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Trypanosoma cruzi: Altered parasites after in vitro treatment with gangliosides, a therapeutic agent in experimental Chagas' disease

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ABSTRACT

Biochemical and structural modifications were investigated in axenic cultured *Trypanosoma cruzi* after treatment with gangliosides. Fluorescence anisotropy showed dose dependent increments in parasite membranes of ganglioside treated epimastigotes. NADP-GDH activity increased in parasites treated at day 4 (13%), 7 (137.2%), and 14 (28.50%) while NAD-MDH but decreased from day 7 to 21 (−5.74%, −32.22%, −27.92%). Treated parasites presented electron-lucent vacuoles opposite to the cytostoma, multilamellar bodies and dilated mitochondrion cristae, disorganized kinetoplast and altered heterochromatin structure. Gangliosides inhibited fusogenic ability (80%) and PLA2 activity (>75%) from the parasite. The same occurred with anti-PLA2 antibodies. Trypomastigotes suffered loss of cytoplasmic material and organelles when GM1 was present in culture medium. We propose that exogenous gangliosides produced: altered lipid order, inhibited membrane enzymes, the parasite energy source shifted from glucose to amino acids, ending on a structural transformation which signals parasite cell death.

1. Introduction

Trypanosoma cruzi (*T. cruzi*) is the unicellular parasite that causes the South American wide spread Chagas' disease. The evolution of the disease presents an acute phase comprising the first month upon infection, an intermediate or latency stage of undefined extension (may be years) and a chronic illness, presenting cardiac or gastroenterological affections (viscerous dilatation, for example) 20–30 years after the infection. The acute infection is stated with detectable parasitemia, local acheless inflammation (when in eye called "Romaña sign"), and sometimes, with a pertinacious grippe condition: violent headaches, muscles pain and ganglion acheless inflammation. Nervous system is also involved, producing meningitis and meningeoencephalitis, with a lethal effect or serious sequels (Tanowitz et al., 1992). Heart disease is an outstanding point, since irreversible chagasic panmiocarditis is developed in almost 600,000 persons of 30–40 years old (Bonet et al., 1968; Sosa Estani, 1994) out of 2,000,000 infected only in Argentina (Carlomagno et al., 1989). Chemotherapy for Chagas' disease is based on nifurtimox and benznidazole, which are not very effective, are mutagenic and may produce adverse reactions and toxic effects (Castro et al., 2006; Moll et al., 2007). Besides, *T. cruzi* strains resistant to these drugs have also been described (Murta et al., 1998; Murta and Romanha, 1998; Villarreal et al., 2005). In the search for new therapeutic agents, we reported the beneficial effects of ganglioside treatment on mice. Treatment of mice infected with a lethal amount of parasites, with a mixture of total bovine brain gangliosides (sialic acid containing sphingolipids, TBBG), decreased parasitemia and increased mice survival (Bronia et al., 1999; Lujan et al., 1993). In a posterior paper we presented evidence that GM1 (a minor component of TBBG) was the principal active component. The therapeutic effect required the whole molecule, and survival correlated with recovery of normal electrocardiographic records and normal production of antibodies (Cossy Isasi et al., 1999). These results let us question whether gangliosides act on the parasite (trypanocidal), on the host (immunomodulation) or both. Since *T. cruzi* expresses gangliosides of the gangliotetraose series (Avila et al., 1998), it seems possible that parasite metabolism may be altered with the incorporation of gangliosides in the plasma membrane. The complete route of ganglioside synthesis and participation in signaling processes of the parasite remains to be fully elucidated.

In this work we analyze biochemical, physicochemical and structural modifications of the parasite after incorporation of exogenous gangliosides in plasma membrane. Epimastigotes or trypomastigotes of *T. cruzi* were incubated with exogenous gangliosides. The activities of glutamate dehydrogenase NADP dependent (GDH-NADPH) and malate dehydrogenase NAD dependent

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(MDH-NADH) which reflect the metabolic stage of the parasite (Carneiro and Caldas, 1983; Cazzulo, 1992); were determined during treatment with gangliosides. Always focused on membrane events, we simulated parasite–host cells interaction, measuring fusion in red cells incubated with epimastigotes of *T. cruzi*. Ultrastructural modifications were studied in epimastigotes and trypomastigotes treated with gangliosides. The activity of a parasite phospholipase A2 (PLA2), an enzyme still not characterized was studied also.

2. Materials and methods

2.1. Substrates

Oxaloacetic acid and α-ketoglutaric acid as well coenzymes NADH and NADPH, Dulbecco's MEM, hemin and buffer Tris were purchased from Sigma. Brain Heart Infusion medium was purchased from Britania (Buenos Aires, Argentina). Ammonium chloride, potassium chloride, sodium phosphate glucose and tryptose were from Merck. Rabbit polyclonal IgG anti-cPLA$_2$ was from Santa Cruz Biotechnology and goat polyclonal IgG anti-porcine sPLA$_2$ was from Southern Biotechnology. Silica gel plates Watman Lab. Division, New Jersey, USA, (10 × 10 cm). 1-palmitoyl-2-(1^{14}C) arachidonoyl, L-3-phosphatidylcholine (55 mCi/mmol, 50 μCi/mL) -PC (sn2-*A)-, and 1-palmitoyl-2-(1^{14}C) oleoyl,L-3-phosphatidylcholine (55 mCi/mmol, 25 μCi/mL) -PC (sn2-*O)-from DuPont NEN were the substrates in the radioactive assay.

