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Note

Metabolic labelling and partial characterisation of a sulfoglycolipid in *Trypanosoma cruzi* trypomastigotes

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Trypanosoma cruzi, a parasitic protozoan, is responsible for American trypanosomiasis (Chagas' disease). The parasite has a complex life cycle[1]. The infective trypomastigote circulates in the blood of vertebrate hosts, penetrates the host cells, and transforms into the dividing amastigote form. When trypomastigotes are ingested by the insect vector, the parasite replicates in the midgut as a non-infective epimastigote but is transformed in the hindgut into metacyclic trypomastigotes which are deposited in the insect faeces and can invade the mammalian host.

The chronic phase of Chagas' disease is associated with autoimmune mechanisms[2]. A monoclonal antibody which cross reacts with sulfated lipids from epimastigotes and antigens of mammalian brain has been described[3] and a positive reaction for sulfate has been obtained for the acidic lipid fraction[4]. Many functions have been suggested for sulfated glycolipids[5,6] and their involvement in cell adhesion has been studied recently[7–9]. We now report on the structure of acidic glycolipids previously detected[10] in cell-culture trypomastigotes.

The trypomastigote form was labelled metabolically with [^{3}H]palmitic acid, then extracted with chloroform–methanol, and the acidic lipids were isolated by chromatography on DEAE-Sephadex (AcO^- form). Further purification on Unisil revealed only one major component (STc) that was homogeneous by two-dimen-

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sional HPTLC and fluorography (data not shown). The mobility of STc in TLC (R_F 0.5, solvent *A*) was similar to those of analogous fractions obtained by labelling with $Na_2{}^{35}SO_4$ and D-[^{14}C]glucose and to that of galactosylceramide 3-sulfate and suggested a sulfoglycolipid structure for STc. When whole parasites were labelled by the galactose oxidase–NaB^3H_4 method, STc was the main radioactive acid glycolipid detected which indicated its location on the surface.

Methanolysis of the lipid moiety of STc labelled with [^{3}H]palmitic acid gave (TLC) mainly methyl palmitate (R_F 0.94, solvent *B*) and a trace of sphingosine (R_F 0.49), the identity of which was confirmed when the chromatogram was monitored for radioactivity (Fig. 1a). As expected, palmitic acid is more easily incorporated into the fatty acid of the ceramide than into the long-chain base. Reverse-phase HPLC of the fatty acid methyl esters showed the main component to be methyl palmitate together with some methyl stearate (Fig. 1b). The same result was obtained by reverse-phase HPTLC (Fig. 1c).

Desulfation of STc with methanolic hydrogen chloride and saponification gave a product, the R_F value of which in HPTLC was only slightly lower (0.44, solvent *A*) than that of STc. A minor component (R_F 0.63), which was not further investigated, was also detected. The same glycolipid was obtained by solvolysis with methyl sulfoxide–methanol containing 9 mM sulfuric acid. There are examples in the literature[11–12] where the mobility of a desulfated glycolipid is only slightly different from that of the parent.

Hydrolysis of STc, labelled by the galactose oxidase–NaB^3H_4 method, with 2 M hydrochloric acid gave (PC) 2-acetamido-2-deoxygalactose and galactose (Fig. 2a). When STc obtained from non-labelled parasites was hydrolysed under similar conditions and the neutral sugars were analysed by GLC as the alditol acetates, derivatives of glucose and galactose in the ratio 1 : 1 were detected.

When STc labelled with [^{3}H]palmitic acid was hydrolysed with 4 M hydrochloric acid and the products were reduced with NaB^3H_4, paper electrophoresis gave the result shown in Fig. 2b. The major peak (B) was neutral, peak A corresponded to 2-amino-2-deoxygalactitol, and peak D to gulonic acid. When the component in peak D was desalted and lactonised, PC revealed gulonolactone. When the component in peak C was treated with β-glucuronidase, paper electrophoresis showed that all the radioactivity was in the neutral product, which suggested that the parent was an acid-resistant oligosaccharide.

