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Structural and immunological characterization of sulphatides: relevance of sulphate moieties in *Trypanosoma cruzi* glycoconjugates

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SUMMARY

Sulphoglycosphingolipids, present on the surface of diverse cells, participate in the regulation of various cellular events. However, little is known about the structure and the role of sulphoglycosphingolipids in trypanosomatids. Herein, sulphated dihexosylceramide structures – composed mainly of sphingosine as the long chain base acylated with stearic acid – have been determined for the first time in Trypanosoma cruzi *epimastigotes by UV-MALDI-TOF-MS analysis. Interestingly, inhibition ELISA assays using cruzipain as antigen and polyclonal rabbit antibodies specific for cruzipain, the major cysteine proteinase of* T. cruzi*, or for its C-terminal domain, have demonstrated (i) that sulphate epitopes are shared between cruzipain and sulphatides of* T. cruzi*, (ii) that cross-reactivity maps to the C-terminal domain and (iii) the existence of other antigenic determinants in the glycolipidic structures. These features provide evidence that sulphate groups are antigenic in sulphate-containing parasite glycoconjugates. Furthermore, IgG2 antibody levels inversely correlate with disease severity in chronic Chagas disease patients, suggesting that IgG2 antibodies specific for sulphated epitopes might be associated with protective immunity and might be considered as potential surrogates of the course of chronic Chagas disease.*

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INTRODUCTION

Chagas disease, one of the major health problems in Latin America, is caused by the kinetoplastid protozoan *Trypanosoma cruzi* (1). Transmission occurs mainly by triatomine insect vectors. The problem is amplified by the fact that most of the available therapeutics were discovered decades ago and suffer from a variety of problems such as acute toxicity and short duration of action. Current drugs are effective in treating congenital infection by *T. cruzi*, acute Chagas disease and children in indeterminate phase of Chagas disease. Little evidence exists as to the effectiveness of treatment in adults (2,3). Therefore, new active trypanocidal compounds, with low toxicities and increased efficacies during the indeterminate and chronic phases, are urgently needed. The Pan American Health Organization (PAHO) estimates that 7·7 million persons currently have *T. cruzi* infection in the 21 endemic countries, which have a total population of 532 million people. The PAHO also estimates that approximately 41 200 new-vector borne cases of *T. cruzi* infection arise per year and that 14 400 infants are born with congenital Chagas disease annually (4). The disease has also emerged as a public health problem elsewhere in the world owing to infected people migrating to other regions such as the United States of America, Europe, Australia and Japan (5,6).

Chagas heart disease develops in 10–30% of infected individuals and is a common cause of fatal dilated cardiomyopathy (7). The pathogenesis of Chagas disease is controversial but irrespective of the eventual contribution of an autoimmune component (8), pathology has been related to the persistence of *T. cruzi* parasites in the affected organs where they induce chronic inflammation (9). Moreover, it

has been postulated that the persistence of the parasite in conjunction with immune responses against multiple myocardial antigens might contribute to heart damage (10).

Although acidic glycosphingolipids (AGSLs) are ubiquitous in eukaryotic cells, very little is known about their roles in parasites. Among acidic glycolipids, gangliosides (containing sialic acid) and sulphoglycolipids or sulphatides that contain sulphate monoesters are usually distributed on the cell surface in brain, kidney and gastrointestinal tract (11). AGSLs are active participants in adhesion processes in many cellular systems and appear to be involved in the regulation of cell proliferation, differentiation, host interaction and other developmental cellular events. Biosynthesis of sulphatides requires the transfer of a sulphate group to the glycolipid moiety, which is catalysed by a unique cerebroside sulphotransferase (CST) (12). The physiological role of sulphated glycosylceramides has been investigated. Several studies have provided evidence that sulphatides expressed on the surfaces of different cells exert biological functions through mediating interactions with extracellular matrix proteins (13), adhesion molecules (14,15), bacteria (16) and viruses (17). These molecules play a role in a variety of biological processes such as cell growth, protein trafficking, signal transduction, cell-cell recognition, neuronal plasticity and cell morphogenesis (18).

In trypanosomatids, glycosphingolipids have been detected in epimastigotes of *T. cruzi* (19–21) and characterized in *Trypanosoma mega* (22). Cross-reacting galactosyl ceramides and sulphoglycosylceramides have also been isolated from *T. cruzi* and sulphated glycolipidic structures have been described representing common antigens on the surface of the parasite and mammalian cells (23). In parasites, lipid metabolism has been attracting more attention with respect to basic biology and applications for chemotherapeutic purposes. In fact, neutral glycosphingolipids have been implicated as cross-reactive antigens in Chagas disease autoimmunity (24); however, very little information is available on sulphoglycosphingolipids structure and their roles in protozoan parasites so far (25), and to our knowledge, nothing is known about complete structures and biological role of sulphatides in *T. cruzi*.

On the other hand, cruzipain (Cz), the major cysteine proteinase of *T. cruzi*, identified as a promising candidate for both vaccine development and target for chemotherapy of Chagas disease (2,3), has been extensively studied as antigen and protease (26). Our research group has revealed the presence of sulphated high-mannose type oligosaccharides on the unique *N*-glycosylation site (Asn 255) of the C-Terminal domain (C-T) of Cz as a new striking feature in this glycoprotein (27). Furthermore, we have demonstrated that sulphate groups are absolutely required for eliciting IgG2b responses to Cz and that sulphate-bearing glycoproteins in trypanosomatids are targets for specific immune responses (28). In addition to the fact that the humoral immune response to Cz was associated with the severity of chronic Chagas disease (29), we reported that subjects chronically infected with *T. cruzi* mount specific humoral immune responses to this sulphated glycoprotein. Interestingly, our results showed that IgG2 antibody responses to sulphate groups on Cz inversely correlate with human severe chronic Chagas disease (28).

