

Glycosphingolipid antigens from *Leishmania (L.) amazonensis* amastigotes. Binding of anti-glycosphingolipid monoclonal antibodies *in vitro* and *in vivo*

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Abstract

Specific glycosphingolipid antigens of *Leishmania (L.) amazonensis* amastigotes reactive with the monoclonal antibodies (MoAbs) ST-3, ST-4 and ST-5 were isolated, and their structure was partially elucidated by negative ion fast atom bombardment mass spectrometry. The glycan moieties of five antigens presented linear sequences of hexoses and N-acetylhexosamines ranging from four to six sugar residues, and the ceramide moieties were found to be composed by a sphingosine d18:1 and fatty acids 24:1 or 16:0. Affinities of the three monoclonal antibodies to amastigote glycosphingolipid antigens were also analyzed by ELISA. MoAb ST-3 reacted equally well with all glycosphingolipid antigens tested, whereas ST-4 and ST-5 presented higher affinities to glycosphingolipids with longer carbohydrate chains, with five or more sugar units (slow migrating bands on HPTLC). Macrophages isolated from footpad lesions of BALB/c mice infected with *Leishmania (L.) amazonensis* were incubated with MoAb ST-3 and, by indirect immunofluorescence, labeling was only detected on the parasite, whereas no fluorescence was observed on the surface of the infected macrophages, indicating that these glycosphingolipid antigens are not acquired from the host cell but synthesized by the amastigote. Intravenous administration of ¹²⁵I-labeled ST-3 antibody to infected BALB/c mice showed that MoAb ST-3 accumulated significantly in the footpad lesions in comparison to blood and other tissues.

Key words

- Glycosphingolipids
- Monoclonal antibodies
- *Leishmania (L.) amazonensis*
- Fast atom bombardment mass spectrometry
- Carbohydrate antigens

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We have recently described the isolation and structure of a new glycosphingolipid (GSL) antigen specific for *Leishmania (L.) amazonensis* amastigotes (1). This new antigen is a III^F β -GalGb₃Cer (Gal-globoside) with the structure Gal β 1 \rightarrow 3Gal α 1 \rightarrow 4Gal β 1 \rightarrow 4Glc β 1 \rightarrow Cer (band 1), which differs from the human erythrocyte P antigen by the substitution of the terminal residue of GalNAc β 1 \rightarrow 3 for Gal β 1 \rightarrow 3 (2,3). In addition to III^F β -GalGb₃Cer, sixteen other GSL antigens (bands 2 to 17) were detected, most of them in very low amounts (4). We analyzed five antigens by negative ion fast atom bombardment mass spectrometry (⁻FAB/MS) and determined their hexose and N-acetyl hexose sequences as well as their ceramide moiety composition. ⁻FAB/MS was performed using a JEOL HX-110 mass spectrometer/DA-5000 data system (Jeol Ltd., Tokyo, Japan). Samples (5-10 μ g) were deposited on the target in a matrix of triethanolamine containing the cyclic polyether 15-crown-5 (5,6) and bombarded with a 6-kV Xenon beam source. The mass range *m/z* 100-3600 was acquired in 1.5 min by linear upward scan, with an acceleration voltage of 10 kV and a resolution of 3000, using a 100-Hz filter. Sodium iodide in glycerol was used as the calibration standard.

Of the seventeen antigenic GSLs isolated from *L. (L.) amazonensis* amastigotes (4), five glycosphingolipids, termed bands b2, b3, b4, b5 and b6, were purified by a combination of HPLC and preparative HPTLC in solvent chloroform/methanol/0.2% aqueous CaCl₂, as described by Straus et al. (1) and analyzed by ⁻FAB/MS. The MW of the pure GSL antigens b2, b3, b4, b5 and b6, recognized by the monoclonal antibodies (MoAbs) ST-3, ST-4, and ST-5 (directed against carbohydrate moieties of GSLs), was 1346 atomic mass units (a.m.u.), 1498 a.m.u., 1388 a.m.u., 1660 a.m.u. and 1550 a.m.u., respectively. The hexose and N-acetyl hexose sequences of the bands analyzed were as follows: band 2: Hex-O-Hex-O-Hex-O-Hex-O-

