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Changes in the structural organization of the cytoskeleton of *Tritrichomonas foetus* during trophozoite-pseudocyst transformation

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ABSTRACT

Tritrichomonas foetus is a parasite that causes bovine trichomonosis, a major sexually transmitted disease in cattle. It grows in axenic media as a trophozoite with a pear-shaped body, three anterior flagella, and one recurrent flagellum. However, under some well-controlled experimental conditions *in vitro*, as well as *in vivo* in infected bulls, the parasite acquires a spherical or elliptical shape, and the flagella are internalized but the cells do not display a cyst wall. This form, known as the endoflagellar or pseudocystic form, is viable, and can be transformed back to trophozoites with pear-shaped body. We used confocal laser scanning microscopy, and high resolution scanning electron microscopy to examine the changes that take place in the protozoan cytoskeleton during trophozoite-pseudocyst transformation. Results confirmed previous studies and added new structural information to the organization of cytoskeletal structures during the transformation process. We observed that changes take place in the pseudocysts' axostyle and costa, which acquired a curved shape. In addition, the costa of multinucleated/polymastigont pseudocysts took variable conformations while curved. The costa accessory structure, as well as a network of filaments connecting this structure to the region where the recurrent flagellum associates to the protozoan body, was not seen in pseudocysts. In addition, the axostyle was fragmented during trophozoite-pseudocyst transformation.

1. Introduction

The flagellated protist *Tritrichomonas foetus* is a parasite that causes bovine trichomonosis, a major sexually transmitted disease in cattle (Rae and Crew, 2006). It usually presents a pear-like shape, known as a trophozoite, characterized by the presence of three anterior flagella and one recurrent flagellum. Under some well controlled experimental conditions, including changes in temperature (Granger et al., 2000; Pereira-Neves et al., 2012) or when the cytoskeleton is modified due to action of drugs such as colchicine (Madeiro da Costa and Benchimol, 2004), the trophozoite acquires a rounded or elliptical shape and internalizes the flagella, transforming into an endoflagellar form (EFF), also known as a pseudocyst because a cyst wall is not formed (Pereira-Neves et al., 2003). Initially, pseudocysts were considered to be a degenerative stage of the protozoan (Powell, 1936; Samuels, 1959). However, several studies have shown clearly that they are living cells able to undertake nuclear division to form multinucleated cells, able to interact with and provoke damage to host cells (Mariante et al., 2004; Pereira and Almeida, 1940; Pereira-Neves et al., 2003; Pereira-Neves and Benchimol, 2009). In addition, once stimuli to pseudocyst induction are removed they transform again into piriform trophozoites (Pereira-Neves and Benchimol, 2009). Clear evidence that pseudocysts form *in vivo* is their presence in naturally infected bulls (Pereira-Neves et al., 2011).

During pseudocyst formation, several morphological changes occur in the cytoskeleton. Previous studies used light microscopy and conventional scanning (SEM) and transmission electron microscopy (TEM) to show that axial structures such as the axostyle and costa, assumed a curved shape (Mariante et al., 2004; Pereira-Neves et al., 2003, 2011; Pereira-Neves and Benchimol, 2009). In this study we further characterize changes to the cytoskeleton using the laser scanning confocal microscopy (LSCM), and high

Abbreviations: EFF, endoflagellar form; SEM, scanning electron microscopy; TEM, transmission electron microscopy; LSCM, laser scanning confocal microscopy; PBS, phosphate-buffered saline; BSA/PBS, bovine serum albumin/PBS.

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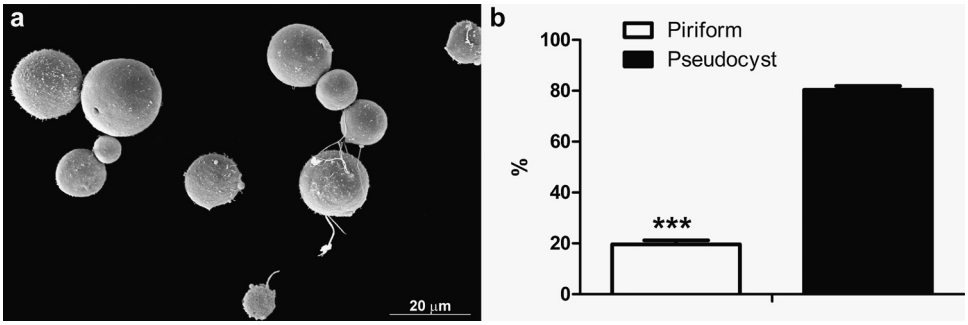

Fig. 1. (a) Scanning electron microscopy of the parasites obtained after the pseudocyst induction, where the trophozoites assumed the spherical form and internalized the flagella. (b) Quantitative analyses of the parasites that were transformed into pseudocyst at the end the induction process.

resolution scanning electron microscopy, thus extending to the pseudocyst form previous studies carried out with trophozoites (de Andrade Rosa et al., 2013).