The above data indicate STc to be a sulfoglycosphingolipid and this is the first report of its presence in the infective form of *Trypanosoma cruzi*. Furthermore, 2-acetamido-2-deoxy-D-galactose and D-glucuronic acid have not been described hitherto as components of glycoconjugates from *Trypanosoma cruzi*. A recent report[13] on the localisation of sulfated glucuronyl glycolipids in human dorsal root and sympathetic ganglia suggests that STc may be a putative antigen involved in the autoimmunity processes[14] in Chagas' disease. Acidic glycosphingolipids are shed by trypomastigotes into the culture medium[15].

EXPERIMENTAL

General methods.—Radioactivity was determined in a 1214 RackBeta Wallack liquid scintillation counter, using Optiphase' Hi Safe 3 scintillation cocktail (LKB) for aqueous and non-aqueous samples. For silica-gel scrapings, Cab-O-Sil (Packard) was added as a thixotropic agent.

For fluorography, TLC plates were coated with 2-methylnaphthalene and 2,5-diphenyloxazole[16], and were exposed to Kodak X-Omat AR films.

Parasites.—Cell-culture trypomastigotes were obtained from infected LLC-MK_2 epithelial cell monolayers maintained in Dulbecco's modified Eagle's medium (DME) containing 2% of foetal calf serum (FCS)[17]. Parasites were washed three times with medium 199 at 800*g* for 10 min.

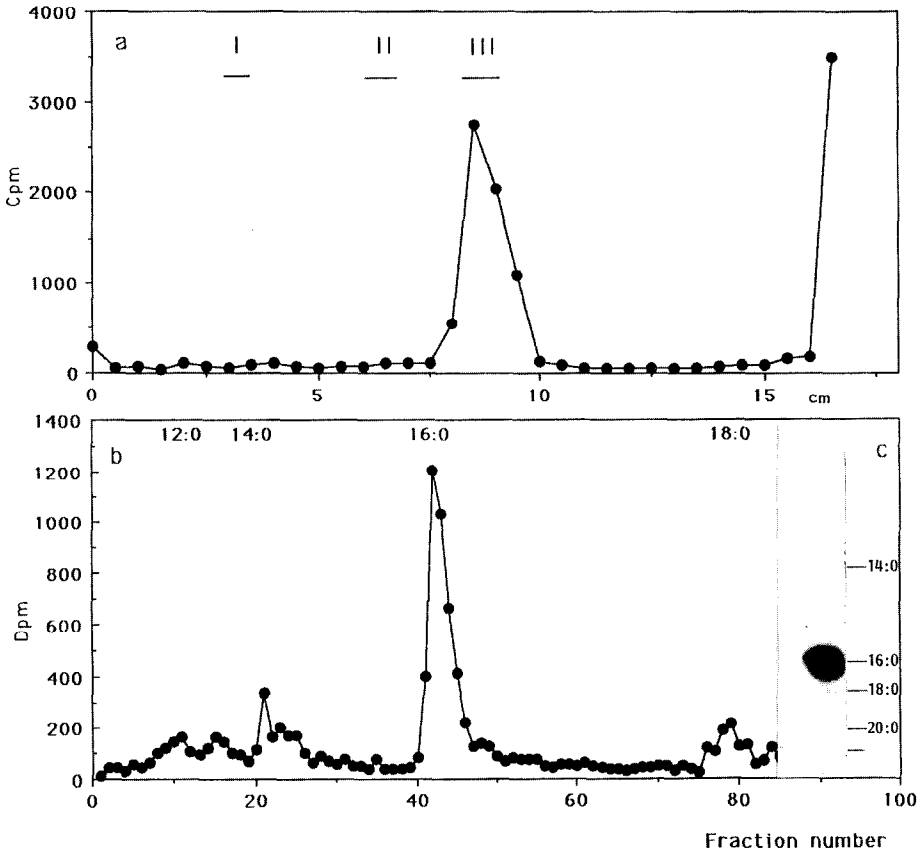

Fig. 1. (a) TLC of the long-chain base component of STc in solvent *B* (double development): I, 4-OH sphinganine (phytosphingosine); II, sphinganine (dihydrosphingosine); III, 4-sphingenine (sphingosine); (b) reverse-phase HPLC of the fatty acid methyl ester components of STc (see Experimental); (c) reverse-phase HPTLC (solvent *D*) of the fatty acid methyl ester components of STc.

