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Trichomonas vaginalis virulence against epithelial cells and morphological variability: the comparison between a well-established strain and a fresh isolate

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Abstract The FMVI strain of *Trichomonas vaginalis* was freshly isolated from an asymptomatic patient, and its morphological properties and virulence in vitro compared with the well-established JT strain. The morphological variability of the parasites was assessed by differential interference microscopy and both scanning and transmission electron microscopy. The FMV1 strain presented nearly 20% amoeboid cells whereas the JT strain presented high percentages of ellipsoid but no amoeboid cells. The FMV1 morphotype population was unaltered after at least 1 year of subculturing. Electron microscopy revealed that this strain produced numerous pseudopod structures which mediated intimate contact and interdigitation among trophozoites. Dead FMV1 parasites were often phagocytosed by conspecific cells. We also compared the cytolytic capacity of these two populations against epithelial MDCK cells and its contact dependence. The FMV1 strain rapidly adhered to plastic or glass surfaces and to MDCK monolayers. This strain destroyed about 93% of the epithelial cells in 90 min whereas the cytolytic activity of the JT parasites was very much lower (about 41%). Parasite supernatants displayed no cytolytic activity, indicating contact-mediated lysis. The protozoan virulence in vitro did not correlate well with the clinical observations. The implications of these results are discussed.

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Introduction

The parasitic protozoan *Trichomonas vaginalis* is the causative agent of human trichomoniasis, the most frequent, non-viral, sexually transmitted disease, accounting for approximately 180–200 million cases annually worldwide (Petrin et al. 1998). Nearly 50% of infections in women are asymptomatic whereas the remaining ones may present vulvar pruritus, fetid, frothy or homogeneous vaginal discharge with leukorrhea and signs of colpitis macularis (Catterall 1972). Pregnant patients are predisposed to rupture of the placental membranes, premature labor and low-birthweight infants (Minkoff et al. 1984; Petrin et al. 1998). Trichomoniasis is also associated with cervical cancer (Gram et al. 1992; Zhang and Begg 1994), atypical pelvic inflammatory disease (Heine and McGregor 1993) and infertility (Grodstein et al., 1993).

Newly isolated trichomonads maintained in axenic culture usually present highly pleiomorphic populations. After subculturing for prolonged periods, most of the cells tend to display an ellipsoid or drop-like morphology. Such *T. vaginalis* morphotypes are rarely found in either vaginal secretions or urine (Honigberg and Brugerolle 1990; Petrin et al. 1998). Conversely, amoeboid parasites are predominantly found in vivo, being easily detected in human vaginal secretions (Heath 1981; Arroyo et al. 1993). The amoeboid forms of *T. vaginalis* were reported to be highly adherent microorganisms (Alderete et al. 1995). The adhesion and morphological properties, as

well as the survival of the protozoan, were shown to require iron (Arroyo et al. 1993; Alderete et al. 1995; Garcia et al. 2003).

The cellular mechanisms underlying *T. vaginalis* pathogenesis are still not fully understood. In vitro, binding and subsequently parasite recognition by the host epithelial cells occur in a highly specific manner, involving ligand molecules localized on both the parasite and epithelial cell surfaces (Arroyo et al. 1992, 1993), as well as the secretion of parasitic cysteine proteinases (Arroyo and Alderete 1989).

The cytotoxicity exerted by *T. vaginalis* is primarily a contact-dependent mechanism (Heath 1981; Alderete and Pearman 1984; Krieger et al. 1985; Silva-Filho and De Souza 1988). However, the involvement of a parasite cytotoxic contact-independent mechanism deserves further consideration since some parasite hydrolases with cytotoxic potential are released by *T. vaginalis* (Alderete and Garza 1985; Arroyo and Alderete 1989; Jesus et al. 2002).

In this study, the morphological diversity among microorganisms of a fresh isolate of *T. vaginalis* was characterized and compared to that of a long-term culture. In addition, the influence of culture conditions in parasite morphology was determined and the cytotoxicity exerted by the different parasite populations was also investigated.

