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Trypanosoma cruzi: metabolic labeling of trypomastigote sialoglycolipids[1]

A.S. Couto, B. Zingales*, R.M. de Lederkremer and W. Colli*

*Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires (Argentina), and *Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, C.P. 20780, São Paulo (Brazil),*
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Summary. Trypomastigote forms (infective) of *Trypanosoma cruzi* incorporate (^{3}H)-palmitic acid and D-(^{3}H)-galactose into glycolipids. Palmitic acid-labeled acidic glycolipids were partially hydrolyzed with neuraminidase. The labeling of these compounds when the intact cell surface was labeled with galactose oxidase plus NaB^3H_4 indicates the membrane location of the sialoglycolipids.
Key words. Trypanosoma cruzi; trypomastigote forms; sialoglycolipids; neuraminidase; galactose incorporation.

Trypanosoma cruzi is the etiological agent of Chagas' disease, a chronic condition affecting approximately 20 million people in the New World. The biological cycle of the parasite is rather complex, involving at least three well-defined differentiation stages[2]. The trypomastigote stage, found in the feces of the insect vector and in the bloodstream of the vertebrate host, is particularly important since it is capable of invading vertebrate cells. The other stages are the epimastigote, found in the midgut of the insect vector and the amastigote, a dividing form encountered inside vertebrate cells. The first indications that sialic acid might be present on the surface of *T. cruzi* came from observations of the binding of cationized ferritin, and from measurements of cellular electrophoretic mobility[3]. Further work on parasite agglutination by wheat germ and *Limulus polyphemus* agglutinins reinforced this conclusion[4]. Treatment of *T. cruzi* with neuraminidase abolished the lectin-induced agglutination[4], reduced the negative surface charge[5], and stimulated the uptake of the parasites by macrophages[6]. Sialidase-sensitive molecules have also been implicated in the escape of trypomastigotes from recognition by the alternative pathway of complement, a mechanism that readily lyses epimastigote forms[7]. No data, however, were available on the nature of the sialoglycoconjugates.

Recently, sialoglycolipids have been characterized in the epimastigote forms of *T. cruzi*. The ganglioside nature of these compounds was demonstrated by the incorporation of palmitic acid into sphinganine and sphingenine in sialoglycolipids which were partially hydrolyzed by neuraminidase[9]. In the present report the existence of sialoglycolipids in the trypomastigote stage of *T. cruzi* is described. These compounds have been characterized by the incorporation of ^{3}H-palmitic acid and ^{3}H-galactose in neuraminidase-sensitive and insensitive molecules.

Materials and methods. Trypomastigotes were obtained from infected LLC-MK_2 epithelial cell monolayers maintained in Dulbecco's modified Eagle's medium (DME) containing 2% fetal calf serum (FCS)[10]. Parasites were washed once with the same medium before experimental use. Epimastigotes were cultivated as previously described[11]. Parasites (total, 4.5×10^8) were resuspended at a density of 50×10^6 cells/ml in DME containing 100 µg/ml of D-glucose, 3 µg/ml of D-galactose and 5% FCS. The cell suspension was maintained at 37°C for 30 min in a humidified atmosphere containing 5% CO_2 followed by the addition of 20 µCi/ml of D-(1-^{3}H) galactose (Amersham, 10.4 Ci/mmole; 1 mCi/ml), and incorporation proceeded for 6 h. Palmitic acid (9,10(n)-^{3}H), originally obtained in toluene (40 µl) from Amersham (500 mCi/mmole; 5 mCi/ml) was dried and resuspended in 40 µl of chloroform/ethanol (1:1, v/v). A suspension of 4.5×10^8 trypomastigotes (50×10^6 cells/ml in DME-5% FCS) was added to the radio-

active fatty acid (20 μCi/ml, final) and incubation proceeded for 7.5 h at 37 °C. After incubation the cells were washed three times with medium 199 and the final pellets were frozen. Parasites were also labeled on their surface by a slight adaptation of a method previously described[11]. The parasites (4.5×10^8) were resuspended in 0.5 ml PBS containing 14 units of galactose oxidase (Sigma, type V) and incubated at 30 °C for 40 min, followed by the addition of 500 μCi of NaB^3H_4 and incubation at room temperature for an additional 10 min. Cold $NaBH_4$ (70 μg/ml) in 10 ml of medium 199 was then added and after 30 min the cells were washed twice with 10 ml of the same medium.