In this work, we report for the first time by extraction, purification and UV-MALDI-TOF mass spectrometry analysis, the structural characterization of sulphoglycosphingolipids present in epimastigote forms of *T. cruzi*. In addition, ELISA inhibition assays performed with sera specific for Cz and C-T, after adsorption with increasing amounts of purified epimastigote sulphatides prior to and after desulphation treatment, have shown strong evidence (i) of a sulphate epitope that is common to both Cz and sulphatides of *T. cruzi*, (ii) that this cross-reactivity is found in the C-T domain of Cz and (iii) of the existence of other oligosaccharidic determinants in the glycolipidic structures suggesting that these groups could be also responsible for the mentioned cross-reactivity. Moreover, sera from chronically *T. cruzi*-infected subjects with mild disease (G0/G1) displayed higher levels of IgG2 antibodies specific for sulphatides compared with those in more severe forms of the disease (G2/G3). Our study provides evidence that *T. cruzi* sulphate moieties are present as antigenic epitopes in parasite glycosphingolipids indicating that sulphate groups are antigenic independently of the type of parasite sulphate-containing glycoconjugate and suggests that antibodies specific for these moieties are potentially associated with protective immunity related to mild and stable early stages of chronic Chagas disease. These specific antibodies might play a role as predictors of stability from the early stages of chronic Chagas disease.

MATERIAL AND METHODS

Materials

Lipid standards were purchased from Sigma (Sigma-Aldrich Co., St Louis, MO, USA). All solvents were of analytical or HPLC grade. TLC was performed on silica gel 60 precoated plates (Merck KGaA, Darmstadt, Germany). Matrix [2,5-dihydroxybenzoic acid (DHB), Sigma-Aldrich, Co.] and calibrating chemicals for UV-MALDI-TOF analysis were purchased from Sigma-Aldrich Chemical Co. Solvents such as methanol, ethanol and acetonitrile (Sigma-Aldrich-Co.) and trifluoroacetic acid (TFA; Merck, KGaA Co., Darmstadst, Germany) were used as purchased without further purification. Water of very low conductivity

(MilliQ grade, 18 MΩ; Millipore, Bedford, MA, USA) was used.

Parasites

Trypanosoma cruzi epimastigotes were grown in brain–heart infusion-tryptose medium (BHT) at 28°C supplemented with 10% heat-inactivated foetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin and harvested as described previously (30).

Immunization of animals

Rabbits (New Zealand white lineage) were immunized with purified Cz extracted from polyacrylamide gels according to the procedure of Acosta *et al.,* (28), or from C-T domain purified according to Barboza *et al.,* (31), using doses of 50 μg protein given intradermally in each case. All experimental procedures were conducted in the INP, Dr Mario Fatala Chaben, Ministerio de Salud de la Nación Argentina, in accordance with the ethical and regulatory entities, established in Argentina and International Guides for care and use of laboratory animals.

Study population

The diagnosis of Chagas disease is currently assessed by indirect hemagglutination, indirect immunofluorescence and enzyme immunoassay using whole homogenates of the epimastigote form of *T. cruzi* as antigen (32). An individual is considered infected if at least two of the three tests were positive. Sera from chronic Chagas disease patients aged from 28 to 58 with different degrees of cardiac dysfunction as determined by the Kuschnir grading system (33) were provided by the Clinical, Pathology and Treatment Department, National Institute of Parasitology. Group 0 (G0, $n = 9$) comprised seropositive individuals showing a normal chest X-ray and a normal electrocardiography (ECG); group 1 (G1, $n = 9$) seropositive patients had a normal chest X-ray with abnormalities in the ECG, group 2 equal to G1 plus cardiomegaly on chest X-ray (G2, $n = 5$) and group 3 (G3, $n = 15$) seropositive patients had ECG abnormalities, cardiomegaly and clinical signs of heart failure. The uninfected control group, provided by Diagnostic Department, INP, was found to be negative for *T. cruzi* by serologic testing as well as for Cz recognition. Individuals with hypertension, vascular, ischaemic or congenital heart disease, cancer, syphilis, HIV, diabetes, arthritis or serious allergies were excluded from the present study. Human stored sera used from Chagas and non-Chagas disease patients were always codified and anonymized. Blood to be used for serum component analysis was obtained from patients and control subjects by venipuncture, allowed to coagulate at 4°C and centrifuged at 1000 *g* for 15 min. Nonhaemolysed serum was separated, and aliquots were stored at 70°C until use. The study was evaluated and approved by the Ethical Committee of the Institute Fatala Chaben. The informed consent was signed for all enrolled patients.

Isolation and purification of glycosphingolipids

Pellets of epimastigotes were extracted with chloroform/methanol (1 : 1, v/v, 3 × 1 mL). Each extract was fractionated by anionic exchange chromatography on a DEAE-Sephadex A-25 (acetate form) column, which was eluted with chloroform/methanol/water (30 : 60 : 8, v/v/v) to recover neutral GSLs (NGSLs) and zwitterionic lipids. Anionic lipids were bulk eluted with chloroform/methanol/0·8 M NaAcO (30 : 60 : 8, v/v/v), evaporated to dryness and treated with 0·1 M NaOH in methanol (500 μL), at 37°C for 3 h. The mixture was neutralized with 1 M HCl in the presence of 1 M phosphate buffer pH 7 (50 μL) to avoid over-acidification. After evaporation, salts were removed by reverse-phase chromatography, using a Sep-Pack C-18 cartridge (Worldwire monitoring, Bellefonte, PA, USA). For UV-MALDI-TOF-MS analysis and dot blot assays, part of the sample obtained after the Sep-Pack C-18 step was dried and loaded into a column of Unisil (7 × 50 mm) which was eluted with chloroform, chloroform/methanol (98 : 2, v/v) and chloroform/methanol (1 : 3, v/v). The purified acidic fraction was analysed by TLC in *n*-propanol/NH_3/water (75 : 5 :5, v/v/v). Spots were developed with I_2. Spots corresponding to the sulphoglycolipids were scraped from the plate and extracted with chloroform/methanol/water (10 : 10 : 1, v/v/v) to be subjected to UV-MALDI-TOF-MS analysis.

Chemical treatments

Acid methanolysis and methylation

The sample was hydrolysed for 18 h at 80°C with 12 M HCl/MeOH/water (3 : 29 : 4, v/v/v) and dried. The acid was eliminated by several evaporations with addition of water. Fatty acids were analysed by RP-TLC (Merck) using acetonitrile/acetic acid (1 : 1, v/v) as solvent.