2.2. Parasites

Epimastigotes of *T. cruzi*, Tulahuen strain, were grown in BHI (brain–heart infusion) supplemented with 10% fetal calf serum, at 28 °C (Calderon et al., 1986; Calderon et al., 1989). After 4, 7, 14, and 21 days in culture (initial, middle, end of logarithmic phase and declining phase, respectively) they were harvested and washed twice with isotonic phosphate buffer 0.01 M (PBS), pH 7.3. For ganglioside treatment the cells were resuspended in Dulbecco's modified medium without serum, separated in control and treated with 300 μM of total bovine brain gangliosides and cultured for 18 h. Again they were harvested and washed twice. For fluorometric experiments the parasites were resuspended in PBS but for enzymatic determinations they were resuspended in the appropriate buffer and mechanically homogenized with an Ultra Turrax T25 (IKA Labortechnic). To obtain the cell free extract the homogenates were centrifuged at 10,000g 20 min. The resultant supernatant (0.5–3 mg of protein) was used for enzymatic and protein determinations. Parasite mobility was checked by optical microscopy before and after each harvesting and was found to be greater than 90%.

Trypomastigotes were obtained from Vero infected monolayers, cultured in Dulbecco's modified, serum free Eagle's medium. Medium was changed at day 3 post-infection to remove parasites that did not enter the cells. Simultaneous culture flasks received fresh culture medium or medium with 50 μM GM1. Culture medium was not changed until harvesting. At 5, 6, and 7 days post-infection, parasites were harvested and the flasks were discarded. After harvesting, parasites were washed twice with isotonic phosphate buffer 0.01 M (PBS), pH 7.3, and finally pelleted at 3000g for 10 min. The dried pellet was fixed and mounted for electron microscopy.

2.3. Enzyme assays

Two enzymes were assayed, glutamate dehydrogenase NADP dependent (NADPH-GDH) and malate dehydrogenase NAD dependent (NADH-MDH). The enzyme activities were determined at 340 nm in a DU 70 Beckman spectrophotometer at 30 °C. The reaction mixtures consisted of 50 mM buffer Tris–HCl, pH 7.6, 90 mM NH_4Cl, 0, 12 mM NADPH, and 34 mM α-ketoglutarate for GDH, or 0.01 mM phosphate buffer, pH 8, 0.12 mM NADH, and 30 mM oxalacetate for MDH. The reactions were initiated by the addition of enzyme. One enzyme unit was defined as the amount of enzyme that catalyzed in 1 min the conversion of 1 μmol of coenzyme under the assay conditions stated (Carneiro and Caldas, 1983).

2.4. Total protein

Total protein was determined according to Smith et al. (1985).

2.5. Membrane microviscosity

Harvested parasites as it is stated above were resuspended in PBS and labeled with DPH by adding a small quantity (1% of the final volume of incubation) of the probe in chloroformic solution (9 mg/ml) and incubating 2 h at 28 °C. After washing the cells were resuspended and kept in isotonic PBS. The optical fluorescence anisotropy measurements were done essentially according to the method described by Lakowicz (2006). The light beam was polarized using Glan–Thompson calcite prism polarizers and made incident on the sample cuvette (10 × 10 × 45 mm) housed in a variable temperature unit. The fluorescence was collected at 90° to the incident beam. Fluorescence intensities were measured parallel and perpendicular to the vertically polarized incident beam. The G factor was determined before studying each sample. This ensured reliable data as any optical component could bring about its own optical effects. Anisotropy was calculated using the following relationship:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad \text{where } G = I_{HV}/I_{HH},$$

I_{VV} represents fluorescence intensity parallel to vertically polarized excitation beam and I_{VH} is that of perpendicular component. I_{HV} and I_{HH} are the perpendicular and parallel intensities when the incident light is horizontally polarized. The equipment was checked with a concentrated solution of glycogen and r was found to equal 0.998.

2.6. Fusion assay

Parasites were harvested by centrifugation (3000g, 20 min), washed three times in buffer Krebs–Ringer–Tris (KRT), pH 7.2–7.4, according to Garrido et al. (2000), and resuspended in the same buffer at a concentration of 2–4 × 10^8 cells/ml. Freshly drawn mice blood was mixed (4:1 v/v) with heparin. Erythrocytes were washed three times in Hanks saline, pH 7.55, containing 5 mM EGTA. Then, they were resuspended in KRT. Cell contact was studied by incubating a total of 2–4 × 10^8 cells/ml of each cell type in Hanks, pH 7.2, at 37 °C in the presence of 2 mM Ca^{2+}. For the inhibition assay, TBBG 300 (μM), antibodies anti-sPLA2 (1:1000) or anti-cPLA2 (1:1000), were added to the fusion medium just at the onset of the assay in separate tubes. Aliquots were obtained at different time intervals and examined at the optic microscope. Fusion ability was measured as the number of fusion bodies determined in ten randomly chosen fields.

2.7. Electron microscopy

The analyses were performed on pellets of cultured trypomastigotes or epimastigotes of *T. cruzi*. Pellets were fixed with Karnovsky solution (Karnovsky, 1965) for 2 h at room temperature, rinsed with 0.1 M cacodylate buffer, pH 7.35, and post-fixed with 1% osmium tetroxide for 2 h. Samples were dehydrated with acetone and included in Durcupam plastic (Polysciences Inc., Warrington, PA). The ultrathin sections were mounted in a 200-mesh grid and stained with uranyl acetate and lead citrate. Observations of