereas, adenosine kinase activity is stimulated by ~ 50-fold (Hart and Coombs 1982). Moreover, plasma membranes of amastigotes differ from those of promastigotes in antigenic properties (Ilg *et al* 1995; Pinto *et al* 2000) and polysaccharide composition (Glasser *et al* 1991; Guha-Niyogi *et al* 2001). It was found that the 78 kDa protein is present in both the amastigote and promastigote stages of the organism (figure 4). Results also indicated that the antigen is present in nearly equal amounts in both stages (data not shown). Therefore, the 78 kDa protein is neither stage-specific nor differentially regulated. During the *in vitro* growth of promastigotes, transition from the logarithmic to the stationary phase is accompanied by expression of various macromolecules (Muskus *et al* 1997). No such changes were observed in the case of 72 kDa protein, as evident from the analysis of parasite antigens by the immunoblot experiment (figure 5).

Cell surface proteins of *Leishmania* have already been purified by immunoaffinity chromatography using species-specific monoclonal antibodies (Chang and Chang 1986; Jaffe and Zalis 1988). Affinity purified 78 kDa protein gave a single band of 78 kDa without any noticeable contaminants after SDS-PAGE (figure 6). Immunoprecipitation with [^{125}I]-surface labelled promastigotes (figure 7) and subcellular fractionation (figure 8) revealed that the protein was solely present on the surface membrane of the promastigotes. Previous reports suggested that the purified surface membrane of *L. donovani* promastigotes could be clearly resolved into nearly 40 polypeptides (12–220 kDa) when subjected to SDS-PAGE analysis, the most prominent polypeptide being tubulin of attached microtubules (Dwyer 1980). Among these 40 surface membrane proteins of *L. donovani* promastigotes, nearly 20 were glycoproteins in nature. However, analysis of the affinity purified 78 kDa antigen after periodic acid oxidation, followed by silver staining indicated that unlike many

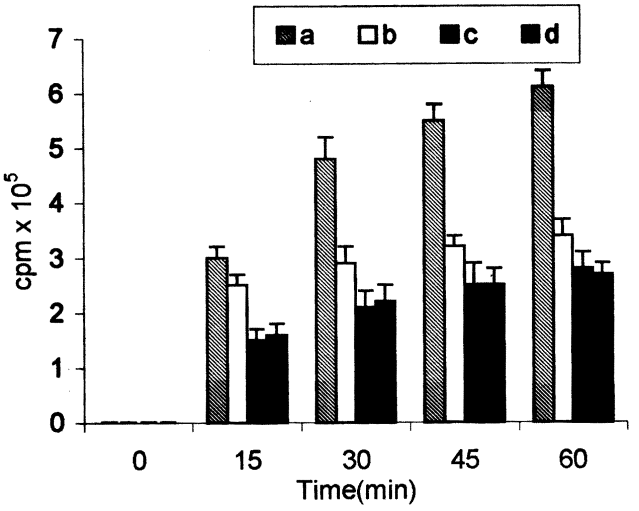

Figure 10. Effect of 78 kDa protein on attachment of promastigotes. Macrophages were pretreated with 5 μg of BSA (a), 0·5 μg (b), 1·0 μg (c) and 2·0 μg (d) of 78 kDa protein respectively and incubated with [^{3}H]leucine labelled promastigotes in the ratio of 1 : 10. [^{3}H]leucine counts are taken as extent of promastigote attachment to macrophages. Values represent mean ± SD of two experiments.

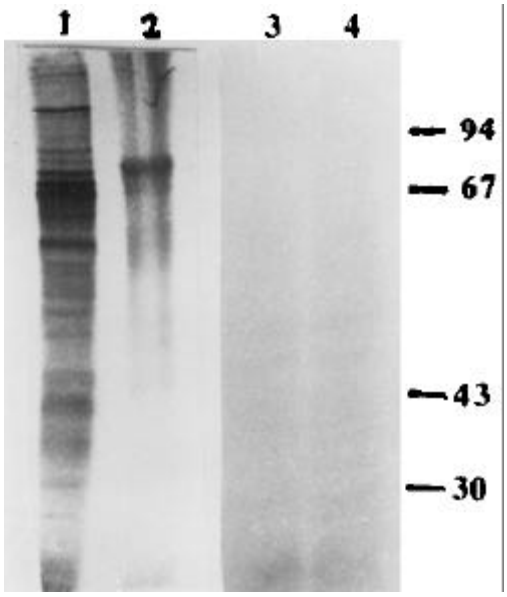

Figure 11. Immunoblot analysis of the purified 78 kDa protein using KA sera. Lanes 1,3 and 2,4 represent 20 μg of promastigote and 2·5 μg of 78 kDa protein respectively. Separated proteins were immunoblotted with 25 times diluted pooled KA (lanes 1, 2) and normal (lanes 3, 4) sera.