Hex-O-Cer; band 3: Hex-O-HexNAc-O-Hex-O-Hex-O-Hex-O-Cer; band 4: Hex-O-HexNAc-O-Hex-O-Hex-O-Hex-O-Cer; band 5: Hex-O-Hex-O-HexNAc-O-Hex-O-Hex-O-Hex-O-Cer; and band 6: Hex-O-Hex-O-HexNAc-O-Hex-O-Hex-O-Hex-O-Cer. Their ceramide moieties contained only one type of long chain base, a sphingosine d18:1, whereas their fatty acid (f.a.) composition was: band 2, f.a. 16:0; band 3, f.a. 24:1; band 4, f.a. 16:0; band 5, f.a. 24:1 and band 6, f.a. 16:0.

Eight different GSL antigens were purified as described above (bands 1, 3, 4, 5, 8, 9, 12, and 13) (Figure 1A), and analyzed by ELISA regarding their affinity for MoAbs ST-3, ST-4 and ST-5. The GSL antigens were double-diluted with the first well containing 100 ng of the antigen (1,7). MoAb ST-3 showed the same affinity for the eight GSL antigens tested. MoAb ST-4 showed higher affinity for bands 9, 12 and 13 and lower affinity for bands 1, 3, 4, 5 and 8. MoAb ST-5 presented higher affinity for glycosphingolipid antigens 4 to 13 and lower affinity for bands 1 and 3. These data indicate that the fine specificity of these three MoAbs presents subtle differences depending on the structure and size of the glycan moiety of GSL antigens.

¹²⁵I-labeled ST-3 antibody was used to determine the *in vivo* binding capacity of the anti-glycosphingolipid MoAbs. The radiolabeled MoAb was injected intravenously into BALB/c mice infected with *Leishmania (L.) amazonensis*. After 72 h their blood was removed by cardiac puncture and the mice were sacrificed. Samples from the tissues and lesions were washed with cold hypotonic PBS (10-fold diluted PBS) and weighed and the radioactivity was counted. Tissue distribution of ¹²⁵I-labeled ST-3 is reported as the ratio of radioactivity in the tissue to that present in the blood [(cpm/g tissue)/(cpm/g blood)]. Antibody distribution was determined in three infected BALB/c mice after injection of ¹²⁵I-labeled antibody as

described above. The data are shown in Figure 2A and represent values obtained 72 h after injection of 3.0 µg (10 µCi) of ¹²⁵I-labeled ST-3. The highest level of radioactivity was observed in the footpad lesions, with almost three times more radioactivity being detected in the lesion when compared to blood and lung. An irrelevant antibody (CU-1) used as control (8) showed no sig-

nificant binding.

The data presented in this study clearly show the importance of glycosphingolipid antigens recognized by MoAbs ST-3, ST-4 and ST-5 as specific markers of *Leishmania (L.) amazonensis* amastigotes. These glycosphingolipid antigens, different from those reported in amastigotes of *Leishmania donovani* and *Leishmania mexicana* (9), are

