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# An amphipathic sulphated glycoconjugate of *Leishmania*: characterization with monoclonal antibodies

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**A major glycoconjugate of *Leishmania tropica major* identified by two monoclonal antibodies was shown to be an externally oriented, amphipathic membrane antigen shed into the culture medium in which the parasites grow. This molecule could be labelled metabolically with [$^3$H]glucose, [$^3$H]-galactose, [$^{32}$P]phosphate and [$^{35}$S]sulphate. It migrated as a polydisperse band upon electrophoresis in SDS-polyacrylamide gels, spanning the region of the gel corresponding to an apparent mol. wt. of 20 000 – 67 000 daltons. An apparently identical family of molecules could be labelled on the surface of living promastigotes using galactose oxidase and [$^3$H]-sodium borohydride. This molecule was shown to be released into the supernatant over a period of several hours. Detection of the $^3$H- or $^{35}$S-labelled molecule required several days exposure of autoradiographs, but a novel blotting technique using nitrocellulose coated with monoclonal antibody allowed rapid detection of the molecule in charge shift electrophoresis, Western blotting and dot blotting. The electrophoretic mobility of the glycoconjugate in agarose relative to its mobility in Triton X-100 was increased in the presence of deoxycholate, and decreased in the presence of cetyl trimethylammonium bromide, indicating amphipathic properties consistent with insertion into the lipid bilayer of the membrane. Using the dot-blotting technique the glycoconjugate was detected in all virulent and avirulent clones of LRC-L137 and in two additional isolates of *L. tropica major* (LRC-L287 and LRC-L251), but not in *L. donovani* or *L. mexicana*, consistent with the previously described specificity of the antibodies. However, the general approaches used in this paper showed that *L. donovani* (LRC-L52) and *L. mexicana* (LRC-L94) synthesize a similar, but antigenically distinct glycoconjugate.**

*Key words:* amphipathic/excreted factors/glycoconjugate/polymorphic membrane antigens/sulphated polysaccharide

## Introduction

*Leishmania* are digenetic protozoa, alternating between the promastigote, a free-living flagellate in the gut of the vector sandfly, and the amastigote, the obligatory intracellular form, which resides in phagolysosomes in mammalian macrophages. Little is known about the mechanism of parasite survival in the sandfly gut, or in the phagolysosomal vacuole. Also, little is known about why different *Leishmania* species home to reticuloendothelial cells in different organs, causing diseases as different as cutaneous leishmaniasis and visceral leishmaniasis. The possible role of leishmanial released antigens (termed excreted factors, by Schnur *et al.*, 1972, 1973) in the biology of the parasite and its survival in the vector and mammalian host has been proposed by many (reviewed in Hernandez, 1983). Some *Leishmania*-released antigens appear to be common to the promastigote and amastigote form of the parasite, and to be present on infected macrophages (Schnur *et al.*, 1972; de Ibarra *et al.*, 1982). These antigens have been shown to be part of a polymorphic family of carbohydrate-rich molecules present in all *Leishmania*. However, each molecule was shown to be species-specific and this has formed the basis for a serotyping system for the diagnosis, demography and epidemiology of *Leishmania* (reviewed by Schnur, 1982). The number and nature of the antigens released by *Leishmania* remains to be determined (Slutzky and Greenblatt, 1977,1979; El-On *et al.*, 1979; Slutzky *et al.*, 1979; Hernandez *et al.*, 1980; Semprevivo and Macleod, 1981; Kaneshiro *et al.*, 1982), but it is clear that a major antigen is a negatively charged glycoconjugate, which may or may not contain protein (reviewed by Hernandez, 1983; Turco *et al.*, 1984).

We have begun a biochemical analysis of the *L. tropica major* glycoconjugate. In this study we have characterized this molecule using two previously described monoclonal antibodies (Handman and Hocking, 1982; de Ibarra *et al.*, 1982; Greenblatt *et al.*, 1983).

## Results

### *A glycoconjugate present in cells and culture supernatants of L. tropica major can be labelled with [$^3$H]glucose, [$^3$H]galactose, [$^{35}$S]sulphate and [$^{32}$P]phosphate*

Two monoclonal antibodies, previously described in the literature, were used for the isolation and characterization of a *L. tropica major* glycoconjugate. These were WIC-79.3 (de Ibarra *et al.*, 1982) and L-5-16 (Handman and Hocking, 1982). Competitive radioimmunoassay shows that the two monoclonal antibodies recognize the same epitope, or epitopes that are extremely close to each other (data not shown). These studies have also shown that the affinity of WIC-79.3 for its target antigen is ~30-fold higher than that of L-5-16, and thus we have used WIC-79.3 in most experiments.