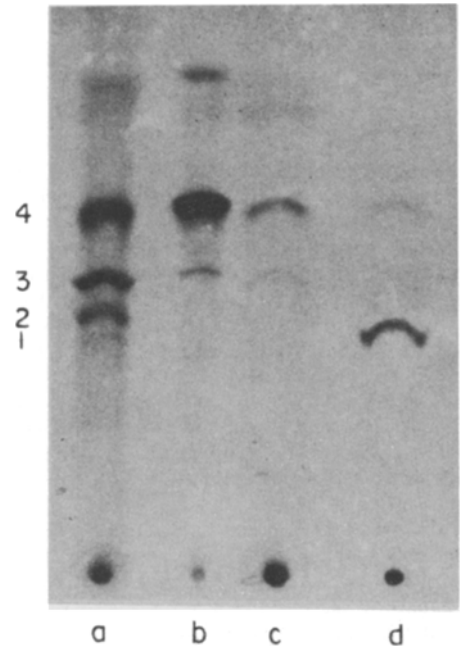
The frozen pellets were resuspended in water, subjected to five cycles of freezing and thawing and lyophilized. The dried material was twice extracted with chloroform/methanol (2:1, v/v). The combined extracts were fractionated on a DEAE-Sephadex acetate column by the method recommended by Ledeen et al.[12] for the purification of gangliosides. Neutral lipids were eluted with 30 ml of methanol/chloroform/water (60:30:8, v/v/v) after which acidic lipids were obtained with 20 ml of methanol/chloroform/0.8 M sodium acetate (60:30:8, v/v/v). The latter fraction was dialyzed, lyophilized and the residue was solubilized in chloroform/methanol (1:1, v/v) and applied onto a column of silicic acid (Carlo Erba). Elution was performed with 20 ml of chloroform/methanol (8:2, v/v) followed by 20 ml of chloroform/methanol (1:1, v/v).

TLC was performed on 0.25 mm silica gel 60 precoated plates (Merck) with n-propanol/28% NH_4OH/water (75:5:5, v/v/v). For fluorography the plate was coated with 2-methyl naphthalene and 2,5-diphenyloxazole[13], and exposed to Kodak X-OMAT AR film. The silica gel scrapings corresponding to the detected glycolipids were twice extracted with 1 ml of chloroform/methanol/water (5:5:1, v/v/v). The supernatants were dried and subjected to neuraminidase treatment. Sialoglycolipids were treated with two additions of 0.1 units of *C. perfringens* neuraminidase (Sigma, type IX) over a period of 48 h, in 200 μl of 50 mM acetate buffer, pH 5.2, at 37 °C[14].

Fractionation of labeled glycosphingolipids from *Trypanosoma cruzi* trypomastigotes*

Fractions	Counts × min^{-1} × 10^{-4}		
	(^{3}H)-Palmitic acid	D-(^{3}H)-Galactose	Galactose oxidase + NaB^3H_4
Chloroform/methanol (1:1, v/v) extract	994	29.2	16.0
DEAE-Sephadex column chromatography			
Neutral lipids	376	16.8	9.0
Acid lipids	293	3.3	2.0
Silicic acid column chromatography			
Chloroform/methanol (8:2, v/v)	146	1.6	1.0
Chloroform/methanol (1:1, v/v)	22	0.6	0.9

* The values correspond to the total incorporation in 4.5×10^8 trypomastigotes.



TLC of *T. cruzi* glycolipid fractions. The chloroform/methanol (1:1, v/v) eluates from the silicic acid columns were chromatographed on a silica gel plate with propanol/ammonia/water (75:5:5, v/v/v) as developing solvent; *a* palmitic acid labeled glycolipids from epimastigotes; *b* palmitic acid labeled glycolipids from trypomastigotes; *c* NaB^3H_4/galactose oxidase labeled glycolipids from trypomastigotes; *d* galactose labeled glycolipids from trypomastigotes.