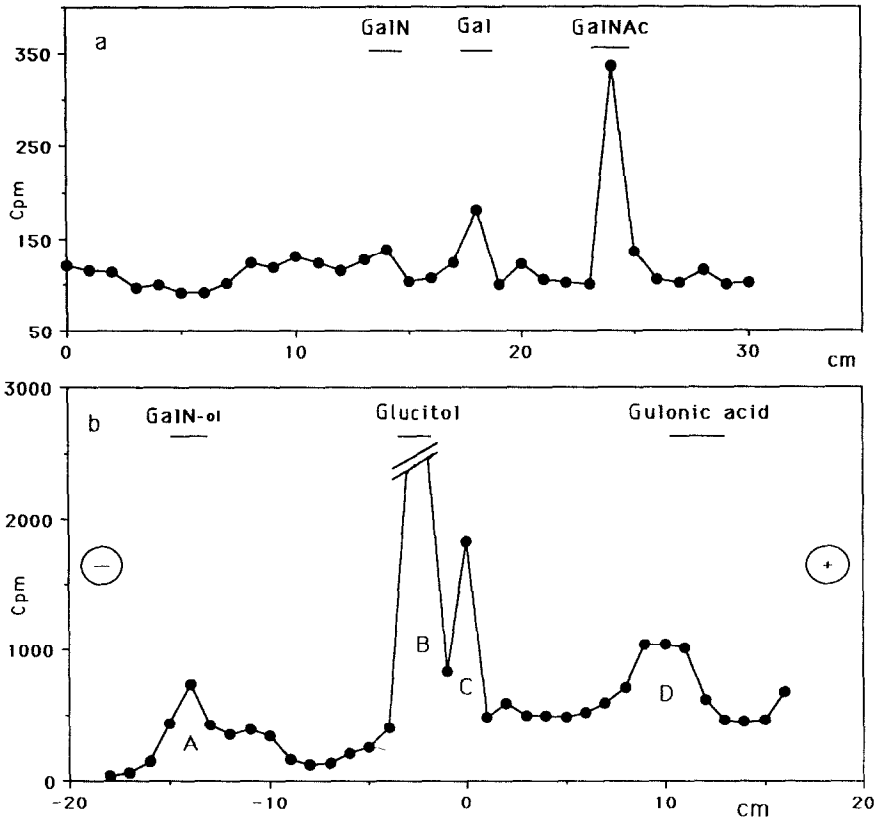

Fig. 2. (a) PC (solvent *A*) of the sugar components obtained from STc labelled by the galactose oxidase–NaB^3H_4 method: GalN, 2-amino-2-deoxy-D-galactose; Gal, D-galactose; GalNAc, 2-acetamido-2-deoxy-D-galactose. (b) PE of the sugar components of STc obtained by acid hydrolysis and reduction with NaB^3H_4.

Labelling of the parasites.—Parasites (1×10^9) were labelled metabolically with [9,10(n)-^{3}H]palmitic acid (Amersham, 500 mCi/mmol, 1.95 mCi/mg, 5 mCi/mL) or with D-[U-^{14}C]glucose (230 mCi/mmol, 50 mCi/mL) as reported[10,18]. Surface labelling was performed by the galactose oxidase–NaB^3H_4 method[10].

Parasites (2×10^8) were labelled metabolically by incubation with $Na_2{}^{35}SO_4$ (500 μCi) in DME containing 2% of FCS for 5 h at 37°.

After incubation, the cells were washed three times with medium 199, and the final pellet was frozen.

Isolation and purification of the sulfoglycolipid (STc).—The labelled cells were extracted with 2:1 (2×1 mL) and 1:1 (2×1 mL) $CHCl_3$–MeOH. The product in the combined extracts was fractionated on a column of DEAE-Sephadex (AcO^- form)[19]. Neutral lipids were eluted with MeOH–$CHCl_3$–H_2O (60:30:8, 50 mL), and the acidic lipids with MeOH–$CHCl_3$–0.8 M NaOAc (60:30:8, 50 mL). The latter fraction was dialysed, then lyophilised, and a solution of the residue in

$CHCl_3$–MeOH (8:2) was added to a column of Unisil (200–325 mesh, Clarkson Chem. Co.) and eluted with 8:2 (50 mL) and then 1:1 (50 mL) $CHCl_3$–MeOH.