Promastigotes of clone V121 of *L. tropica major* (LRC-L137) were biosynthetically labelled with [$^3$H]galactose. Cell lysates and culture supernatants were immunoprecipitated with monoclonal or polyclonal antibodies and the immunoprecipitates analysed by SDS-PAGE. Figure 1 (lanes A and C) shows that monoclonal and polyclonal antibodies immunoprecipitated from cell lysates a species of considerable heterogeneity, spanning an area of the gel corresponding to apparent mol. wt. 67 000 – 20 000. This material was not recognized by another anti-*L. tropica major* monoclonal antibody L-5-28 (Figure 1, lane D), or by serum from mice bearing NS-1 tumours (data not shown). Several distinct bands were present in the smear. The same material was also detected in parasite culture supernatants by the monoclonal antibodies (Figure 1, lane B). The *L. tropica major* glycocon-

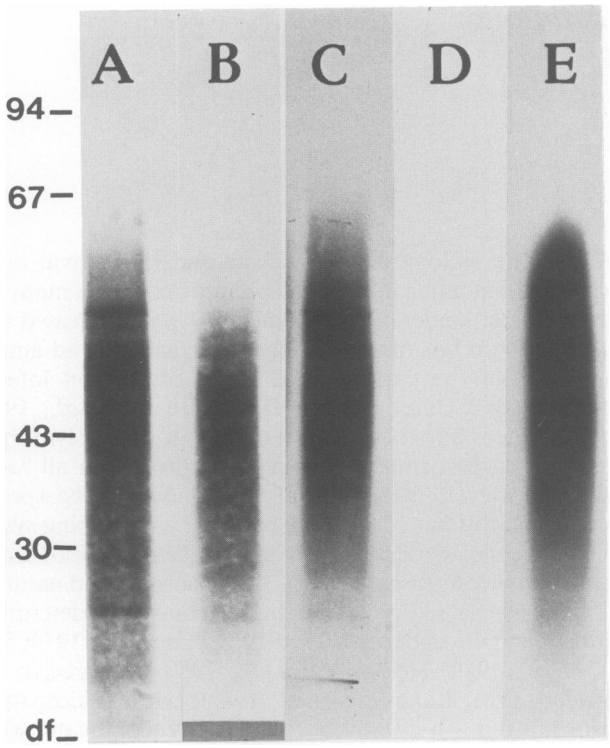


**Fig. 1.** The pattern of the *L. tropica major* glycoconjugate on SDS-PAGE. **(A)** Antigen bound by WIC-79.3 in [$^3$H]galactose-labelled cell lysate, **(B)** antigen bound by WIC-79.3 in culture supernatants from [$^3$H]galactose-labelled cells, **(C)** antigen bound by rabbit anti-clone V121 antibodies in [$^3$H]galactose-labelled cell lysate, **(D)** antigen bound by control monoclonal antibody L-5-28 in cell lysate, **(E)** antigen from unlabelled cell lysate bound on a two-site-Western by radioiodinated WIC-79.3. Numbers represent rhodamine-conjugated mol. wt. standards phosphorylase B (94 000), bovine serum albumin (67 000), ovalbumin (43 000) and carbonic anhydrase (30 000).

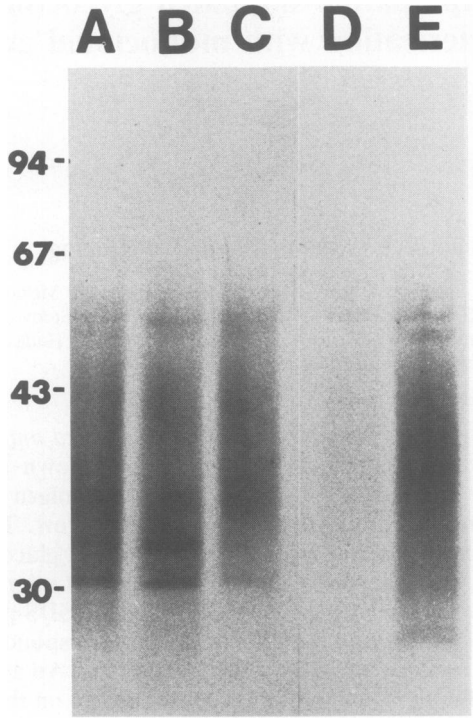


**Fig. 2.** Disappearance of galactose oxidase, [$^3$H]sodium borohydride-labelled *L. tropica major* glycoconjugate from the cell surface and its recovery in the supernatant as detected by immunoprecipitation with monoclonal antibody WIC-79.3. **(A)** Material in cell lysate immediately after labelling. **(B)** Material in cell lysate 6 h after labelling. **(C)** Material in supernatant 6 h after labelling. **(D)** Material in cell lysate 24 h after labelling. **(E)** Material in supernatant 24 h after labelling. Numbers represent mol. wt. standards as in Figure 1.

jugate could also be labelled biosynthetically with [$^3$H]glucose, but not [$^3$H]mannose. [$^{35}$S]sulphate and [$^{32}$P]phosphate were also incorporated into the glycoconjugate. The synthesis of the molecule was partially inhibited by tunicamycin at a concentration of 5 $\mu$g/ml, but lower concentrations had no detectable effect. At this concentration of drug, protein synthesis was not impaired (data not shown).