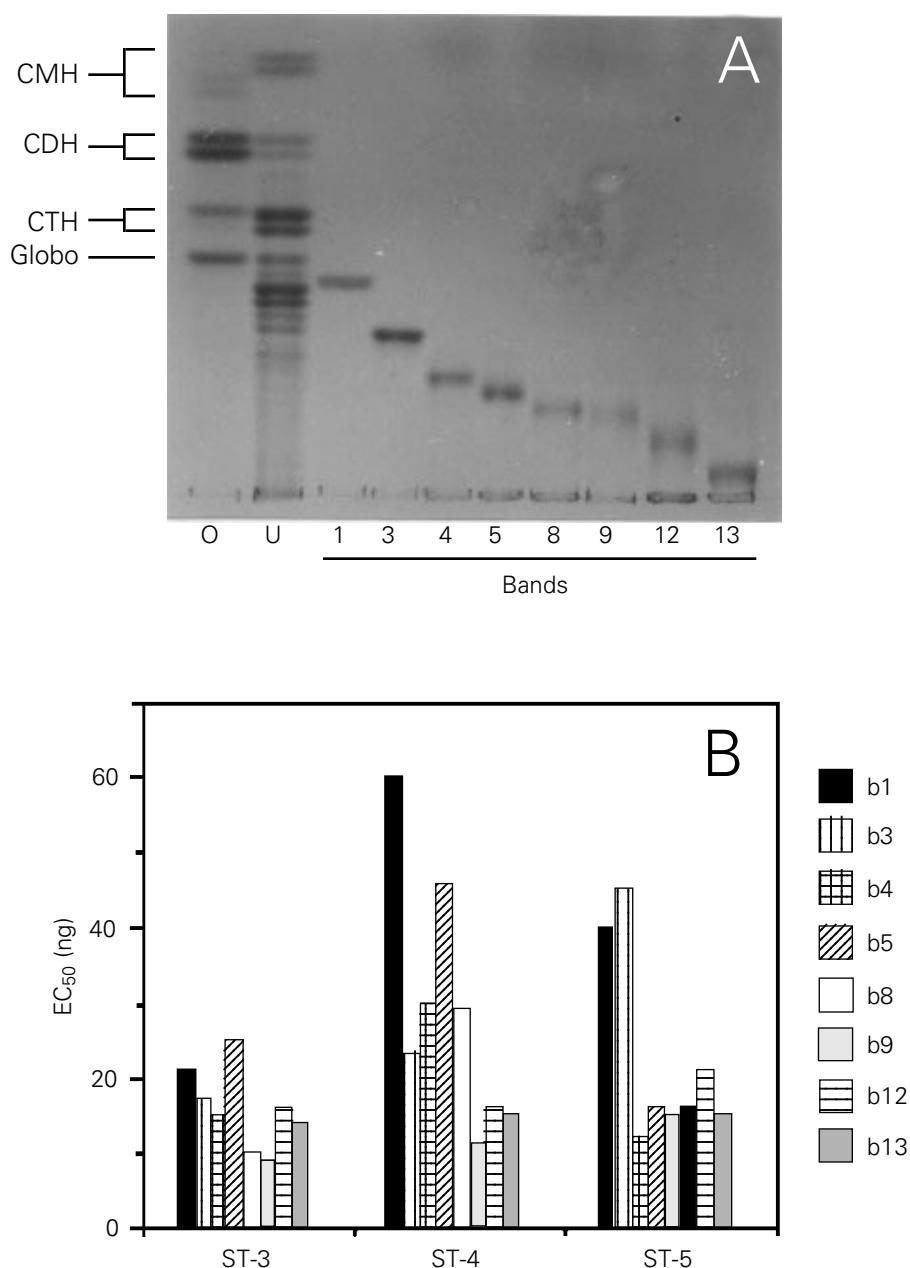
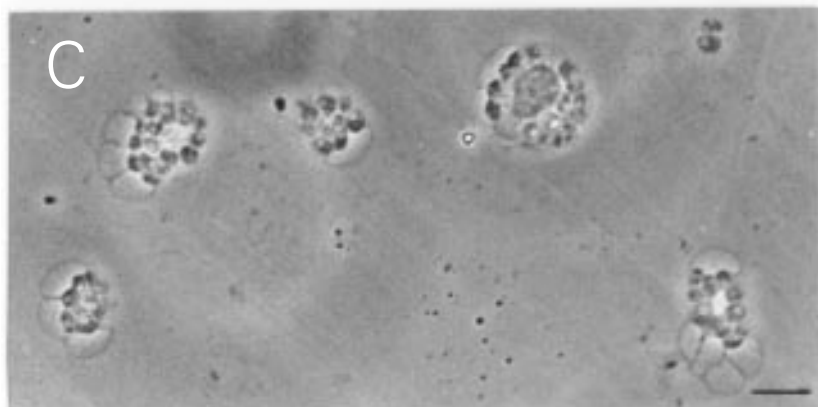