Results and discussion. Maximal incorporation of (^{3}H)-galactose in trypomastigotes was achieved after 4–5 h incubation (1.04×10^4 cpm/10^7 cells) while (^{3}H)-palmitic acid incorporation was much higher, reaching maximum levels after 7.5 h (22×10^4 cpm/10^7 cells). The chloroform/methanol extracts of trypomastigotes labeled with the two isotopes, as well as those from parasites which had been labeled at the cell surface with galactose oxidase – NaB^3H_4, were fractionated as recommended for the isolation of gangliosides[12] (table). Galactose was preferentially incorporated into neutral glycolipids, compounds that have not been investigated, as yet, in *T. cruzi* trypomastigotes. TLC of the fraction eluted with chloroform/methanol (1:1, v/v) from the silicic acid column showed different incorporation patterns of palmitic acid when epimastigote (fig., a) and trypomastigote (fig., b) forms were compared. Bands 3 and 4 (fig., c) appeared labeled when intact trypomastigotes were labeled at the surface with NaB^3H_4 after galactose oxidase treatment of intact cells, suggesting that at least these two glycolipids are membrane-located. Incorporation of (^{3}H)-galactose (fig., d) was more significant in a compound (band 1) which did not label with palmitic acid in trypomastigotes. We can only infer that band 1 might be an acidic glycolipid but further studies are needed to establish its structural nature. Bands 3 and 4 in the figure b, were eluted from the plate, treated with neuraminidase and rechromatographed. The chromatographic migration of band 3 was noticeably altered and band 4 was partially hydrolyzed with the appearance of a new spot, as measured by the increase in Rf (not shown). This result indicates the presence of sialic acids in the isolated glycolipids. The molecular structures of these sialoglycolipids are at present under study.

Sialoglycolipids have been implicated in ligand-receptor recognition in other cell systems[15]. Further studies on the sialoglycolipids of *T. cruzi* may elucidate whether they participate in biological phenomena involving sialic acids[4–7]. Also, the endogenous labeling of these macromolecules should facilitate studies on their function and help to follow the effect of different factors on their biosynthesis. In work in progress sialic acid has also been detected in a surface membrane glycoprotein isolated from trypomastigotes.

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Effect of long acting somatostatin-analogue, SMS 201 995, on gut hormone secretion in normal subjects

M.E. Kraenzlin, S.M. Wood, M. Neufeld, T.E. Adrian and S.R. Bloom

Department of Medicine, Hammersmith Hospital, London (England), and Clinical Research Division, Experimental Therapeutics Department, Sandoz Ltd, CH-4002 Basel (Switzerland), 30 July 1984

Summary. SMS 201 995 is a new long acting analogue of somatostatin. We have investigated its effect on basal and meal stimulated secretion of gut hormones and have shown that after a single s.c. injection of 50 µg it lowers significantly the basal plasma levels of pancreatic polypeptide, secretin, motilin, pancreatic glucagon and insulin, it also effectively suppresses the postprandial release of pancreatic polypeptide, gastrin, secretin, gastric inhibitory peptide, pancreatic glucagon and insulin. Except for the usual brief discomfort of an injection, no symptoms or untoward effects were observed.
Key words. Somatostatin analogue; gut hormones.

Somatostatin (SRIF), a tetradecapeptide initially isolated from the hypothalamus because of its inhibition of growth hormone secretion[1] has subsequently been shown to be present in the D cells throughout the gastrointestinal tract and pancreatic islets[2]. It has widespread actions including the suppression of growth hormone and TSH release from the pituitary[1,3], and of most hormones from the gut and pancreas[2]. It also potently inhibits gastric acid and pepsin secretion, pancreatic exocrine secretion, gut motility and nutrient absorption by mechanisms independent of its inhibition of hormone secretion[2,4].