cell surface polypeptides, it is not a glycoprotein (data not shown).

Success of leishmaniae as infective agents depends on their ability at one stage to colonize the sandfly gut, and at another stage to establish intracellular parasitism within the macrophage of the mammalian host. Several cell surface macromolecules of leishmaniae promastigotes have been implicated in attachment of the parasites to host macrophages. Handman and Goding (1985), showed that binding of the lipid containing glycoconjugate of *L. major*, and also the intact promastigotes to the macrophages *in vitro* is specifically inhibited by Fab fragments of WIC-79·3 (a monoclonal antibody specifically directed against the glycoconjugate). The extent of inhibition in the presence of the Fab fragments was nearly 80%. Moreover, it was observed that the glycoconjugate binds only to the macrophages and not to the T or B lymphoid cells. Therefore, the researchers concluded that the glycoconjugate is the *Leishmania* receptor for macrophages. In contrast, Russel and Wilhelm (1986), showed that univalent antibody fragments derived from monospecific antiserum raised against gp63 reduced
L. major mexicana promastigotes-macrophage binding to 30–35% of the control binding level. However, uptake of gp63 containing proteoliposome was suppressed by over 90% by anti-gp63 Fab indicating that gp63 plays an important role in the attachment of promastigotes to macrophages. The use of Fab fragments was essential to prevent opsonization of parasites and their binding to macrophages via the macrophage Fc receptor. Silveira *et al* (2001), report that monoclonal antibodies SST-2, SST-3 and SST-4 raised against *Leishmania* (*Viannia*) *braziliensis* promastigotes recognized antigens which were present on the surface of the parasite. Fab fragments of SST-3 and SST-4 significantly inhibited the infectivity of *Leishmania* (*Viannia*) *braziliensis* promastigotes to mouse macrophages. In the present study, since the 78 kDa protein was localized on the cell surface, its role in parasite-macrophage interaction was studied. Results indicated that promastigote attachment was reduced by more than 50% when promastigotes were preincubated with 0·5 mg/ml of anti-78 kDa Fab fragments (figure 9). However, with increasing concentration of the antibody, no increase in the inhibition of attachment was observed. Similarly when the murine macrophages were preincubated with different amounts of purified protein, with 1 μg of the protein, more than 60% of reduction in parasite attachment was observed (figure 10). Furthermore, when macrophages were preincubated with increasing amount of the protein, increase in inhibition of parasite attachment to macrophages was not observed. Though this antigen is non-stage-specific, inhibition results indicate a clear association of the protein in host–parasite interaction. Thus it appears that the 78 kDa antigen, like other antigens mentioned earlier, plays a significant role in the process of infection. Whether this host–parasite binding is mediated by a specific or a non-specific mechanism is unknown. The inhibition is achieved by preincubation of macrophages with the purified antigen, suggestive of preoccupation of macrophage "receptors" (if they exist) for leishmanias as a possible mechanism. Resolution of the complexity of such cell-cell binding depends on the purification of the putative "receptors" from macrophages, and the study of their interaction with purified *Leishmania* surface antigens. The purification of the 78 kDa protein from *L. donovani* now makes available suitable material to achieve these ends and to investigate other aspects of host–parasite interactions, many of which are apparently dictated by surface molecules of interacting cells. Further studies are going on to elucidate the detailed mechanism of the action of this protein in the process of infection.

The presence of antibodies directed against the 78 kDa protein in kala-azar sera (figure 11), provides an additional practical application of the purified antigen which includes its possible use as a reagent for serodiagnosis. It can also be considered as an immunoprophylactic tool to combat Leishmaniasis.

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