Desulphation treatment

Solvolysis.

For chemical desulphation, samples were passed over 0·5 mL of AG50W-X8 resin (H^+) and the column washed with water (2 mL). After the addition of pyridine (0·015 mL), the sample was lyophilized, dissolved in dim-

ethylsulphoxide/methanol (9 : 1 v/v, 0·2 mL), adjusted to pH 4 with dilute HCl, heated at 100°C for 2 h and freeze dried (34).

UV-MALDI-TOF-MS analysis

Measurements were performed using an Ultraflex II TOF/-TOF mass spectrometer (Bruker Daltonics GmbsH, Bremen, Germany) equipped with a high-performance solid-state laser (λ = 355 nm) and a reflector. Calibrating chemicals used were β-Cyclodextrin (cyclohexaamylose, MW: 972·9), β-cyclodextrin (cycloheptaamylose, MW: 1135·0), β-cyclodextrin (cyclooctaamylose, MW: 1297·1), angiotensin I (MW: 1296·49), neurotensin (MW: 1672·96) and bovine insulin (MW: 5733·5) purchased from Sigma-Aldrich. The system is operated by the FLEXCONTROL 2.4 software package (Bruker Daltonics GmbsH). The mass spectra reported are the result of 100 laser shots. All samples were measured in the linear and the reflectron mode, and as routine in both positive and negative polarity. The samples were loaded onto a ground steel sample plate (MTP 384 ground steel; Bruker Daltonics GmbsH) using the classic dried drop method: a sample/matrix solution mixture, 1 μL (1 : 1, v/v), was deposited on the target plate and let to dry at room temperature. 2, 5-DHB was used as matrix to obtain the best signal/noise relationship.

Determination of total IgG and IgG subclasses in rabbits and/or human sera

ELISA inhibition assays were performed according to Acosta *et al.* (35) with some modifications. After blocking, plates (Nunc-immuno™ 9 microwell™, Maxisorp™ Thermo Fischer Scientific Inc, Waltham, MA, USA) were coated with Cz (1 μg) and incubated with polyclonal rabbit sera specific for Cz and C-T, or human sera, respectively. Rabbit (1/6400) and human (1/50) sera were adsorbed with AGSLs prior to and after desulphation treatment and incubated 2 h at 37°C. The amounts of GSLs used were estimated taking into account the original parasite number used to purify them ($0–8 \times 10^7$ parasites for rabbit sera and 1×10^8 parasites for patient sera inhibition assays, respectively). Solvent was evaporated prior to incubation. In rabbit sera, total IgG specific for Cz and C-T were detected by incubation with biotinylated horse anti-rabbit IgG (H + L) (1 : 2000 dilution) (Vector Laboratories, Inc., Burlingame, CA, USA), and in patients' sera, total IgG levels and IgG2 and IgG1 subclasses were detected with biotinylated mouse anti-human total IgG, IgG2 and IgG1 isotypes (PharMingen, BD Biosciences, San Diego, CA, USA) at a final concentration of 3·5 μg/mL, followed by incubation with HRP-streptavidin (Vector Laboratories, Inc., Burlingame, CA, USA), respectively. The reaction was developed with *o*-phenylenediamine dihydrochloride and stopped with sulphuric acid. Plates were read at 490 nm with an ELISA reader (MR 700; Dynatech Laboratories, Alexandria, VA, USA). The results represent at least three experiments with data performed as triplicates. The percentage of inhibition of Cz recognition was determined by comparing rabbit serum reactivity in the presence and absence of the inhibitor and was calculated in accordance with the following formula: % Inhibition = (OD_{490nm} without I − OD_{490nm} with I) 100/Abs_{490nm} without I. The value corresponding to 100% recognition = 0% Inhibition of Cz recognition is obtained in the absence of any GSL used as inhibitor. NGSLs, which do not contain sulphate groups, were also subjected to desulphation treatment and were used as controls to verify whether desulphation techniques affect lipid structure. In parallel, preimmune sera used as controls were incubated with increasing amounts of the different lipidic fractions prior to and after desulphation treatment and were processed in the same conditions. In experiments with human sera, the percentage of remnant IgG affinity to Cz for each serum was calculated as [OD_{490nm} with AGSL/OD_{490nm} without inhibitor] × 100 or [OD_{490nm} with desulphated AGSLs/OD_{490nm} without inhibitor] × 100 (b), using untreated (a) or desulphated (b) AGSLs, respectively.

Dot blot assays

Dot blots were performed using the Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Philadelphia, PA, USA) following the manufacturer's instructions. Briefly, similar amounts of acidic and NGSLs purified from 4×10^8 parasites and taken from TLC plates were seeded in triplicate, probed with polyclonal rabbit sera specific for Cz and C-T (comparable titres: 1/6400) or sera from five chronic Chagas disease patients (1/50), followed by incubation with a HRP-horse anti-rabbit IgG (H + L) diluted 1 : 2000 or HRP-goat anti-human IgG (H + L) at a 1 : 2000 dilution), respectively, and visualized using ECL (Amersham Biosciences, Cambridge, UK) enhanced chemiluminescence's reagent. Spots were visualized using a FujiLAS1000 densitometer equipped with IMAGE GAUGE 3.122, software (Fuji Film, Tokyo, Japan). PVDF rather than nitrocellulose membranes were used as they are resistant to organic solvents.

Statistical analysis

Values are presented as means ± SD of triplicate determinations from a minimum of three or more independent

experiments. Pair-wise comparisons between groups were evaluated with one-way analysis of variance, followed by Tukey test in GRAPH PAD PRISM 5.0 software (GraphPad software, Inc., San Diego, CA, USA). Values of $P < 0{\cdot}05$ were considered significant.