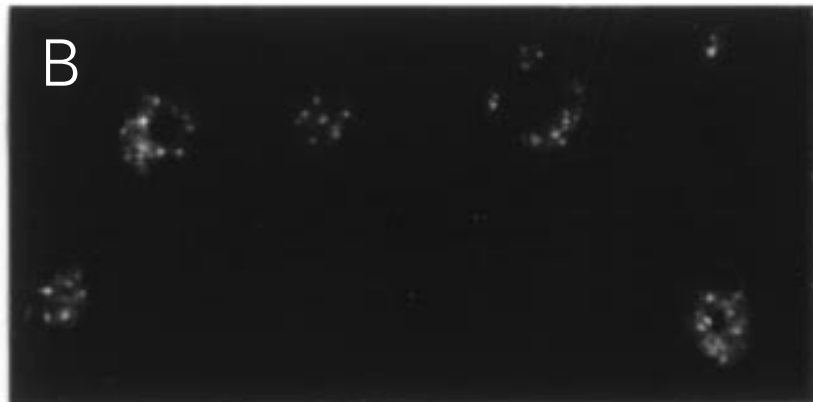
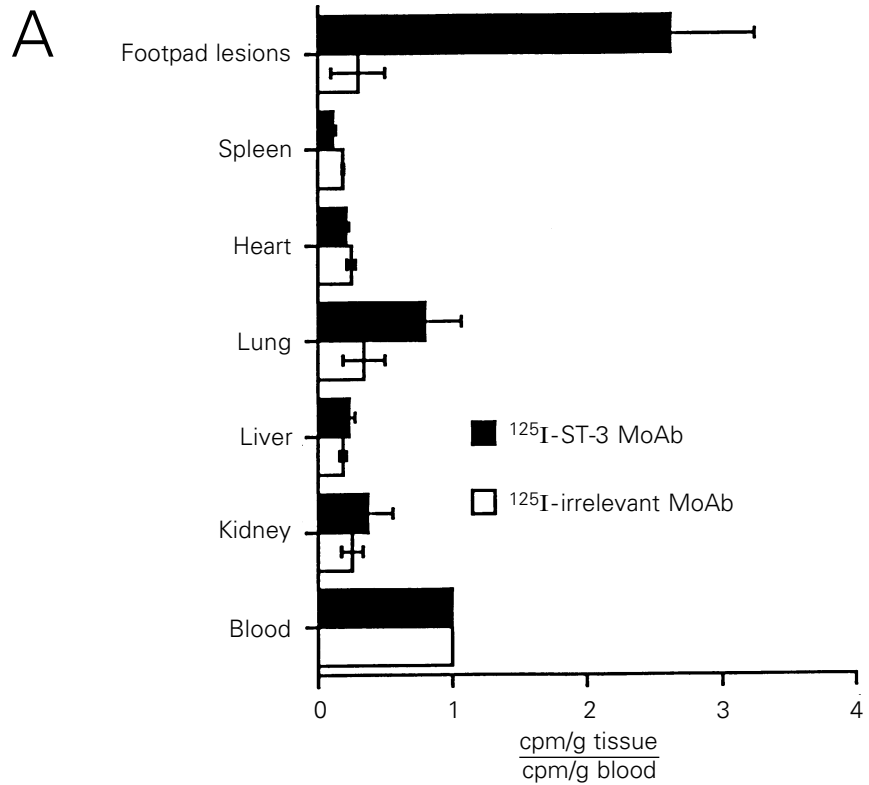