Radiolabelled cell lysates and supernatants were also incubated with a panel of lectins bound to agarose. The same pattern of migration on SDS-gels observed for the WIC-79.3 antigen was observed for the material that bound to ricin-agarose and peanut lectin-agarose (data not shown). After passage of 200 $\mu$l [$^3$H]galactose-labelled cell lysate or 800 $\mu$l culture supernatant over a 500 $\mu$l ricin-agarose column, immunoprecipitation of the flow-through material with WIC-79.3 failed to detect any reactivity. Under the same conditions, the effluent from a Sepharose 4B control column contained the WIC-79.3 antigen.

The glycoconjugate identified by WIC-79.3 antibody in the galactose-labelled material was also detected in an unlabelled cell lysate of promastigotes by radioiodinated WIC-79.3 in a two-site Western blot (Figure 1, lane E). The two-site Western blot allowed the detection of this molecule after a 4 h autoradiographic exposure of the nitrocellulose compared to 1 week necessary for the metabolically labelled antigen.

### *The L. tropica major glycoconjugate is present on promastigote surface membrane and is released into the culture supernatant*

Since WIC-79.3 and L-5-16 were initially characterized as directed to cell surface antigens, it was important to elucidate the relationship between the carbohydrate-rich molecule recognized by WIC-79.3 and L-5-16 in culture supernatants and the antigen recognized on cell membranes. The lectin binding experiments suggested that terminal galactosyl residues were present on the glycoconjugate. Promastigotes were therefore surface labelled using galactose oxidase and [$^3$H]-sodium borohydride. Immunoprecipitation of the cell lysate with WIC-79.3 and L-5-16 revealed the same pattern spanning the mol. wt. region of 67 000 – 20 000 daltons (Figure 2, lane A). In a few experiments, evenly spaced, discrete, fine bands were observed in the smear.

When surface-labelled promastigotes were incubated in RPMI-1640 medium at 26°C for various periods of time, the labelled molecule gradually disappeared from the cell surface and was recovered in the supernatant (Figure 2, lanes B,C, D,E). The process seemed to be essentially complete after 24 h (Figure 2, lanes D and E). Analysis of total antigenically active glycoconjugate by modified Western blotting revealed similar kinetics, suggesting that shedding from the cell surface is the major mechanism of release (data not shown). Since the labelled material could not be removed by extensive washing of the cells, it appeared that antigen WIC-79.3 is a cell surface

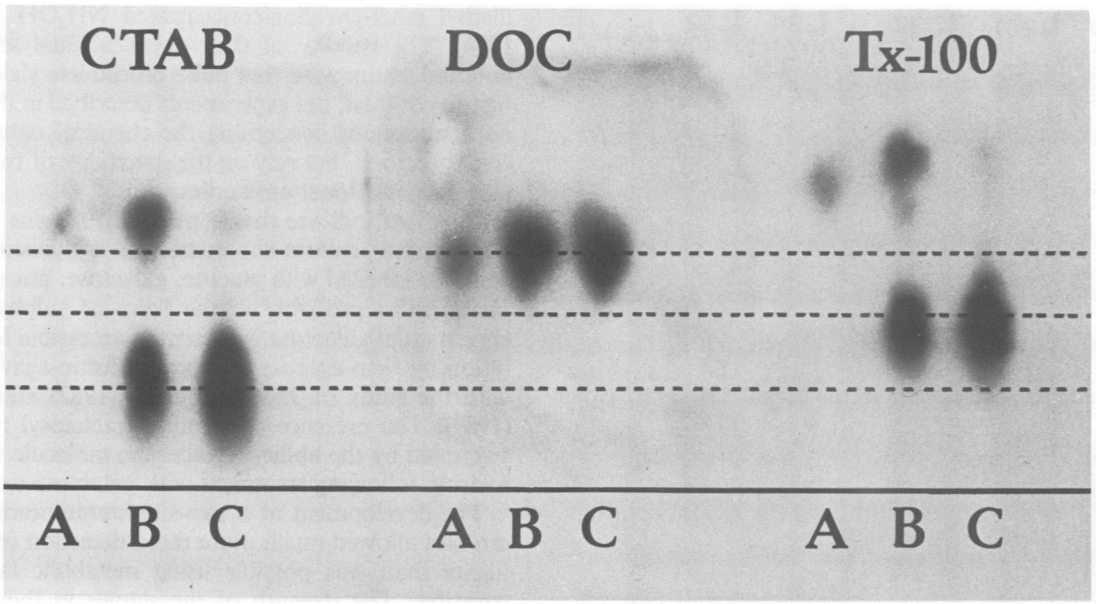


**Fig. 3.** Charge shift electrophoresis of *L. tropica major* cell lysate and culture supernatant detected in a two-site immmunoradiometric assay by WIC-79.3. In each panel, **(A)** consists of culture supernatant, **(B)** consists of cell lysate treated with pronase for 1 h at 37°C, and **(C)** consists of untreated cell lysate. CTAB, electrophoresis in 0.5% Triton X-100 plus 0.05% cetyl trimethylammonium bromide; DOC, electrophoresis in 0.5% Triton X-100 plus 0.25% sodium deoxycholate; Triton X-100, electrophoresis in 0.5% Triton X-100 alone. The solid line marks the point of sample application, at the cathode end of the slide. The dotted lines mark the migration of the glycoconjugate in the various detergents.

component released or shed by the parasite under normal culture conditions.