RESULTS AND DISCUSSION

Acidic and neutral glycosphingolipids (GSLs) obtained from epimastigotes of *T. cruzi* were purified by DEAE-Sephadex chromatography followed by alkaline hydrolysis. After passage over Sep-Pack C18 to eliminate salts, the acidic components were subjected to TLC and ELISA prior to and after desulphation treatment. AGSLs were further purified over a UNISIL column and analysed by TLC and UV-MALDI-TOF mass spectrometry. Although TLC can be used for qualitative analysis of complex sulphoglycosphingolipids such as sphingomyelin or ceramides (36), it does not offer sufficient structural specificity to guarantee homogeneity within a single spot of lipid using detection by iodine vapour. Therefore, the structures of AGSLs have been determined using UV-MALDI-TOF mass spectrometry, a modern and ultrasensitive technique.

Structural characterization

TLC analysis of AGSLs purified from epimastigotes of Trypanosoma cruzi

TLC analysis of the AGSLs using *n*-propanol/NH_3/water (75 : 5 : 5, v/v/v) as developing solvent showed a spot (*Tc*S) with lower mobility than the SM4s; the galactosylsulphatide used as control (Figure 1). To verify the fatty acid composition of this GSL, the fraction was subjected to methanolysis and analysed by RP-TLC. Spots co-migrating mainly with C18:0 in addition to minor amounts of C14:0 and C16:0 were detected (data not shown), in accordance with previous reports (37–39). To confirm the sulphoglycosphingolipidic nature, the sample was subjected to chemical solvolysis and the product was analysed by TLC. As expected, after treatment, a compound migrating faster than *Tc*S1, owing to the loss of the sulphate group, was obtained (Figure 1b). The desulphated product presents a double band characteristic of sphingolipids showing heterogeneity in the ceramide moiety.

Structural determination by UV-MALDI-TOF mass spectrometry analysis

To determine the chemical structure of the sulphoglycolipidic compound obtained, an additional purification step using Unisil column chromatography was performed. Mass spectrum analysis of the purified lipid performed in positive lineal mode (Figure 3), allowed the identification of sulphatides, presenting a glycosidic common portion linked to the ceramide moiety (Table 1). Interestingly, the glycosidic part is constituted by a sulphated disaccharide composed of hexuronic acid linked to a hexose unit. Although the hexose was not characterized, glucose or galactose is probably present as both of these sugars are known to be transferred to the ceramide moiety as the first step of the biosynthesis of complex glycosphingolipids (38–41). Glycolipids containing sulphated hexuronic acid are not common but have been identified in human dorsal root and sympathetic ganglia (42–44). In fact, a glucuronic acid containing sulphoglycosphingolipid has been previously detected in trypomastigote forms of *T. cruzi* (21).

Regarding the mass spectrum (Figure 2), at *m/z* 1055·7 (calc. *m/z* 1056·6) a signal consistent with a $NaSO_3$-HexA-Hex oligosaccharide linked to a d20:1-C18:0 ceramide as an $[M + Na]^+$ adduct is shown. Accordingly, a signal at *m/z* 1053·8 (calc *m/z* 1054·6) may be ascribed to a similar structure bearing an additional double bond in the fatty acid. The HSO_3-HexA-Hex backbone bearing the same ceramides as $[M + H]^+$ would account for signals at *m/z* 1013·2 (calc. *m/z* 1012·6) and 1011·2 (calc. *m/z*

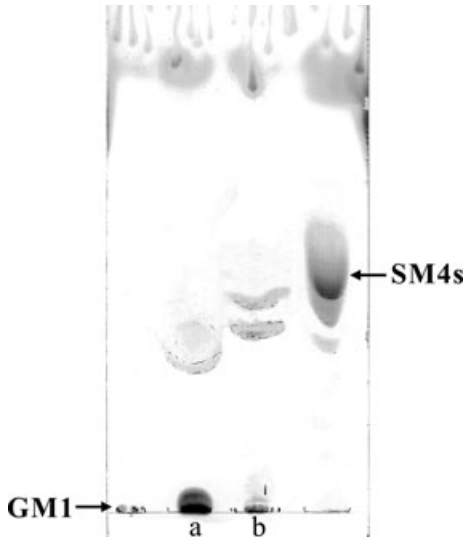

Figure 1 TLC analysis of purified acidic glycosphingolipids (AGSLs) of *Trypanosoma cruzi* prior to and after desulphation treatment. The acidic fraction obtained from the Sep-Pack C18 column was subjected to chemical desulphation treatment. AGSLs prior to (a) and after (b) desulphation treatment were loaded and run using propanol/NH_3/H_2O (75 : 5 :5, v/v/v) as mobile phase. A spot corresponding to acidic (TcS1) can be observed in lane (a). A higher mobility in lane (b) as consequence of the lower polarity owing to the loss of sulphate when compared with the sample without treatment is observed. The ganglioside GMI and the sulphatide SM4s were used as control ceramides.

Table 1 Proposed structures of the sphingoid-fatty acid combinations of ceramides present in the sulphoglycosphingolipid purified from epimastigote forms of *Trypanosoma cruzi* after MALDI-TOF mass spectrometry analysis in the positive ion mode (sugar backbone: HexA-Hex)

$Mass_{calc}$ $[M + NaSO_3 + Na]^+$	$Mass_{meas}$ $[M + NaSO_3 + Na]^+$	$Mass_{calc}$ $[M + HSO_3 + H]^+$	$Mass_{meas}$ $[M + HSO_3 + H]^+$	Proposed ceramide structure
1056·6	1055·4	1012·6	1013·2	d20:1-C18:0
		1014·6	1015·2	d20:0-C18:0
1054·6	1053·8	1010·6	1011·2	d20:1-C18:1
		1042·6	1042·9	d20:0-C20:0
		1040·6	1040·9	d20:1-C20:0
		986·6	987·4	d20:0-C16:0
				d18:0-C18:0

calc., calculated monoisotopic masses; meas., measured monoisotopic masses.