Figure 1 - Panel A, HPTLC pattern of 8 pure GSL antigens from *L. (L.) amazonensis* amastigotes developed in chloroform/methanol/aqueous CaCl₂ (60:40:9; v/v/v). O, Human erythrocyte neutral GSLs from Folch's lower phase; U, total neutral GSLs from *L. (L.) amazonensis* amastigotes. CMH, Ceramide monohexoside; CDH, ceramide dihexoside; CTH, ceramide trihexoside; Globo, globoside. The plate was stained with orcinol/H₂SO₄. Panel B, Affinity of MoAbs ST-3, -4 and -5 for eight GSL antigens (bands 1, 3, 4, 5, 8, 9, 12 and 13). Antigens were serially double-diluted in ethanol from 100 ng/well to 0.4 ng/well and adsorbed on 96-well plates. Plates were blocked with 1% BSA/PBS (2 h) and incubated overnight at 4°C with the MoAbs. Antibody binding was determined after incubation with rabbit anti-mouse IgG (2 h) and 10⁵ cpm of ¹²⁵I protein A in 1% BSA (1 h). The radioactivity of each well was measured with a gamma counter and the results are reported as EC₅₀ (glycosphingolipid antigen necessary to achieve 50% of maximum binding).

Figure 2 - *Panel A*, Distribution of ^{125}I -labeled monoclonal antibody ST-3 in tissues from infected BALB/c mice. Three infected BALB/c mice were injected with $3.0\ \mu\text{g}$ ($10\ \mu\text{Ci}$) of ^{125}I -labeled ST-3 MoAb. In control experiments, infected mice were injected with a labeled irrelevant MoAb (CU-1 MoAb) and sacrificed 72 h later. The radiolabeled MoAb distribution is reported as the mean \pm SEM ratio of radioactivity in the tissue to that present in the blood [(cpm/g tissue)/(cpm/g blood)]. *Panel B*, Immunofluorescence of the ST-3 MoAb in a mouse lesion imprint. Mouse imprints were fixed with 2% formaldehyde, blocked with normal mouse serum (1:50) and incubated with fluorescein-isothiocyanate (FITC)-labeled ST-3 MoAb. Only amastigotes inside the macrophages are reactive with MoAb ST-3. Bar = $20\ \mu\text{m}$. *Panel C*, Phase-contrast of a mouse lesion imprint.



not acquired from the host (10) but rather synthesized by the parasite (1). Figure 2B shows MoAb ST-3 immunofluorescence of *Leishmania (L.) amazonensis* present in macrophages isolated from BALB/c mouse footpad lesions. Labeling was detected on the parasite and, as expected, no fluorescence was observed on the surface of the infected macrophages.

Anti-GSL monoclonal antibodies can greatly reduce the invasion of macrophages by the parasites. Nevertheless, no anti-GSL antibodies were detected in sera from patients with cutaneous leishmaniasis. Taken together, these data are consistent with the hypothesis that the lack of anti-GSL antibodies may render amastigote forms more effective in binding and invading host macrophages. Analysis by ⁺FAB/MS of five GSL antigens showed that they present linear carbohydrate structures and their ceramides range in mass from 536 a.m.u. to 646 a.m.u. Structural characterization of twelve other

GSL antigens is currently in progress. The complete structural elucidation of these antigens together with competitive assays using MoAbs ST-3, ST-4 or ST-5 may provide information concerning the fine specificity of these MoAbs as well as a more detailed vision of the GSL antigens directly related to binding/invasion of macrophages. One hypothesis currently under investigation in our laboratory is the presence of a unique UDP-galactose: Gal α 1 \rightarrow 4Gal β 1 \rightarrow 3galactosyltransferase, responsible for the synthesis of Gal-globoside in these organisms using globotriosylceramide (Gal α 1 \rightarrow 4Gal β 1 \rightarrow 4Glc β 1 \rightarrow Cer) as substrate. The mechanism of precursor synthesis or transfer from the macrophage to the parasite is under investigation. Since Gal-globoside was found to be an amastigote-specific antigen which is not present in mammals, it may prove to be a new valuable target molecule for treatment and/or diagnosis of leishmaniasis caused by *L. (L.) amazonensis*.

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