Materials and methods

Parasite isolation and axenization

Trophozoites of *T. vaginalis* were collected from a 45-year-old female patient, who attended the University Hospital of the Medical School of Valença (Rio de Janeiro, Brazil) for routine gynecological examination, and designated the FMV1 strain. The patient was asymptomatic and not previously exposed to nitroimidazole treatment. Parasites were collected on sterile swabs from both the endocervix and the posterior fornices of the vagina, seeded in TYM medium (Diamond 1957) supplemented with 1,000 U/ml penicillin, 200 μg/ml gentamicin and 10% fetal bovine serum, and maintained at 37°C for 24 h. Parasite samples were subjected to axenization by daily washing in serum-free medium supplemented with antibiotics, as described above. Axenic trophozoites were maintained either cryopreserved or by inoculation in fresh antibiotic-free culture medium up to 15 times as described elsewhere (Farris and Honigsberg 1970). Prolonged culturing leads to loss of in vitro cytotoxicity. The well-established *T. vaginalis* FF28JT-Rio strain was maintained in the same conditions. The viability of both strains was assessed before and after all assays.

Scanning electron microscopy

Parasites from the FMV1 and JT strains were collected by centrifugation (1,400 *g*) at 4°C, washed twice with cold 0.01 M phosphate-buffered saline (PBS) pH 7.4 and fixed in 2.5% glutaraldehyde and 0.2% picric acid in 0.1 M sodium cacodylate buffer pH 7.4. After washing, the cells were attached to poly-L-lysine-coated coverslips and post-fixed in 1% OsO_4, 0.8% $K_3Fe(CN)_6$ and 5 mM $CaCl_2$ in the same buffer for 1 h. Samples were then dehydrated in an ethanol series (40–100%), critical point dried in CO_2, mounted on metal stubs, covered with an about 20-nm-thick gold layer and observed in a Jeol 940 scanning electron microscope at 20 kV.

Transmission electron microscopy

Parasites were fixed in 2.5% glutaraldehyde (Sigma, grade II) in 0.1 M sodium cacodylate buffer pH 7.2, post-fixed as above, dehydrated in an acetone series and embedded in Polybed resin (Polysciences). Thin sections were stained with uranyl acetate and lead citrate and observed under a Zeiss 109 transmission electron microscope.

Light microscopy

T. vaginalis grown for 24 h at 37°C in TYM medium were harvested by centrifugation, washed in PBS pH 7.2 and fixed in 2.5% glutaraldehyde in the same buffer. Samples were rinsed with PBS and the morphology was determined by differential interference contrast microscopy. Morphotype counting in a Neubauer chamber was performed in triplicate ($n = 1{,}200$ cells per assay)

Parasite-host cell interactions: measurement of cytotoxicity

MDCK cells were grown in 24-well plates until confluence. Cultures were washed and incubated in 300 μl serum-free TYM medium for 30–180 min with either FMV1 or JT strain parasites (5×10^5 cells/well) at 37°C. Controls were carried out in the same conditions in the absence of parasites. Monolayers were fixed in 2.5% glutaraldehyde, washed in PBS and stained in 1% methylene blue in 0.1 M borate buffer pH 8.7 as described previously (Orozco et al. 1978). After washing, incorporated stain was extracted in 0.1 N HCl (30 min at 37°C) and the resulting cell labeling was spectrophotometrically measured at 660 nm. Cytotoxicity was expressed as the percentage of recovered stain before and after interaction with the parasites. Additional assays were performed in order to evaluate the role played by parasite secreted products in epithelial cytotoxicity. Trophozoite culture supernatants (5×10^5 parasites/ml), obtained after incubation in serum-free TYM medium for different periods, were ultracentrifuged (16,500 *g* at 4°C) and added to MDCK cell monolayers for 90 min. Cell lysis was assessed as described above.

Results

The cytological appearance of *T. vaginalis* strains

Observations of fresh FMV1 parasites collected from axenized cultures resulted in the detection of highly polymorphic microorganisms including elongated, spherical, pear-shaped and amoeboid cells (Fig. 1A). Conversely, we did not observe such morphological variability among parasites collected from axenic JT cultures. The latter were mainly found as pear-shaped forms (Fig. 1B). FMV1, but not the JT strain, quickly (10 min) adhered to glass and plastic surfaces.

The ultrastructure of the *T. vaginalis* strains

The ultrastructure of the FMV1 and JT strains of *T. vaginalis* was examined by scanning and transmission electron microscopy. Most of the FMV1 parasites presented a profuse formation of pseudopodia such as lamellipodia and fillopodia. Many of these pseudopodia emerged from the rear portion of the cells near the axostyle (Fig. 2A) and often mediated adhesion among parasites (Fig. 2B). Contrarily, JT parasites did not present significant pseudopod formation or membrane ruffling (Fig. 3A–C). Most of these parasites were oval or spherical (Fig. 3A, B) and some resembled pseudocysts (Fig. 3C). However, in the FMV1 strain, pseudopodia were present and usually mediated adhesion among neighboring parasites (Fig. 3D).