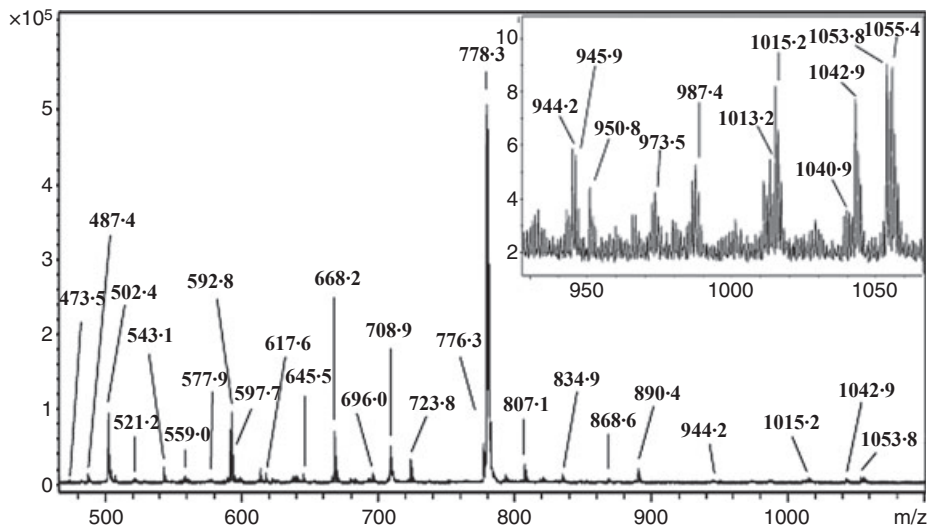

Figure 2 UV-MALDI-TOF mass spectrum of the purified acidic lipid obtained from epimastigote forms of *Trypanosoma cruzi* performed in the positive ion mode using gentisic acid (GA) as matrix.

1010·6). In addition, at *m/z* 1040·9 (calc. *m/z* 1040·6) and 1042·9 (calc. 1042·6), signals corresponding to the same backbone carrying a d20:0-C20:0 ceramide with and without a double bond as $[M + H]^+$ were detected. Similarly, signal at 987·4 (calc. *m/z* 986·6) corresponds to the analogous ion bearing a d20:0-C16:0 or d18:0-C18:0 ceramide. Loss of a $^{\cdot}SO_4H$ (Δ 97 mu) from *m/z* 1040·9 and 1042·9 would give rise to *m/z* 944·2 (calc. *m/z* 943·6) and 945·9 (calc. *m/z* 945·6). Analogously, peak at *m/z* 950·8 (calc. 951·8) may be ascribed to the loss of $^{\cdot}SO_3Na$ (Δ 102 mu) from *m/z* 1053·8, and 890·4 (calc. *m/z* 889·6) matches with the loss of SO_4H (Δ 97 mu) from *m/z* 986·6. Interestingly, the main peaks at *m/z* 778·3 (calc. *m/z* 777·6) and 776·3 (calc. 775·6) correspond to the loss of a sulphated hexuronic acid ($NaSO_3HexA$, Δ 279 mu) from *m/z* 1055·4 and 1053·8, respectively. Furthermore, *m/z* 807·1 corresponds to the analogous cleavage (HSO_3HexA, Δ 257 mu) from *m/z* 1042·9 as $[M + Na]^+$. Similarly, loss of the whole oligosaccharidic moiety from *m/z* 1055·4 would give rise to *m/z* 592·8 (calc. 593·5). On the other hand, cleavage of the oligosaccharidic moiety from *m/z* 987·4 with concomitant migration of the sulphate group to the ceramide moiety would be consistent with *m/z* 670·2 (calc. *m/z* 670·5) as sodium adduct (45). Similarly, species bearing an additional double bond would give rise to *m/z* 668·2 and 645·5 as sodium or proton adducts, respectively. Finally, the signal at *m/z* 617·6 (calc. *m/z* 617·4) would correspond to the loss of the oligosaccharide from d20:0-18:0 ceramide as sodium adduct. A scheme of one of the structures and its main cleavages is presented in Figure 3.

UV-MALDI-TOF mass spectrometry has been applied to the study of serum biomarkers of infectious diseases such as African trypanosomiasis, fascioliasis, cysticercosis and Chagas disease (46). Such studies have focused on

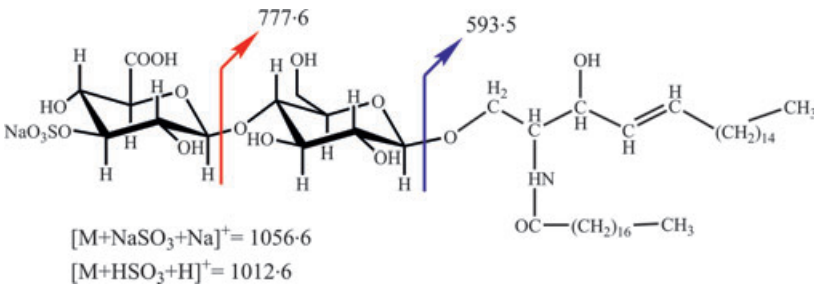

Figure 3 Scheme corresponding to the characterization of the glycosphingolipid structure by UV-MALDI-TOF. Mass spectrum analysis: the ceramide d20:1-18:0 taking into account the predicted cleavages in the text. Red: sodium adduct; Blue: Proton adduct.

identifying a distinctive configuration of circulating serum proteins that are indicative of a specific pathophysiological state, a so-called 'proteomic fingerprint' (47). Also, assays based on MALDI-TOF MS allowed the identification of *Plasmodium falciparum* dihydropteroate synthetase genes associated with pyrimethamine resistance, an antimalarial drug for treatment of malaria in several areas where this disease is endemic (48). On the other hand, novel glycoconjugates related to schistosomiasis were identified in urine of *Schistosoma mansoni*-infected individuals using a combination of glycopeptide separation techniques and in-depth mass spectrometric analysis (49), and in *Trichomonas vaginalis*, the ceramide phosphoinositol glycan core released by mild acid that defines molecular domains of the parasite surface glycol-conjugate lipophosphoglycan with distinct functions in the host immunoinflammatory response was determined by MALDI-TOF MS (50). Although GSLs have been identified and purified two decades ago (19–21), this work shows for the first time the structural characterization of sulphated GSLs present in epimastigote forms of *T. cruzi*. UV-MALDI-TOF mass spectrometry analysis allowed the identification of an acidic structure carrying a ceramide portion based mainly in d20:1-18:0 sharing a sulphate-containing glycosidic moiety formed by a hexuronic acid-hexose disaccharide. These results are in line with the presence of a glucuronic acid containing sulphatide in trypomastigote forms of *T. cruzi* (21). In this regard, some patients with demyelinating neuropathy possess immunoglobulin M proteins that react with carbohydrate determinants shared by myelin-associated glycoprotein (MAG) and two peripheral nerve acidic glycolipids, termed sulphoglucuronosylglycosphingolipids (SGGLs). Rabbit antisera generated against sulphoglucuronosylparagloboside, a major SGGL in peripheral nerves, have the same or similar antigenic specificity as those of the anti-MAG M proteins from patients with the mentioned neuropathy, suggesting that an autoimmune response against the sulphoglucuronosyl residue may participate in the immune pathogenesis of this type of neuropathy (51). Interestingly, common sulphoglucuronyl-glycolipid antigens are present at high concentrations in mammalian nervous system and in *T. cruzi* trypomastigotes (52).

