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Original article

Trypanosoma cruzi: parasite shed vesicles increase heart parasitism and generate an intense inflammatory response

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Abstract

Trypanosoma cruzi trypomastigotes continuously shed into the medium plasma membrane fragments sealed as vesicles enriched in glycoproteins of the gp85 and trans-sialidase (TS) superfamily and α-galactosyl-containing glycoconjugates. Injection of a vesicle fraction into BALB/c mice prior to *T. cruzi* infection led to 40% of deaths on the 16th day post-infection and 100% on day 20th whereas 20% of untreated animals survived for more than 30 days. The vesicle-treated animals developed severe heart pathology, with intense inflammatory reaction and higher number of amastigote nests. Analysis of the inflammatory infiltrates 15 days after infection showed predominance of $TCD4^+$ lymphocytes and macrophages, but not of $TCD8^+$ cells, as well as a decrease of areas labeled with anti-iNOS antibodies as compared to the control. Higher levels of IL-4 and IL-10 mRNAs were found in the hearts and higher IL-10 and lower NO levels in splenocytes of vesicles pretreated animals. Treatment of mice with neutralizing anti-IL-10 or anti-IL-4 antibodies precluded the effects of pre-inoculation of membrane vesicles on infection. These results indicate that *T. cruzi* shed membrane components increase tissue parasitism and inflammation by stimulation of IL-4 and IL-10 synthesis and thus may play a central role in the pathogenesis of Chagas' disease acute phase.

Keywords: Tissue parasitism; Cytokines; *T. cruzi* infectivity

1. Introduction

Trypanosoma cruzi, a flagellate protozoan causing Chagas' disease, is carried from the infection site via lymphatic and blood vessels to all organs or tissues. The heart and secondarily the autonomic nervous system of the esophagus and large intestine are mostly affected. Virtually any cell type can be invaded by *T. cruzi*. The parasite multiplies in the cytosol as amastigotes an eventually differentiating into trypomastigotes that are released in the extracellular space upon rupture of the host cell.

The parasite elicits intense innate and specific immune responses and focal areas of inflammation are seen especially in the cardiac tissues [1]. In the beginning of infection, control of parasitism relies on the innate immunity, with the synthesis of cytokines and activation of several cell types, including natural killer cells (NK) and macrophages [2]. Parasitism is eventually controlled by several immune mechanisms: cytokine-activated macrophages, opsonization by antibody and T cell-mediated cytotoxicity.

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Cells from the innate or specific immune system produce cytokines and other mediators. Among the cytokines, IL-12, IFN-γ, TNF-α [3] and type I IFN [2] participate in the resistance to the parasite. Conversely, both TGF-β and IL-10 prevent the protective effect of IFN-γ [4,5]. Treatment of mice with anti-IL-10, anti-TGF-β or anti-IL-4 antibodies may decrease parasitemia in the acute phase and/or increase survival of infected animals [6]. However, IL-10 is also important to control excess inflammation as evidenced by the increased mortality of IL-10 deprived mice [4].

Infective trypomastigote forms of *T. cruzi* are covered by mucin-like molecules with O-linked oligosaccharides containing terminal sialic acid and α-galactosyl residues [7] as well as members of the gp85/trans-sialidase (TS) family [8], all being glycosylphosphatidylinositol (GPI) anchored glycoproteins [7,9]. These GPI-anchored glycoconjugates are present in the plasma membrane vesicles that are continuously shed by the parasites and which also contain lipids [10], Tc-85 and TS. The half-life of these shed membranes is approximately 3.5 h, as judged by the half-life of Tc-85 [11], which is released from the parasite with its GPI anchor [12].

The importance of secreted membrane vesicles has been stressed in a variety of cells and physiological conditions. They can act as communicators between cells without the need of direct cell to cell contact and also act on the tissue microenvironment thus regulating the evasion of tumor cells from the immune system and the process of metastasis [13,14].