We employed transmission electron microscopy to further characterize the parasite's homophilic adhesion. We observed intimate contact among cells where cortical cytoplasmic organelle exclusion was observed (Fig. 4A). Parasites were often seen endocytosing the debris of the dead conspecifics (Fig. 4B–D).

In order to investigate whether the procedure employed to axenize parasite cultures could induce alterations in the protozoan morphology, we studied the freshly isolated parasites carefully collected from the cotton swabs. These cells were inoculated into tubes containing TYM medium, immediately harvested by centrifugation, resuspended in PBS and prepared for transmission and scanning electron microscopy. As can

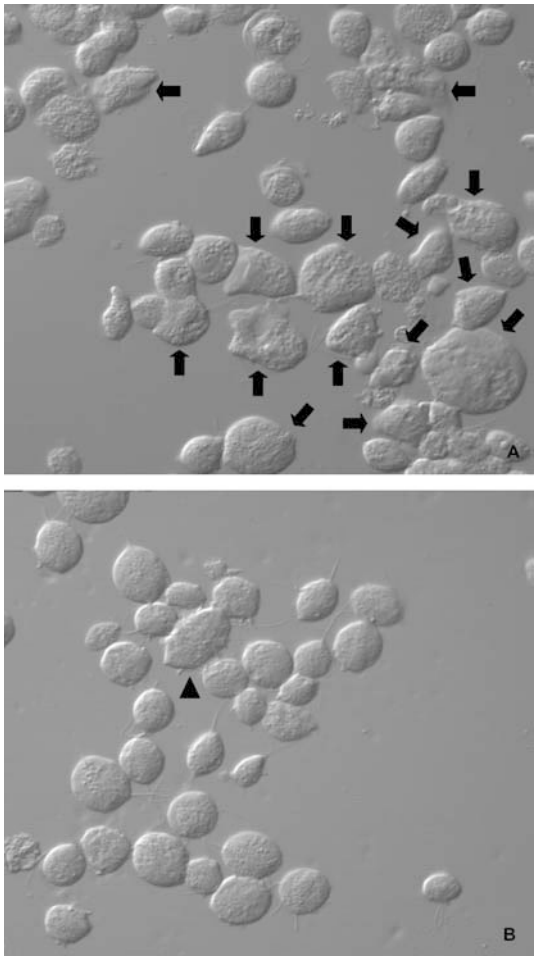

Fig. 1A, B Interferential contrast microscopy of *Trichomonas vaginalis* grown in axenic culture medium for 24 h. **A** General morphology of a fresh clinical isolate of *T. vaginalis* named FMV1. Note the high morphological diversity illustrated by the large number of amoeboid cells (*arrows*). **B** General morphology of long-term cultured *T. vaginalis* JT strain presenting homogenous oblong or pear-shaped forms. *Arrowheads* indicate trophozoites during the process of division

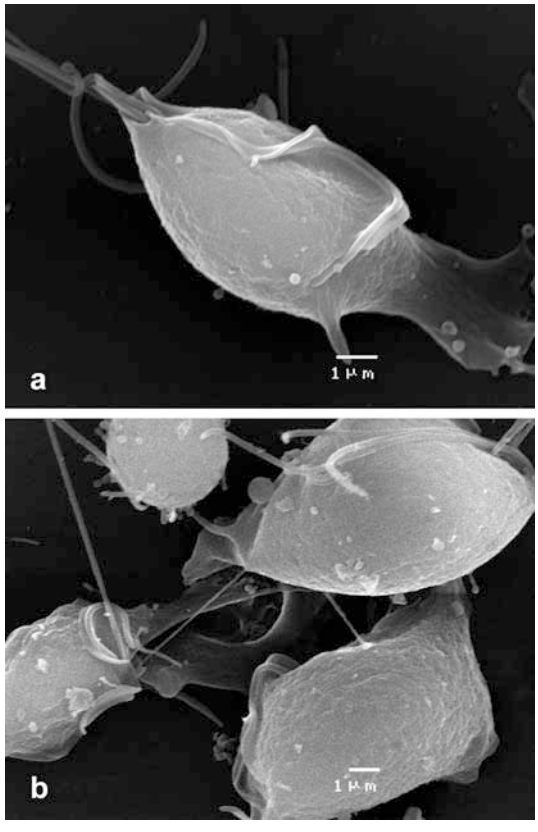

Fig. 2A, B Scanning electron micrographs of the fresh *T. vaginalis* isolate (FMV1 strain) grown in axenic culture for 24 h. The amoeboid forms grown in suspension as **A** isolated or **B** aggregates of three to six parasites associated with each other by pseudopodia