2. Material and methods

2.1. Cell culture

The K strain of *T. foetus* was isolated by Dr. H. Guida (Embrapa, Rio de Janeiro, Brazil) from the urogenital tract of a bull, and it has been maintained in culture since the 1970s. Cells were cultivated in TYM Diamond's medium (Diamond, 1957) supplemented with 10% fetal calf serum and grown for 24 h at 36.5 °C.

2.2. Pseudocyst induction

Cultures of *T. foetus* grown for 30 h at 37 °C in TYM medium were cooled to 4 °C for up to 4 h, without changing the medium. The pseudocyst formation was followed by phase contrast microscopy. To quantify pseudocysts, at least 100 cells were examined by scanning electron microscopy in three independent experiments in triplicate. The results were expressed in percentage.

2.3. Cytoskeleton preparation

Using 10 μL 0.01% poly-L-lysine (mol wt 150,000–300,000, Sigma, USA) on a glass coverslip and allowed to stand for at least 15 min, then rinsed in distilled water after which a 20 μL sample of living cells (10^7 cells/mL) was added. After 15 min, samples were rinsed in phosphate-buffered saline (PBS), pH 7.2, to remove non-adherent cells. Cytoskeletons were prepared by treating with 2% Triton X-100 and 2% NP-40 in a modified buffer previously designed for cytoskeleton preservation (IC buffer: 10 mM Tris Base, 2 mM EDTA, 2 mM DTT, 2 mM $MgSO_4$, 150 mM KCl, 30% glycerol, pH 7.4) for 30 min or 1 h (Palm et al., 2005).

2.4. Immunofluorescence microscopy

The cytoskeleton of both piriform trophozoites and pseudocysts were fixed for 1 h with 4% freshly prepared formaldehyde in phosphate buffer (0.1 M, pH 8.0). Samples were allowed to adhere to glass coverslips previously coated with poly-L-lysine, and cells were permeabilized with 0.1% Triton X-100. The free aldehyde groups were quenched using 50 mM NH_4Cl and 3% bovine serum albumin/PBS (BSA/PBS). Samples were then labeled with monoclonal antibody anti-β-tubulin (Amersham Bioscience, USA), and diluted 1:10 in 1% BSA/PBS. The coverslips were incubated for 1 h with an Alexa Fluor 488-conjugated anti-mouse antibody diluted 1:100 in BSA/PBS. Finally, the coverslips were washed and examined using a Leica TCS SP5 confocal microscope equipped with an Argon 488 laser emission bandwidth filter.

2.5. Scanning electron microscopy

Cytoskeletons were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), washed in PBS (pH 7.2), and post-fixed for 30 min in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2), and dehydrated using an ascending series of ethanol washes, ending with 100% ethanol. They were then critical point-dried with CO_2 using a Bal-Tec CPD 030 critical point dryer, and sputter-coated with a thin layer (1–2 nm) of chromium or carbon using a Leica EM SCD 500 or 005 sputter coater. The FEI Nova NanoLab 600 and Magellan field emission SEMs were used at accelerating voltages of 2 and 1 kV, respectively. For better identification of some structures, colors were added to some images using Adobe Photoshop color balance.

2.6. Morphometric analyses

The areas and lengths of thirty axostyles of both forms were measured using iTEM software, as well the lengths of thirty costae of both forms of *T. foetus*. All results were analyzed using the GraphPad Prism 4.

2.7. Statistical analyses

Statistical comparisons were performed using the paired *t* test, and *P* values less than 0.0001 were considered to be statistically significant.

3. Results

3.1. Pseudocyst induction

In previous studies it has been established that incubation of trophozoites at low temperature is a highly efficient and reproducible method to induce the transformation of the pear-shaped parasites into pseudocysts. We used this well-established methodology in the present study and we obtained around 80% of pseudocysts in the cultures (Fig. 1).

3.2. Localization of microtubules

Using a monoclonal antibody that recognizes β-tubulin, we observed that cytoskeletal changes occur during the transformation from piriform to pseudocyst. In piriform parasites, the three anterior flagella and recurrent flagellum are externalized. A typical axial axostyle was also observed by immunolabeling with the β-tubulin-recognizing antibody (Fig. 2) confirming previous observations (Lopes et al., 2001). Intense fluorescence was seen where the microtubule sheet turns upon itself to form the axostylar trunk (Fig. 2). Using LSCM it was possible to observe labeling of