Since *T. cruzi* shed membrane vesicles [15] contain several molecules that interfere with the immune system, the present study was undertaken to examine the effects of these vesicles on the course of infection by *T. cruzi* trypomastigotes.

2. Materials and methods

2.1. Mice

BALB/c female mice (8–10 week-old) were obtained from the animal facility at the Instituto de Química (IQ-USP, Brazil). All the animal handling and procedures were approved by the Institute's Committee on the Ethical handling of laboratory animals.

2.2. Preparation, fractionation and characterization of T. cruzi *vesicles*

2.2.1. Preparation of T. cruzi *vesicles*

Total shed material was obtained from *T. cruzi* trypomastigotes, Y strain, as described [15]. Briefly, trypomastigotes were incubated in RPMI (Life Technologies, Grand Island, NY), supplemented with 5% fetal calf serum (FCS, Invitrogen) for 2 h at 37 °C, centrifuged (15 min, 1000 × *g*) and the supernatant was filtered through 0.45 μm membranes. The filtrate was loaded into a Sepharose CL-4B column (1 × 40 cm, GE Healthcare, Piscataway, NJ) pre-equilibrated with 100 mM ammonium acetate, pH 6.5, followed by elution with the same buffer at a flow rate of 0.2 ml/min. Routinely, fractions of 0.25 ml were collected, and then screened with anti-α-Gal antibodies prepared as described [7] by a chemiluminescent enzyme-linked immune-assay (CL-ELISA). The α-Gal-positive fractions were pooled, concentrated in a vacuum centrifuge and resuspended in PBS. Radiolabeled total shed material was obtained by incubating [^{35}S]-methionine-metabolically labeled parasites (300 μCi/10^9) in culture medium, as described [15].

2.2.2. Western blotting

Protein samples (30 μg per lane) were separated in 9% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with one of the following monoclonal (mAb) or polyclonal antibodies: anti-cruzipain (mAb, provided by Dr. Ana Paula Lima), mAb 39 anti-TS (provided by Dr. Sérgio Schenkman), anti-Tc-85 antibodies (against the recombinant protein Tc85-11) and anti-α-Gal (antibodies purified from a pool of sera obtained from Chagas' disease patients [7]). Reactivity was developed after incubation with the respective peroxidase- or avidin-conjugated secondary antibody (mouse, rabbit or goat anti-human IgG), or streptavidin-peroxidase and ECL (Pierce).

2.3. Evaluation of parasite viability during shedding

Aliquots corresponding to 2.5 × 10^8 parasites under shedding were removed at every 30 min and incubated for 30 min at 37 °C with 50 ng/ml calcein and 1 μM 2-ethidium homodimer (Molecular Probes, Eugene, OR). After centrifugation the parasites were resuspended in 1 ml of PBS containing 4% paraformaldehyde and parasite individuals (gated at FSC *vs.* SSC) were analyzed by fluorescence intensity at 520 nm for incorporated Calcein (FL1-green) and 670 nm for ethidium homodimer (FL-4 red) on a Cytomics-500 flow cytometer (Beckman–Coulter). Viability was calculated as 99% after incubation for 3 h. No changes in motility and parasite number were detected after 3 h incubation of the parasites under shedding conditions.

2.4. Electron microscopy

The parasites submitted to shedding were centrifuged and the vesicle-rich supernatant was fixed for 60 min at room temperature in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, post-fixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Epon. Thin sections were stained with uranyl acetate, essentially as described before [15].

2.5. Experimental design of in vivo experiments

T. cruzi, Y strain, was routinely maintained by weekly intra peritoneal (i.p.) infection of BALB/c mice. Parasitemia was quantified by counting motile parasites in 10 μl of fresh blood sample drawn from lateral tail veins, as described [6]. A total of 5 μg of protein from peak 1 (pool of fractions 56–70, Section 2.2 and corresponding to vesicles from approximately 10^5 trypomastigotes) was injected in mice i.p. 7 days before the i.p. inoculation of 500 blood forms. In control groups, the mice were inoculated with only blood forms or membrane vesicles.