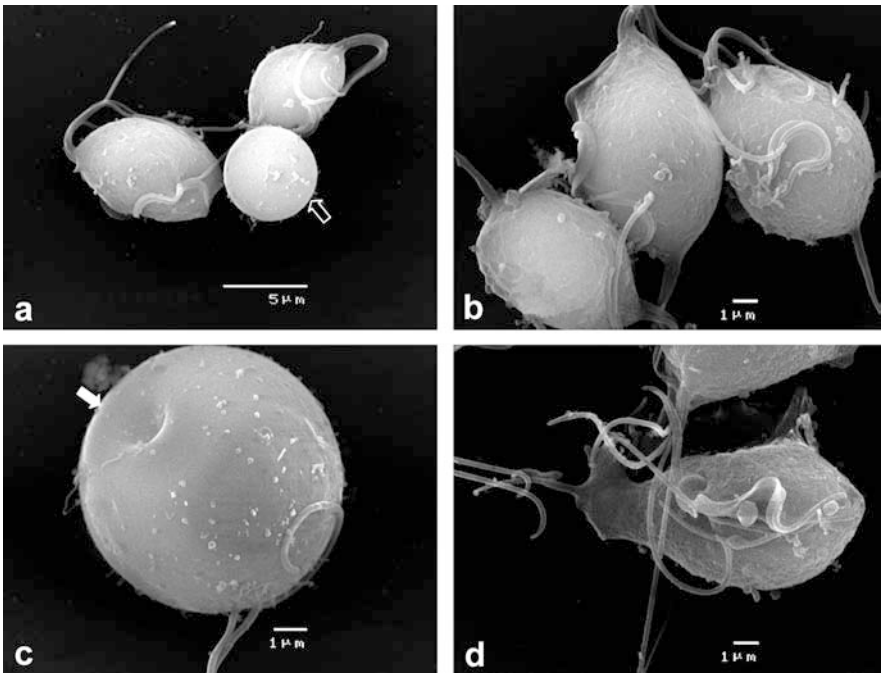

Fig. 3 Scanning electron micrographs of *T. vaginalis* JT (**A**–**C**) and FMV1 (**D**) strains grown in axenic culture for 24 h. Both ellipsoid and spherical forms but no amoeboid forms can be observed among the JT microorganisms. JT parasites, even when contacting each one, did not extrude pseudopodia, in contrast to what was observed for FMV1 (**D**). Some spherical forms of JT strain (**A**, **C**) resemble pseudocysts, since the anterior and/or recurrent flagella, as well as the axostyle, seem to be invaginated (*arrows*)