Many somatostatin analogues have now been synthesized, and several of these have been reported to be more potent, and to have a different spectrum of action to natural somatostatin[5-7]. In the present study we have investigated the actions of a new somatostatin analogue

D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-NH-CH(CH_2OH)CHOHCH_3

(SMS 201 995 Sandoz), a long acting, cystine bridge octapeptide analogue of somatostatin. In vitro and in vivo studies have shown it to be more potent than the native hormone[8]. The present study evaluated the effect of a single s.c. injection of SMS 201 995 on test meal stimulated gut hormone secretion in healthy subjects.

Subjects and methods. Five healthy male subjects (aged 21–27 years) were studied. The test protocol was approved by the Royal Postgraduate Medical School Ethical Committee and informed consent was obtained from all subjects. Following an overnight fast the subjects were given a s.c. injection of SMS 201 995 (50 µg) or placebo in random order. At time 0, 30 min after injection, a standard breakfast was given (60 g white bread, 35 g jam, 10 g butter, 150 ml unsweetened orange juice, 2 eggs; protein 20 g, fat 22 g, carbohydrate 67 g, 530 kcal). Blood samples were taken at −60, −30, −15, −5, 0, +15, +45, and +90 min into heparinized tubes containing 400 KIU aprotinin (Trasylol) per ml blood, centrifuged and the plasma deep frozen within 15 min of sampling. Pulse and blood pressure were measured at the same time intervals. Insulin, pancreatic polypeptide (PP), gastrin, gastric inhibitory polypeptide (GIP), pancreatic glucagon, motilin, cholecystokinin (CCK8), secretin and SMS 201 995 plasma levels were measured using previously described radioimmunoassay[8,9]. The glucose was measured using a standard glucose oxidase method adapted for autoanalyzer. Results are expressed as mean ± SEM. Statistical analysis was carried out using the paired Student t-test. p-value of less than 0.05 was regarded as significant.

Results. The mean peak plasma concentration of SMS 201 995 (2.7 ± 0.12 ng/ml) was reached 15–30 min after its s.c. injection, and then fell slowly over the subsequent 90 min to a value of 1.1 ± 0.1 ng/ml (fig., A).

The effect of SMS 201 995 on gut hormone secretion is shown in the panels of the figure. 15 min after injection there was a significant difference between basal levels of PP for placebo and SMS 201 995 respectively (35.6 ± 5.2 pmoles/l and 10.2 ± 3 pmoles/l, $p < 0.01$). The postprandial release of PP was completely suppressed throughout by SMS 201 995; on placebo the peak concentration 15 min after test meal was 145.2 ± 14.2 pmoles/l, on SMS 201 995 it remained in the same range as the basal values (13 ± 1.5 pmoles/l, $p < 0.005$).

The basal gastrin levels did not differ significantly between SMS and placebo. The postprandial peak on placebo was 44.9 ± 18 pmoles/l, this was significantly suppressed by SMS 201 995 (9.4 ± 5.8 pmoles/l, $p < 0.05$) which was followed by a slight rise (15.3 ± 7.6 pmoles/l) at 45 min.

The postprandial secretin response was significantly suppressed (0.9 ± 0.3 pmoles/l compared with 2.0 ± 0.3 pmoles/l at 45 min, $p < 0.05$). Basal GIP levels were significantly lower 30 min after SMS 201 995 than on placebo (4.0 ± 1.9 pmoles/l v. 9.8 ± 2.3 pmoles/l, $p < 0.05$), and postprandial release of GIP was significantly suppressed throughout. SMS significantly lowered basal motilin levels (27 ± 14 v. 64 ± 12 pmoles/l, $p < 0.01$); there was no postprandial rise in motilin. SMS 201 995 suppressed basal secretion of pancreatic glucagon (1.8 ± 0.6 v. 14.8 ± 3 pmoles/l, $p < 0.01$; 30 min after injection) and inhibited its postprandial release ($p < 0.01$). Basal insulin was markedly suppressed by SMS 201 995 (5.0 ± 0.3 v. 57.6 ± 13 pmoles, 30 min