To investigate whether the glycoconjugate is held in the membrane by a hydrophobic segment, charge shift electrophoresis was used. This technique has been used to determine detergent binding by integral membrane proteins. It is based on changes in the charge of amphipathic molecules after binding anionic or cationic detergents (Helenius and Simons, 1977). The mobility of the *L. tropica major* glycoconjugate was increased in the presence of a mixture of the anionic detergent, deoxycholate (DOC) and the non-ionic detergent Triton X-100, and was retarded in the presence of the cationic detergent, cetyl trimethylammonium bromide (CTAB), and Triton X-100 (Figure 3). In the presence of CTAB and Triton X-100, two populations of molecules could be detected in the cell lysate; a major, slow-moving population, and a minor, fast-moving population (Figure 3, C in panel CTAB and TX-100). Pronase treatment of the cell lysate prior to electrophoresis reduced the intensity of the slow-moving spot and increased the intensity of the fast-moving spot (Figure 3, B in panel CTAB and TX-100). The culture supernatant exhibited mainly the fast-moving spot (Figure 3A). There was some variability in the intensity of the slow-moving spot in different experiments and it became more prominent when the electrophoresis was carried out for a longer time. In the presence of DOC, glycoconjugate from the cell lysate and culture supernatant exhibited similar mobility and appeared as a tight spot (Figure 3A,B,C in panel labelled DOC).

These results indicated that the *L. tropica major* glycoconjugate is an amphipathic molecule with a hydrophobic region. This may be a small hydrophobic protein or covalently bound lipid. Attempts to label the molecule biosynthetically with amino acids have not been successful. Methionine, leucine, serine, proline and threonine were not incorporated in the glycoconjugate although they were successfully incorporated in a variety of parasite proteins. In contrast, we have found that [$^3$H]palmitic acid was metabolically incorporated into the

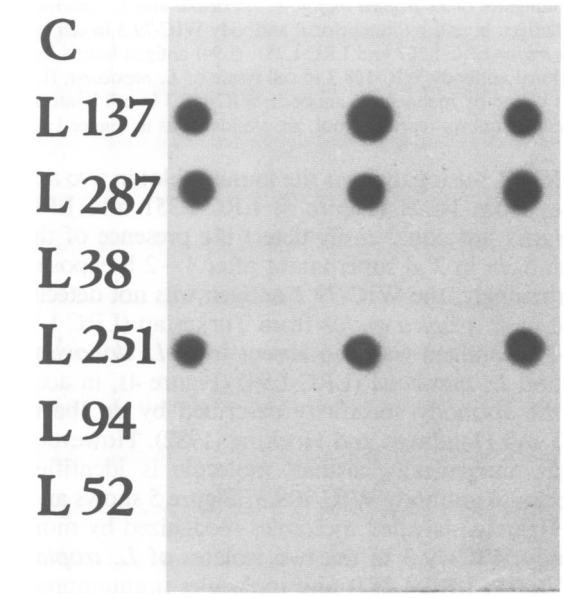


**Fig. 4.** The detection of *L. tropica major* glycoconjugate in a two-site immunoradiometric dot-blot, by monoclonal antibody WIC-79.3. The dots represent triplicate 2 μl culture supernatant from *L. tropica major* (L137, L287, L251, L38), *L. mexicana* (L94), *L. donovani* (L52) and control blood-agar medium overlaid with saline **(C)**.

glycoconjugate immunoprecipitated by monoclonal antibody WIC-79.3 (data not shown), suggesting the presence of a lipid component.

*The glycoconjugate is present on all clones of LRC-L137 and on two other isolates of L. tropica major*

Promastigotes from six avirulent and four virulent clones of LRC-L137 were labelled biosynthetically with [$^3$H]glucose, and detergent lysates immunoprecipitated with WIC-79.3. All exhibited the glycoconjugate antigen recognized by WIC-79.3. The *L. tropica major* glycoconjugate was also detected