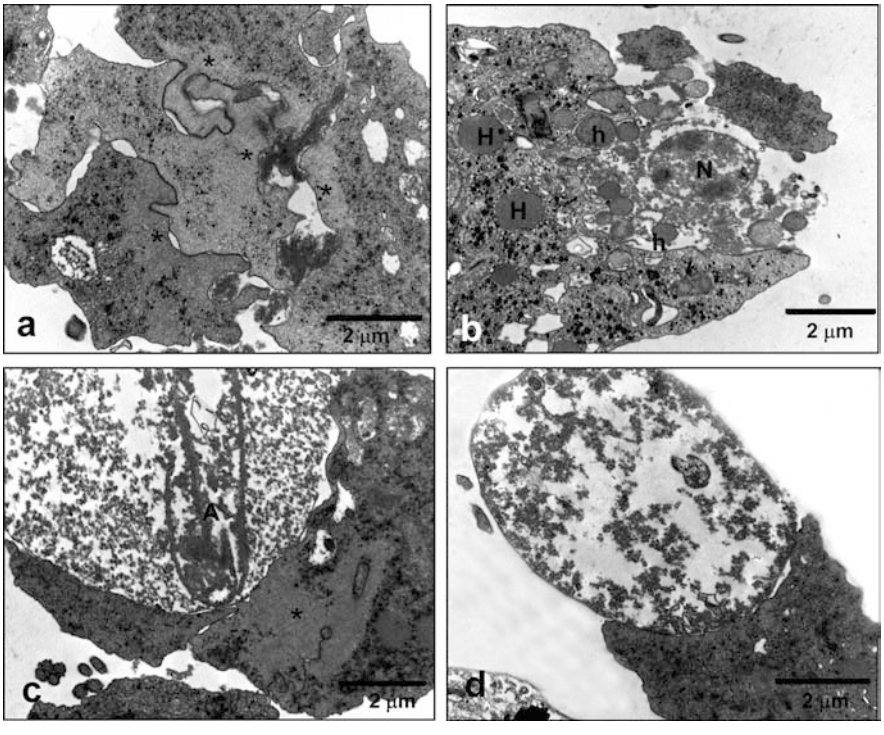

Fig. 4A–D Transmission electron microscopy of FMV1 *T. vaginalis*. **A** Homophilic adhesion among trophozoites mediated by numerous interdigitating pseudopodia which are devoid of organelles (*asterisk*). **B**–**D** *T. vaginalis* ingesting the remnants of its conspecific parasites. **B** Free hydrogenosomes (*h*), nuclei (*N*) and axostiles (**C**, *A*). Even whole cells presenting washed out cytoplasm were phagocytosed (**D**)

be seen in Fig. 5A and B, the surface topography of freshly isolated *T. vaginalis* revealed a morphological appearance similar to that of axenized cultures, including general cell shapes and pseudopod emission. In addition, it was possible to observe physical contact between the flagella and cytoplasmic protrusions of the parasites (Fig. 5B). The fresh isolate, as expected, included some bacteria from the normal vaginal flora (Fig. 5A). In addition, the different morphological subpopulations displayed by JT and FMV1 parasites were estimated (Fig. 6). The FMV1 strain showed about 21.0% amoeboid, 49.2% ellipsoid, 24.0% spherical and 5.7% intermediate (ellipsoid to spherical) forms, whereas the JT strain presented no amoeboid and circa 89.8%, 7.8% and 2.3% ellipsoid, spherical and intermediate forms, respectively.

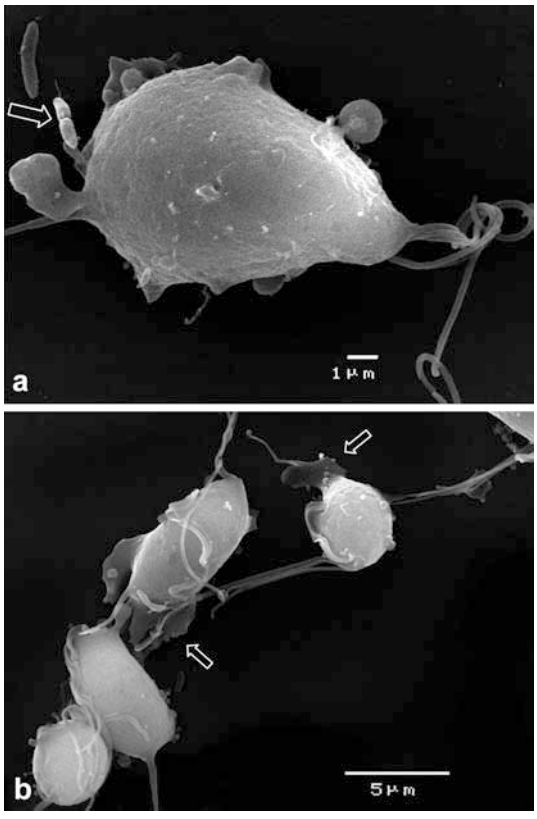

Fig. 5 Scanning electron micrographs of the fresh clinical FMV1 isolate of *T. vaginalis* grown for 24 h just after collection prior to axenization (**A**, **B**). An amoeboid form of the parasite (**A**) is ingesting a vaginal bacterium (*arrow*). Pseudopodia (**B**) can be observed during cell-cell contact as well as in single amoeboid forms (*arrows*)