Evidence of cross-reactivity between cruzipain and sulphatides: involvement of sulphated moieties in this cross-reactivity

There are several reports regarding the antigenicity of parasite GSLs (23,53,54) as well as the presence of antibodies specific for lipidic structures either in Chagas disease patients or in sera from *T. cruzi*-infected animals (52,55,56). In addition, more than two decades ago, the existence of cross-reactivity between antibodies specific for parasitic sulphatides and eukaryotic cells was reported (57,58). In this context, with the aim to study the possible immune cross-reactivity between Cz and AGSLs, and to evaluate the involvement of sulphate moieties in this cross-reactivity, we tested the immune recognition of acidic and NGSLs, obtained from *T. cruzi* epimastigote forms, by confronting them with rabbit polyclonal sera specific for Cz and C-T as well as with sera from chronic Chagas disease patients by dot blot assays. A strong recognition of AGSLs was observed with all the tested sera, demonstrating cross-reactivity between Cz and AGSLs. Although *T. cruzi* contains higher amounts of NGSLs in comparison with AGSLs (19), when GSLs were extracted from a similar quantity of parasites, sera specific for Cz and C-T showed a significantly higher recognition of *T. cruzi* AGSLs rather than neutral ones, indicating differential recognition depending on the presence of an anionic group in the GSL tested. Accordingly, significantly lower recognition of desulphated AGSLs in comparison with untreated controls was observed by dot assays. By contrast, whereas antibodies present in sera from chronic Chagas disease patients were able to recognize GSLs from *T. cruzi* by dot blots, no difference in recognition of either parasite acidic or NGSLs, either prior to or after desulphation treatment of AGSLs, was observed for any of the patients' sera tested (Figure 4).

Interestingly, in addition to the well-known involvement of sugar content in the immune recognition of GSLs (59–64), diglycosylated lipid structures were more antigenic than the monoglycosylated ones, indicating that ceramide glycosylation influences antigenicity in *T. cruzi* (39). On the other hand, *T. cruzi* has been shown to share common lipid sulphated antigens with the mammalian brain, suggesting that the sulphate group of the lipids might be an important part of the antigenic determinant (23,53). To confirm these results and further evaluate the involvement of sulphated moieties in the cross-reactivity between Cz and sulphatides from *T. cruzi*, purified AGSLs were desulphated by solvolysis and used prior to and after desulphation treatment as inhibitors of Cz recognition by polyclonal rabbit sera specific for Cz (Figure 5a) and C-T (Figure 5b) in ELISA inhibition assays. An increase in inhibition of Cz recognition was observed using increasing amounts of either neutral or acidic lipids. However, whereas ELISA inhibition assays showed that sulphatides prior to desulphation treatment were able to inhibit Cz recognition by rabbit sera specific for Cz (Figure 5a) and

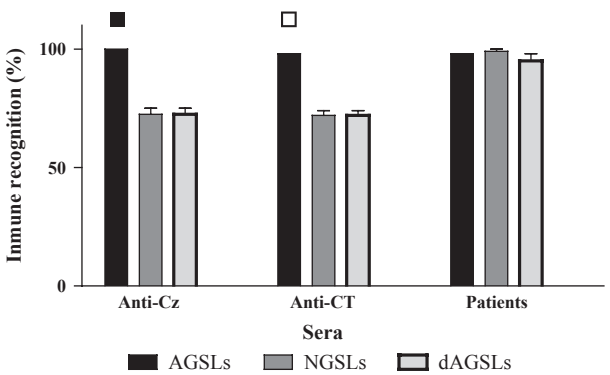

Figure 4 Immune recognition of GSLs purified from epimastigotes of *Trypanosoma cruzi* by polyclonal rabbit sera specific for Cz and C-T, and by chronic patient sera. Quantitative analysis of the immune recognition obtained with polyclonal rabbit sera specific for Cz, C-T or patient sera incubated with neutral or acidic GSLs, and desulphated acidic GSLs, was performed followed by second anti-rabbit or human total IgG coupled to HRP and developed with enhanced chemiluminescence reagent (Amersham Biosciences). Control spot corresponding to Cz recognition was used. PVDF membranes containing similar amounts of GSLs purified from 4×10^8 parasites and taken from TLC plates were probed with polyclonal rabbit sera specific for Cz and C-T as well as by sera from five chronic Chagas disease patients. Densitometric analysis of each dot blot spot was performed considering 100% to the highest recognition value obtained with each serum on the different purified lipidic structures tested. Quantitation was performed with a FujiLAS1000 densitometer equipped with IMAGE GAUGE 3.122 software (Fuji Film). Pair-wise comparison between groups was by one-way analysis of variance, followed by Tukey test in GRAPH PAD PRISM 5.0 software ■ $P < 0{\cdot}05$ vs. neutral GSLs (NGSLs) and dAGSLs; □ $P < 0{\cdot}05$ vs. NGSLs and dAGSLs. AGSLs, acidic glycosphingolipids.