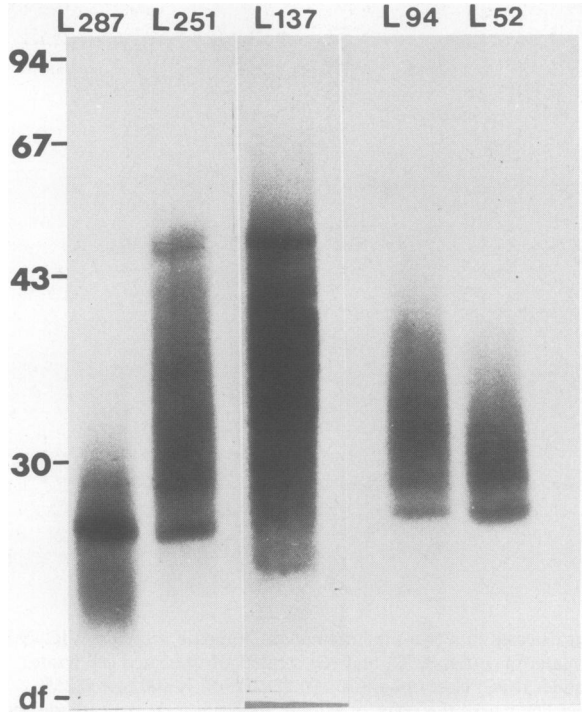


**Fig. 5.** The pattern exhibited on SDS-PAGE by [$^3$H]glucose-labelled glycoconjugates of *L. tropica major*, *L. mexicana* and *L. donovani*. (L287, L251) antigen bound by monoclonal antibody WIC-79.3 in cell lysate of *L. tropica major* LRC-L287 and LRC-L251, (L94) antigen bound by monoclonal antibody WIC-108.3 in cell lysate of *L. mexicana*, (L52) antigen bound by monoclonal antibody WIC-108.3 in cell lysate of *L. donovani*. Numbers represent mol. wt. standards as in Figure 1.

by WIC-79.3 using the two-site immunoblot in two additional isolates from Israel (Figure 4) LRC-L251 and LRC-L287. This rapid test could easily detect the presence of the WIC-79.3 antigen in 2 $\mu$l supernatant after 1–2 h exposure.

Surprisingly, the WIC-79.3 antigen was not detected in an isolate of *L. tropica major* from Turkestan (LRC-L38). The WIC-79.3 antigen was also absent from *L. donovani* (LRC-L52) and *L. mexicana* (LRC-L94) (Figure 4), in accordance with the antibody specificity described by de Ibarra *et al.* (1982) and Handman and Hocking (1982). However, a similar but antigenically distinct molecule is identified using monoclonal antibody WIC-108.3. Figure 5 shows an analysis of [$^3$H]glucose-labelled molecules recognized by monoclonal antibody WIC-79.3 in the two isolates of *L. tropica major* (LRC-L251, LRC-L287) and molecules immunoprecipitated by WIC-108.3 from *L. mexicana* (LRC-L94) and *L. donovani* (LRC-L52). The apparent mol. wt. of the glycoconjugate in isolate LRC-L287 was lower than that observed in the other *L. tropica major* isolates examined. Upon longer exposure of the autoradiograph a component of higher mol. wt. appeared on the film. The fine, evenly spaced bands observed in the *L. donovani* glycoconjugate (Figure 5, lane L52) have been observed in other preparations as well.

## Discussion

Previous studies which have attempted to elucidate the nature of the leishmanial 'excreted factors' have used as the first step chemical purification of the carbohydrate fraction by phenol extraction (El-On *et al.*, 1979; Kaneshiro *et al.*, 1982; Zehavi *et al.*, 1983), methanol-acetate (Slutzky and Greenblatt, 1979), perchloric acid (El-On *et al.*, 1980), or water-ethanol-diethyl ether-pyridine-concentrated $NH_4OH$ (Turco *et al.*, 1984). The validity of their chemical analyses rests on the untested assumption that these procedures yield a pure product. In contrast, the experiments described in this study make no assumptions concerning the chemical nature of the 'excreted factors', but rely on the specificity of two well-characterized monoclonal antibodies.

Our data indicate that a major *L. tropica major* antigen released into culture supernatant is a glycoconjugate which could be labelled with glucose, galactose, phosphate and sulphate, most probably in the form of sulphated sugars. It appears that galactose is present in accessible form for recognition by ricin-agarose and peanut lectin-agarose, consistent with the study of Jacobson *et al.* (1982) and Zehavi *et al.* (1983). The presence of terminal galactosyl residues is also suggested by the ability to label the molecule with [$^3$H]borohydride following treatment with galactose oxidase.

The development of a two-site immunoradiometric Western blot allowed much more rapid detection of the glycoconjugate than was possible using metabolic labelling of the parasites. The strength of the signals in the two-site assay systems described here probably reflects the presence of large numbers of repeating antigenic determinants. This procedure identified the same molecule in unlabelled parasites as in metabolically labelled preparations as determined by SDS-PAGE. On SDS-PAGE the *L. tropica major* glycoconjugate showed considerable heterogeneity, spanning the region of the gel corresponding to proteins of mol. wt. 67 000–20 000. Discrete bands were apparent in the smear and in some experiments they were sharp and evenly spaced (Figure 5, L52) similar to the pattern described for bacterial lipopolysaccharide (Tsai and Frasch, 1982). This heterogeneity could be due in part to the effect of charge contributed by the sulphated carbohydrates and in part by the heterogeneous nature of carbohydrate polymers (Lennarz, 1980; Sharon, 1975). Such heterogeneity on SDS-PAGE has been described for a proteoglycan component of the murine Ia molecular complex (Sant *et al.*, 1984) as well as for a glycoconjugate of *L. donovani* (Turco *et al.*, 1984) and bacterial lipopolysaccharide (Tsai and Frasch, 1982). At present no exact mol. wt. determination can be made for the *L. tropica major* glycoconjugate. Zehavi *et al.* (1983) have provided a mol. wt. estimate of 49 670 daltons for a phenol-extracted, ricin-agarose purified *L. tropica major* 'excreted factor'. This figure was obtained using sedimentation equilibrium centrifugation.