Analytical methods.—TLC and HPTLC were performed on Silica Gel 60 (Merck), using *A*, 1-propanol–aq 28% NH_4OH–H_2O (75:5:5); *B*, $CHCl_3$–MeOH–2.5 M NH_4OH (40:10:1); *C*, hexane–ether (95:5); and *D*, acetonitrile–acetic acid (1:1). Galactosylsulfatide (Supelco) and GM_1 (Sigma) were used as standards.

The silica gel in the areas of each detected glycolipid was removed, washed twice with hexane in order to remove the scintillation mixture, and extracted with $CHCl_3$–MeOH–H_2O (5:5:1, 1 mL). The extract was concentrated and the residue was subjected to enzymic or chemical hydrolysis.

PC was performed on Whatman No. 1 paper, using *A*, 1-butanol–pyridine–H_2O (6:4:3); and *B*, EtOAc–acetic acid–formic acid–H_2O (18:3:1:4).

PE was performed on Whatman No. 1 paper, using 0.1 M pyridine acetate buffer at 1700 V for 90 min.

GLC was conducted with a Hewlett–Packard 5840 A gas chromatograph equipped with a capillary column (15 m × 0.25 mm) of SP-2330 (Supelco) with the following temperatures: column 200°, injector 250°, detector 250°. The carrier gas was N_2 at 2 mL/min. Neutral sugars were analysed as the alditol acetates.

Acid methanolysis.—STc was hydrolysed[20] for 18 h at 70–78° with 12 M HCl–MeOH–H_2O (3:29:4). The hydrolysate was made alkaline (pH 9.0), then extracted with ether. The extract was dried and concentrated, and a solution of the residue in $CHCl_3$ was subjected to chromatography on a column of Unisil. Fatty acid methyl esters were eluted with $CHCl_3$ and the long-chain bases with 1:1 $CHCl_3$–MeOH. The bases were analysed by TLC (solvent *B*).

Determination of the fatty acids.—Silica Gel 60 plates (Merck) were precoated with saturated methanolic $AgNO_3$, activated for 15 min at 100°, and developed[21] in solvent *C*.

Fatty acid methyl esters were analysed by reverse-phase HPTLC on RP-18 F_{254} (Merck), using solvent *D*[22], or by HPLC (Micromeritics equipment with a refractive index detector) using a column (250 × 4.6 mm) of LiChrosorb RP-18 (10 μm) and MeOH–H_2O (9:1) as the mobile phase at 1 mL/min. The sample was co-injected with a standard mixture containing $C_{12:0}$–$C_{18:0}$ fatty acid methyl esters. Fractions (0.5 mL) were counted for radioactivity.

Acid hydrolysis.—STc was hydrolysed with (*a*) 2 M HCl for 3 h at 100° and (*b*) 4 M HCl for 4 h at 100°. Each hydrolysate was repeatedly co-concentrated with water and then lyophilised.

Desulfation.—The following conditions were used. (*a*) Anhydrous 0.05 M HCl in MeOH[23] at room temperature for 4 h; the solution was repeatedly co-concentrated with water and the glycolipid was dissolved in 1:1 $CHCl_3$–MeOH. (*b*) 9 mM H_2SO_4 in 9:1 Me_2SO–MeOH at 80° for 3 h[24]; after neutralisation with dilute ammonia, the mixture was concentrated to dryness and the residue was dissolved in 1:1 $CHCl_3$–MeOH.

Hydrolysis with β-glucuronidase.—The oligosaccharide was dissolved in 0.1 M NaOAc buffer, pH 5.0 (1 mL), β-glucuronidase (Limpet, type L-II, Sigma, 500 U) was added, and the mixture was digested at 37° for 20 h.

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