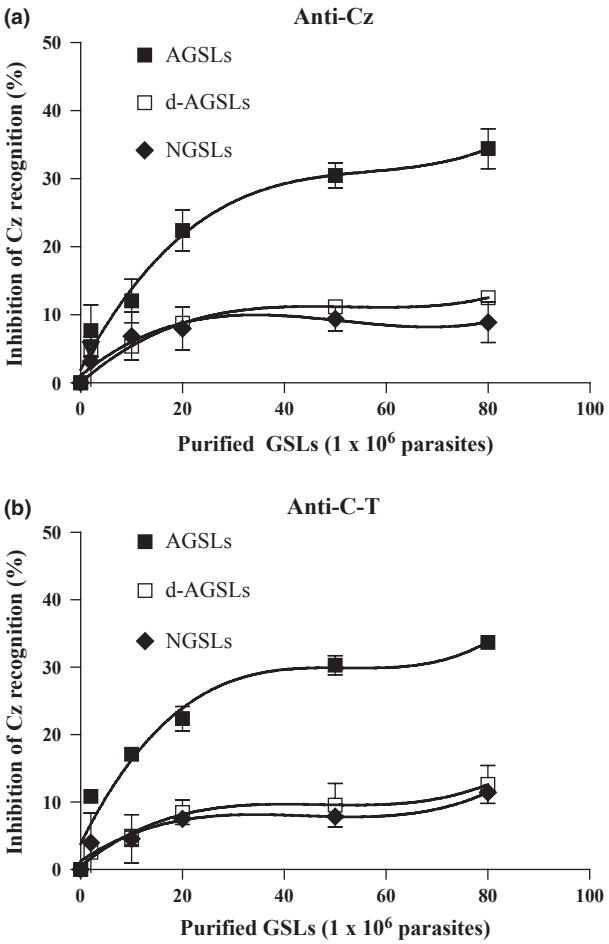

Figure 5 Analysis of the involvement of sulphated moieties from acidic glycosphingolipids (AGSLs) purified from *Trypanosoma cruzi* in the crossreactivity between Cz and sulphatides. Polyclonal rabbit sera specific for Cz and C-T (1/6400) were preadsorbed with increasing amounts of AGSLs prior to and after desulphation treatment, and ELISA inhibition using Cz as antigen was performed. Neutral lipids were used as controls. Total recognition without adsorption corresponds to 0% inhibition of Cz recognition.

C-T (Figure 5B) by about 30%, inhibition of Cz recognition was much lower (approximately 10%) with sulphate-depleted sulphatides. Thus, the sulphate group of the sulphatides is responsible for much of the competition for Cz recognition by both specific sera. Inhibition by sulphate-depleted sulphatides was not significantly different from that obtained with NGSLs used as natural nonsulphated purified controls. The 20% of reduction in the percentage of Cz recognition using sulphate-depleted AGSLs as inhibitor confirms the involvement of sulphate groups in the cross-reactivity observed between AGSLs and Cz (Figure 5a). In addition, the same decrease in inhibition of Cz recognition was obtained when sera specific for the C-T domain (Figure 5b) of Cz were

used, confirming that the common epitopes between sulphated GSLs and Cz are located in the C-T domain of this glycoprotein (27). In addition, the inhibition percentage of Cz recognition observed using sulphate-depleted AGSLs was similar to that obtained with NGSLs providing evidence for the existence of other antigenic determinants in the glycolipidic structures (in addition to the sulphate epitopes), suggesting participation of the sugar moiety in the cross-reactivity. It is worth noting that the acidic lipidic fraction is a mixture of glycosphingolipids. Therefore, other unidentified sulphatides may be also present.

Altogether, these findings provide strong evidence that serum specific for Cz and C-T contains antibodies that recognize common sulphate moieties from sulphatides, and epitopes present in the *N*-glycans from the C-T domain of Cz, demonstrating cross-reactivity between Cz and sulphatides. The fact that *T. cruzi* exposes a common anionic epitope in different glycoconjugates highlights the relevance of the sulphate group in parasite antigenicity.

We have previously demonstrated that sulphated oligosaccharides from Cz are involved in immune responses and that IgG2b antibodies are mainly involved in recognition of sulphate moieties. Taking into account that mouse IgG2b is capable of fixing complement or mediating antibody-dependent cell-mediated cytotoxicity reactions, we have previously suggested that the binding of IgG2b antibodies to specific tissues may be responsible for the damage found in mice immunized with sulphated C-T domain from Cz but not with the desulphated domain (28). Interestingly, IgG2b antisulphatide 'autoantibodies' in sera from rats experimentally infected with *T. cruzi*, which bind to homologous neural tissue, were described as the predominant isotypes of 'autoantibodies' specific for sulphatides, suggesting that specific binding of these rat 'autoantibodies' to sulphocerebroside on cell surfaces might play some detrimental role *in vivo* (54,58,65,66). In accordance with our findings, when cross-reacting lipidic antigens were isolated from *T. cruzi* and the mammalian brain with a specific monoclonal antibody (VESP 6.2), chemical reactions indicated that the sulphate group of the lipids was an important part of the epitope recognized by the monoclonal antibody (23).

Differential humoral immune recognition of sulphated moieties from AGSLs of *Trypanosoma cruzi* among patients with different degrees of Chagas disease

It is known that antibodies from Chagas disease chronic patient's sera are able to recognize sulphatides. We have previously demonstrated that sera from chronically *T. cruzi*-infected subjects with mild disease displayed higher levels of total IgG and IgG2 antibodies specific for Cz sulphated epitopes compared with those in more severe forms of the disease [Acosta *et al.,* (28)]. As expected, serum from healthy control individuals did not react with Cz (data not shown). Herein, we show that AGSLs and Cz share common sulphated epitopes. To determine whether sulphated structures shared by AGSLs and Cz are targets of the humoral immune response in the natural