This study has clarified the relationship between the molecule found in supernatants and the glycoconjugate present in cell lysate. Surface labelling of terminal galactosyl and galactosaminyl residues on the parasite membrane using galactose oxidase and sodium borohydride demonstrated that the glycoconjugate was an externally oriented membrane antigen. This is in agreement with studies showing that antibodies preabsorbed on *L. donovani* membrane preparations failed to react with *L. donovani* 'excreted factors' (Kaneshiro *et al.*, 1982). The antigen is slowly released from the cell surface in a process which is essentially complete by 24 h. This appeared to be the main mechanism of release from the cell, and no evidence was found for additional secretion of the glycoconjugate. The glycoconjugate detected in culture supernatants exhibited the same polydisperse pattern on SDS-PAGE as the parent molecule on the cell surface.

The mobility of the glycoconjugate in relation to its mobility in Triton X-100 was increased in the presence of the anionic detergent DOC and decreased in the presence of the

cationic detergent CTAB, indicating that it possesses amphipathic properties. Two populations of molecules were identified in cell lysates in the presence of CTAB and TX-100. Pronase treatment of cell lysates prior to electrophoresis caused a reduction in the intensity of the slow-moving spot and an increase in the intensity of the fast-moving spot. The glycoconjugate detected in culture supernatants migrated predominantly in the fast-moving spot. In the presence of DOC the glycoconjugate in all preparations appeared as a single spot. The significance of the two populations of molecules with different mobilities is not clear, and the effect of pronase in increasing the mobility was unexpected. Difficulty in interpretation of these results is compounded by the possibility that the enzyme preparation may have been contaminated with phospholipases or glycosidases. Resolution of this point will require use of more highly purified enzymes. Using immunoelectrophoresis of *L. donovani* 'excreted factors', Kaneshiro *et al.* (1982) also identified two bands of different mobilities in supernatants and cell preparations.

Proline, threonine and serine were not incorporated into the glycoconjugate, and neither were leucine and methionine. We have not attempted labelling with [$^3$H]asparagine. These results argue against a peptide component, but not conclusively so. Treatment of the glycoconjugate with streptococcal V8 protease and pronase failed to resolve this question. However, we have recently found that [$^3$H]palmitic acid is incorporated into the glycoconjugate immunoprecipitated by monoclonal antibody WIC-79.3 (unpublished data). The presence of lipid in the 'excreted factors' of *Leishmania* has been suggested by Semprevivo and MacLeod (1981) and Kaneshiro *et al.* (1982), but this is the first indication that lipid may be an intrinsic part of the native molecule and not a contaminant.

Little is known about the mechanism of shedding of integral membrane molecules. Vesicles pinching off the membrane have been observed on promastigotes by Hernandez (1983) and ourselves (unpublished observations). The antigen is present in the top third fraction of the 100 000 *g* supernatant of culture medium, implying it is either water-soluble or contained in vesicles which float due to their low density. The molecule may behave in a water-soluble manner by forming a micellar structure shielding the hydrophobic portion from water, and orienting a highly branched carbohydrate chain outwards (A.Bacic, personal communication).

The *L. tropica major* glycoconjugate has previously been shown to be part of a polymorphic family of molecules present on all *Leishmania* species (Schnur, 1982). In this paper, we have shown that the *L. tropica major* glycoconjugate is identifiable on a number of isolates from cases of cutaneous leishmaniasis as well as in virulent and avirulent clones of *L. tropica major* (LRC-L137).

The role of the glycoconjugate in host-parasite interaction is not understood, although the involvement of 'excreted factor' in parasite survival in the phagolysosome has been suggested (Handman and Greenblatt, 1977). Its presence on infected macrophages makes it a potential candidate for a host-protective antigen (Mitchell and Handman, 1983). Handman *et al.* (1977) could induce protection in the resistant C3H/He mice by immunization with *L. tropica major* 'excreted factors' complexed with homologous rabbit antibodies.

The studies described here have identified similar, but antigenically distinct glycoconjugate families in *L. mexicana* and *L. donovani*. An important unresolved question in leishmaniasis is whether this parasite polymorphism, based on varying glycosylation patterns, plays a role in the parasite interaction with different populations of reticuloendothelial cells and hence may be directly involved in the different organ distribution of the various *Leishmania* species.

