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Acta Tropica 98 (2006) 103–109
ACTA TROPICA
www.elsevier.com/locate/actatropica

Biological aspects of the *Trypanosoma cruzi* (Dm28c clone) intermediate form, between epimastigote and trypomastigote, obtained in modified liver infusion tryptose (LIT) medium

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Received 7 October 2005; received in revised form 21 February 2006; accepted 28 February 2006

Abstract

We describe some biological characteristics of the *Trypanosoma cruzi* intermediate form derived from the transformation of epimastigotes to trypomastigotes obtained from cultivation in modified liver infusion tryptose (LIT) medium. The ultrastructural analysis of the intermediate forms in this medium showed the enlargement of the kinetoplast located adjacent to the flagellate nucleus. Some biological characteristics of the intermediate form are similar to trypomastigotes and others to epimastigotes. Despite displaying a similar trypomastigote surface charge, the intermediate forms, like the epimastigotes, are not resistant to complement-mediated lysis. Moreover, the intermediate forms are unable to infect cultured fibroblasts cells but develop limited infections in macrophages.

Keywords: *Trypanosoma cruzi*; Intermediate form; Biological characteristics; LIT

1. Introduction

The life cycle of *Trypanosoma cruzi*, etiological agent of Chagas' disease (Chagas, 1909), comprises three different morphological and functional forms, namely, amastigotes, epimastigotes and trypomastigotes (Brener, 1973; De Souza, 1984). The transformation of epimastigotes to metacyclic trypomastigotes (metacyclogenesis process) involves differential gene expression (Goldenberg et al., 1984) and deserves special interest as this process occurs naturally within both the triatomine insect vector (Dias, 1934; Carvalho-Moreira et al., 2003) and the anal gland of some opossums (Deane et al., 1984).

Several complex in vitro differentiating systems have been developed allowing the transformation of epimastigotes to metacyclic trypomastigotes (Camargo, 1964; Castellani et al., 1967; Sullivan, 1983; Contreras et al., 1985a). Chemically defined in vitro differentiating conditions have also been established (Contreras et al., 1985b; Goldenberg et al., 1987) supporting the repeata-

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doi:10.1016/j.actatropica.2006.02.006

bility of the in vitro metacyclogenesis methods for *T. cruzi* studies.

Previous analyses of the cell surface of these protozoan stages have discovered differences in the carbohydrate, protein, and lipid compositions (Bourguignon et al., 1998; De Souza, 1995, 1999). Surface components are assumed to play some role in basic biological characteristics, such as resistance to complement-mediated lysis (Kipnis et al., 1981; Joiner et al., 1986; Iida et al., 1989), and recognition of the host cell (Araujo-Jorge, 1989; Jacobson and Doyle, 1996). Previous observations using cell electrophoresis have shown that each developing stage of *T. cruzi* presents a mean characteristic electrophoretic mobility (EPM), and, using this technique, it is possible to distinguish *T. cruzi* from other members of the sub-genus Schizotrypanum, such as *Trypanosoma dionisis* and *Trypanosoma myoti* (Souto-Padron et al., 1984, 1990).

However, in vivo and in vitro metacyclogenesis studies have demonstrated the presence of intermediate forms (Dias, 1934; Brener, 1973; Schaub, 1988; Perlowagora-Szumlewicz and Moreira, 1994; Cortez et al., 2002; Carvalho-Moreira et al., 2003). These intermediate forms are identified morphologically through the position of the kinetoplast relative to the nucleus (Shaub, 1989). Despite the morphological identification, little is known about the biological characteristics of these intermediate forms. In this work, we evaluated some biological aspects (i.e., EPM, complement lysis, and infectivity on Vero and macrophage cells) of *T. cruzi* intermediate forms (Dm28c clone) cultivated in modified-LIT medium. These results in vitro are the first step to future studies about the role of these intermediate forms in the parasite development in the invertebrate host.

2. Materials and methods

Parasite culture. All experiments were performed using *T. cruzi* Dm28c clone, characterized as TcI group (Anonymous, 1999; Kawashita et al., 2001; Araujo et al., 2002). Epimastigote forms were maintained in LIT filtered medium, as described by Camargo (1964). An aliquot of LIT medium was then prepared using autoclaving (modified-LIT medium) for 20 min instead of filtration (unmodified-LIT medium) prior to serum addition (10%). Parasites from filtered medium (LIT) in growth phase were centrifuged ($1000 \times g$/15 min/4 °C) and resuspended in LIT or in modified-LIT medium (autoclaved) in an initial concentration of 5.0×10^5 parasites/ml. Samples of both of these cultures were then used after harvesting at day 2 (see below). Trypomastigotes (>72%) were obtained from LIT medium at day 10 and purified by chromatograph on DEAE-cellulose, according to Pinho et al. (1991).

Cell cultures. Normal peritoneal macrophages were obtained for infection studies, as described previously (Meirelles et al., 1982; Araujo-Jorge, 1989) from Swiss mice previously injected (intra-peritoneal) with 5 ml Hanks solution (pH 7.2). The cell suspension was incubated in 24-well Falcon plates for 1 h. Then this medium was removed with the addition of medium 199 plus 10% fetal bovine serum. These culture flasks were maintained at 37 °C and 5% of CO_2 for 24 h. Infection by parasites was also evaluated in Vero and 3T3 cells (ATTC, CRL-1586™ and CCL-92). Cell cultures were infected with parasites obtained from LIT, modified LIT and from chromatography on DEAE-cellulose (10 parasites/cell). Non-internalized parasites were removed 2 h after assessing intracellular forms in an inverted microscope. The percentage of fibroblasts (Vero and 3T3) or macrophages containing parasites was determined by examining at least 400 randomly selected cells at 400× magnification, using a Zeiss photomicroscope. The endocytic index is represented by percentage of cells containing parasites multiplied by the average number of parasites per cell. All experiments were performed three times.

Optical microscopy analysis. Fresh parasite samples were observed and quantified in Neubauer hemocytometers. To analyze trypanosomes forms, parasites were fixed with methanol and stained with May-Grunwald-Giemsa before light microscopy (Perlowagora-Szumlewicz and Moreira, 1994; Cortez et al., 2002; Carvalho-Moreira et al., 2003).

Transmission electron microscopy (*TEM*). Infected macrophages, control parasites alone, and uninfected macrophages were fixed for 1 h at 4 °C with 1% paraformaldehyde plus 1% glutaraldehyde diluted in 0.1 M cacodylate buffer, pH 7.2, and then washed with the buffer solution. Cells were then post-fixed for 30 min with 1% OsO_4 in 0.1 m cacodylate buffer, dehydrated in graded acetones, and embedded in polybed resin. Ultrathin sections were then stained with aqueous uranyl acetate and lead citrate. Both stained and unstained sections were observed using a Zeiss 900 transmission electron microscope operating at 80 kV.

Resistance to complement-mediated lysis. Cultured parasites (150 μl; 2×10^6 cells/ml) were incubated with fresh human serum (50 μl) at 28 °C for 30 min. Then, parasite lysis was observed with a light microscope, and complement resistant parasites were counted in a hemocytometer (Santos et al., 2004). The incubations were made in triplicate and repeated three times, each time using serum from different donors.