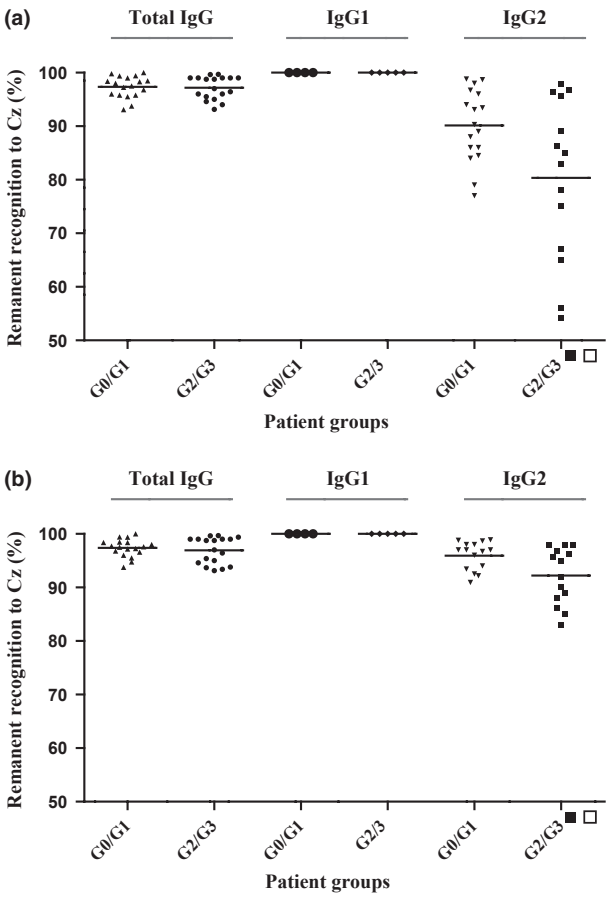

Figure 6 Humoral immune response to sulphated epitopes of acidic glycosphingolipids (AGSLs) purified from *Trypanosoma cruzi* measured in sera from patients with asymptomatic, mild or severe chronic Chagas disease. ELISA inhibition was performed using sera from patients with different degree of cardiac disease previously adsorbed with a fixed amount of sulphatides prior to and after desulphation treatment and Cz as antigen. Each dot represents the median percentage of remnant reactivity to Cz after inhibition using untreated (a) or desulphated (b) sulphatides. Each assay was performed in triplicate. Total IgG, IgG1 and IgG2 values of three experiments are shown. The value of A_{490nm} measured for each serum in the absence of inhibitor corresponds to 100% Cz recognition. The percentage of remnant IgG affinity to Cz for each serum was calculated as [OD_{490nm} with AGSL/OD_{490nm} without inhibitor] × 100 (a) or [OD_{490nm} with desulphated AGSLs/OD_{490nm} without inhibitor] × 100 (b), using untreated (a) or desulphated (b) AGSLs, respectively. ■ $P < 0{\cdot}05$ vs. IgG2 G0/1; □ $P < 0{\cdot}05$ vs. IgG total G0/1 and vs. IgG2 G0/1.

course of chronic Chagas disease, we performed ELISA inhibition assays to determine the capacity of complete or desulphated AGSLs to inhibit the immune recognition of Cz by sera from chronic Chagas disease patients. For this aim, sera from patients with asymptomatic or mild (G0 and G1 clinical groups) or severe (G2 and G3 groups) cardiac forms of Chagas disease were adsorbed with a fixed amount of sulphatides prior to or after desulphation treatment and confronted with Cz. The percentage of remnant recognition to Cz by total IgG, IgG1 and IgG2 isotypes was calculated for each patient sera. Immune recognition to Cz was inhibited when sera were adsorbed with AGSLs (Figure 6a) and this inhibitory effect was considerably reverted after desulphation treatment (Figure 6b) showing that sulphate groups are largely responsible for the cross-reactivity between Cz and sulphatides. The percentage of remnant IgG2 reactivity to Cz after adsorption with either AGSLs or desulphated AGSLs was higher in asymptomatic/mild Chagas disease (G0/G1) patients than in patients with severe disease (G2/G3), indicating that the majority of G0/G1 patients displayed higher antibodies levels specific for sulphate moieties on *T. cruzi* sulphatides compared with those with advanced cardiomyopathy. No differences were observed in total IgG or IgG1 antibody levels specific for sulphated AGSLs (Figure 6). These results confirm that IgG2 levels specific for sulphated moieties inversely correlate with the severity of chronic Chagas disease suggesting that anti-sulphate antibodies may prevent disease pathology. In support of this suggestion, transfer of IgG2a and IgG2b antibodies protects mice from lethal infection with *T. cruzi* parasites (67).

Our observation that Chagas disease patients with severe disease possess lower levels of IgG2 antibodies to sulphate groups compared with patients with less severe disease, in line with previous results obtained with sulphates from Cz (28), suggests a potential role for these antibodies in the control of chronic Chagas disease. Similarly, high IgG2 levels were also associated to an efficient protective immunity during chronic HIV infection (68) and human *P. falciparum* infection (69).

On the other hand, previous data from Avila *et al.,* (52) showed that levels of specific anti-*T. cruzi* as well as anti-sulphatide antibodies decrease with increasing severity of disease. In our study, total anti-*T. cruzi* titres did not differ between disease severity groups (data not shown) suggesting that specific anti-*T. cruzi* antibodies are not associated with progression of chronic Chagas disease. Moreover, specific anti-*T. cruzi* antibody titres remain stable over the evolution of the disease (70) indicating that these antibodies are not involved in the pathogenesis of the disease.

In conclusion, this report shows the determination by UV-MALDI-TOF mass spectrometry analysis of a sulphoglucuronyl-containing sphingolipid of *T. cruzi* mainly composed of d20:1-C18:0 ceramide. Interestingly, although parasite persistence is currently considered as a predominant factor in the pathogenesis of the disease, we provide strong evidence that sulphate is a common epitope between the C-T domain of Cz and sulphatides of *T. cruzi*, and that other sugar epitopes in the glycolipidic structures participate in this cross-reactivity. In addition, we have shown that higher levels of IgG2 antibodies specific for sulphate epitope from sulphatides are associated with less severe disease in chronic *T. cruzi* patients. In summary, we provide evidence that *T. cruzi* sulphate moieties linked to sugars are antigenic and that IgG2 antibodies specific for these epitopes might be considered as markers of progression of chronic Chagas disease.

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