## Materials and methods

*Parasites*

Parasites were obtained from the W.H.O. Reference Centre for Leishmaniasis, Israel. The *L. tropica major* isolate LRC-L137 has been cloned by limit dilution in two separate experiments. In the study of Handman *et al.* (1983) seven clones were isolated, a virulent clone, that has consistently been infective for BALB/c mice (V121), and six avirulent clones which have been consistently non-infective for BALB/c mice (A12, A52, A59, A65, A120, A122). Three additional virulent clones (V11, V7, V12) used in this study were derived from LRC-L137 as a result of a second limit dilution cloning experiment (E.Handman, unpublished). Parasites were maintained in blood-agar cultures (Nicolle, 1908; Tobie *et al.*, 1950) and passaged to fresh cultures weekly. Other *Leishmania* used in this study have been kept as stabilates in liquid nitrogen and thawed out for the limited number of experiments described here. These are *L. mexicana* (LRC-L94), *L. donovani* (LRC-L52) and *L. tropica major* (LRC-L38, LRC-L251, LRC-L287). For the experiments described in this study parasites were used in late logarithmic growth phase, on days 3 – 5 in culture.

*Antisera*

Antibodies to promastigotes of the virulent clone V121 were raised in rabbits by several s.c. and i.m. injections of promastigotes in complete Freund's adjuvant followed by several boosters of intact promastigotes in phosphate buffered saline (PBS). Promastigotes used for immunizations were grown in blood-agar medium containing rabbit blood.

Monoclonal antibody WIC-79.3 was the generous gift of Dr D.Snary. This IgG1 antibody is described in detail by de Ibarra *et al.* (1982) and Greenblatt *et al.* (1983). It was derived from BALB/c mice immunized with glutaraldehyde-fixed promastigotes, and binds specifically to a *L. tropica major* promastigote surface antigen, which is also present on infected macrophages. It was also shown to precipitate in agar a carbohydrate-rich, phenol-extracted antigen (EF) from boiled culture supernatant of *L. tropica major* (Greenblatt *et al.*, 1983). The IgG3 monoclonal antibody L-5-16 (Handman and Hocking, 1982) was derived from chronically infected BALB/c mice. It binds to an antigen present on *L. tropica major* promastigotes, amastigotes and infected macrophages. In one experiment, monoclonal antibody WIC-108.3, which binds to *L. donovani* and *L. mexicana* was used (Greenblatt *et al.*, 1983). As a control in all our studies we used the irrelevant IgG3 monoclonal antibody L-5-28 which recognizes an antigen present on promastigotes and amastigotes of *L. tropica major, L. mexicana, L. donovani* and *L. enriettii* but absent from culture supernatants (Handman and Hocking, 1982). Another control used in these studies was serum from mice carrying the parental NS-1 tumour cells used for the production of the hybridomas.

*Biosynthetic labelling of promastigotes*

Promastigotes from blood-agar cultures were washed three times in PBS, and 5 x $10^7$ parasites in a volume of 5 ml used for labelling with 300 $\mu$Ci of D[5-$^3$H]glucose (sp. act. 24 Ci/mmol), D[6-$^3$H]galactose (40 Ci/mmol) or D[2,6-$^3$H]mannose (60 Ci/mmol) (Amersham Corporation, Arlington Heights, IL). In initial experiments, the labelling was done in glucose-free RPMI-1640 containing 10% foetal calf serum (FCS) for 15 h. Subsequently, the labelling was performed in the absence of FCS for only 6 h at 26°C, with identical levels of incorporation. Promastigotes were also labelled biosynthetically with 500 $\mu$Ci[$^{35}$S]sulphate (sp. act. 1100 – 1300 Ci/mmol (Amersham Corporation, Arlington Heights, IL). For this purpose sulphate-free RPMI-1640 was prepared by substituting $MgCl_2$ for $MgSO_4$ in the RPMI Select-amine kit (Gibco Laboratories, Grand Island, NY). Promastigotes were labelled with 500 $\mu$Ci[$^{32}$P]orthophosphate (in aqueous solution, carrier-free, Amersham, IL) for 3 h at room temperature, in 1 ml phosphate-free RPMI-1640. Biosynthetically labelled parasites were washed three times in PBS and solubilized in 1% Triton X-100 in PBS with 2 mM PMSF and 10 mM iodoacetamide.

*Surface membrane labelling of promastigotes using galactose oxidase and [$^3$H]sodium borohydride*

Promastigotes were washed in a Hepes-buffered balanced salt solution pH 7.2 (BSS) (Shortman *et al.*, 1972). The labelling was performed essentially as described by Gahmberg and Hakomori (1973) using 5 x $10^7$ or $10^8$ promastigotes in 50 $\mu$l BSS. They were treated for 30 min at room temperature with 1 U galactose oxidase (Sigma Chemical Co.). After 30 min, 5 ml BSS were added, parasites pelleted at 1500 *g*. The liquid was then removed and replaced with 100 $\mu$l BSS and 500 $\mu$Ci $NaB^3H_4$ (New England Nuclear, 70 Ci/mmol in 0.01 M NaOH) in a volume of 20 $\mu$l. Parasites were incubated at room tem-

perature for 30 min, washed three times in BSS and solubilized in 1% Triton X-100 in PBS in the presence of 2 mM PMSF, 10 mM iodoacetamide. In preliminary experiments it was ascertained that the pH of the BSS was not affected by the 20 μl $NaB^3H_4$ in 10 mM NaOH. In the absence of galactose oxidase no parasite labelling was detected. The enzyme preparation was tested for contaminating proteases using the Bio-Rad Protease Detection Kit (Bio-Rad Laboratories, Richmond, CA). At a concentration of 10 U/μl no protease activity could be detected.

*Immunoprecipitations*

Detergent-solubilized promastigotes and culture supernatants were centrifuged at 28 000 *g* to remove insoluble material. Aliquots of the cell lysates and supernatants were used for immunoprecipitations. WIC-79.3 is IgG1 and does not bind to protein A. For immunoprecipitation, a second, protein A binding rat anti-mouse kappa chain monoclonal antibody was used (Yelton *et al.*, 1981), followed by *Staphylococcus aureus* of the Cowan 1 strain as described by Kessler (1981).

*SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

Radiolabelled proteins were electrophoresed in 10% acrylamide slab gels as previously described (Handman *et al.*, 1981). Mol. wt. standards (LMW Calibration Kit, Pharmacia Fine Chemicals, Uppsala, Sweden) that had been conjugated to tetramethyl rhodamine isothiocyanate were run on each slab gel, and their position used to determine the approximate mol. wt. of bands on autoradiograms. Gels were fluorographed using 'Amplify' (Amersham, IL) according to the manufacturer's recommendations, dried at 80°C and autoradiographed at −70°C using Kodak X-Omat AR film.

*Charge shift electrophoresis – detection by a two-site immunoradiometric assay*

Charge shift electrophoresis was performed as described by Helenius and Simons (1977) and Goding (1984). Promastigotes were washed twice in PBS and 6 x $10^7$ solubilized in 100 μl 1% Triton X-100 in PBS. Insoluble material was pelleted in an Eppendorf centrifuge at 15 600 *g* for 15 min at 4°C. The supernatant from the same culture was also centrifuged as above immediately before loading on to the agarose gel. A volume of 7 μl was loaded in each well. Agarose slides (Agarose C, Pharmacia Fine Chemicals) were prepared on 75 x 50 mm Gelbond (Seakem, Marine Colloids Division, FMC Corporation; Bio Products, Rockland, Maine 04841, USA). The agarose was dissolved in buffer (0.05 M glycine, 0.1 M NaCl, 0.5% Triton X-100 pH 9.0) containing in addition to Triton X-100 either 0.25% deoxycholate (DOC) or 0.05% cetyl trimethylammonium bromide (CTAB) (Sigma Chemical Co.), or Triton X-100 alone. Electrophoresis was performed in three separate tanks (Shandon Model 600 Electrophoresis Chamber) in the presence of buffer containing the relevant detergent combinations described above. After 2 h at 100 V constant voltage, the current was stopped and the material in the agarose blotted onto nitrocellulose that had been previously coated with 50 μg/ml of monoclonal WIC-79.3 and further incubated in BLOTTO (Johnson *et al.*, 1984) for 60 min to block any remaining protein-binding sites in the paper (BLOTTO = 5% w/v non-fat powdered milk in PBS). The nitrocellulose was covered by a stack of dry paper towels and a heavy book. After 50 min at room temperature, the nitrocellulose was washed in BLOTTO for 30 min and then incubated for 60 min with 3 x $10^5$ c.p.m./ml of radioiodinated WIC-79.3 in BLOTTO. The radioiodination was performed using chloramine-T to a specific activity of 10 μCi/μg. After further washes, the nitrocellulose was blotted dry and autoradiographed at −70°C, in the presence of Cronex Lighting Plus intensifying screens (Laskey and Mills, 1977).

*The two-site-immunoradiometric Western blot*

This is a modification of the procedure described by Towbin *et al.* (1979). The culture supernatant or cell lysate was electrophoresed in 10% acrylamide slab gels and then electroblotted onto nitrocellulose that had been previously incubated with 50 μg/ml of WIC-79.3 antibody followed by BLOTTO. The acrylamide gel was rinsed in water for 30 min prior to blotting. After electrophoretic transfer, the nitrocellulose was washed in BLOTTO for 30 min and incubated with $10^5$ c.p.m./ml of radioiodinated WIC-79.3. After further washes the nitrocellulose was autoradiographed at −70°C in the presence of Cronex Lighting Plus intensifying screens.

A rapid dot-blot assay for detection of the *Leishmania* glycoconjugate was essentially as described above, except that instead of electrophoresis of the parasite material prior to immunoblotting, 2 μl volumes of culture supernatant were dotted directly on to the almost-dry nitrocellulose which had been pre-coated with the monoclonal WIC-79.3. The antigen was then detected with radioiodinated WIC-79.3.

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