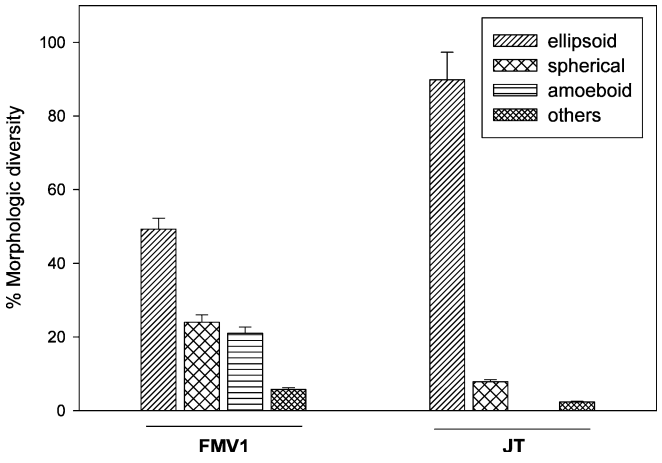

Fig. 6 Measurement of morphological diversity among *T. vaginalis* FMV1 and JT strains. The parasites were axenically cultivated in TYM medium for 24 h and observed by interferential contrast microscopy (400×). The different parasite morphotypes were counted using a Neubauer chamber. Results are expressed as the means(±SE) of three independent cell suspensions ($n \cong 1{,}200$ cells)

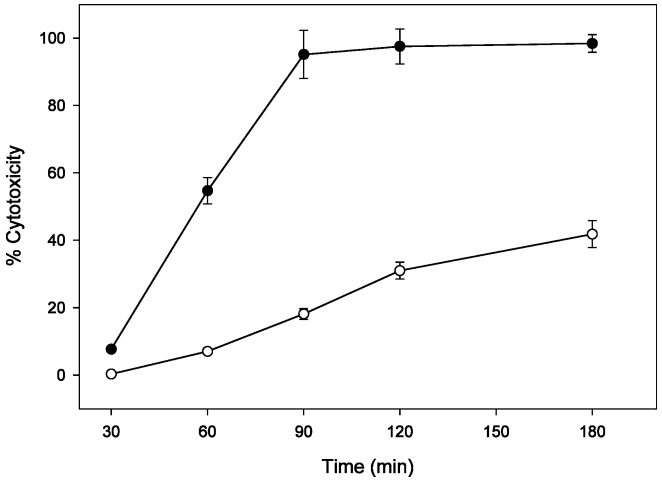

Fig. 7 The time-course of cytotoxicity exerted by *T. vaginalis* on MDCK cells by the FMV1 strain (*filled circles*) or the long-term cultured JT strain (*empty circles*). A total of 5×10^5 parasites were added to the MDCK cell monolayers. Cytotoxicity was spectrophotometrically determined by dye recovery at 660 nm. Control monolayers were not in contact with *T. vaginalis*. The values represent the means(±SE) of at least three independent experiments performed in triplicate

T. vaginalis cytotoxicity for epithelial cells

The cytopathic effects exerted by both the FMV1 and JT parasite strains were evaluated. FMV1 parasites were far more cytotoxic than the JT ones (Fig. 7). In MDCK monolayers kept in contact with FMV1 parasites, most of the epithelial cells were lysed after 90 min (Figs. 7 and 8A, right panel). On the other hand, only 42.0% cytolysis could be detected in MDCK cells incubated with JT parasites, even after 180 min. In 60 min FMV1 parasites disrupted 54.7% of the monolayer cells (Fig. 7).

In order to explore the contact dependence of the cytotoxicity mechanism exerted by FMV1 and JT parasites to MDCK cells, assays were carried out using supernatants from parasite axenic cultures. No significant monolayer disruption or cell lysis was observed in the presence of supernatants from either parasite strain in the conditions employed (Fig. 8A, left panel and B).

Discussion

Morphological variability among pathogenic trichomonads such as *T. vaginalis* is determined by different environmental factors such as temperature, pH, oxygen tension, carbohydrates and contact with other cell types and on the parasite's molecular machinery to respond to these factors (Honigberg and Brugerolle 1990). In axenically grown cells, usually only ellipsoid and spheroid forms have been observed (Honigberg and Brugerolle 1990). On the other hand, during adhesion to host cells or inert surfaces covered or not with extracellular matrix glycoproteins, most parasites become amoeboid (Silva Filho and De Souza 1988; Crouch and Alderete 1999). Such amoeboid forms of *T. vaginalis* are highly cytoadherent and cytotoxic (Alderete et al. 1995). Moreover,

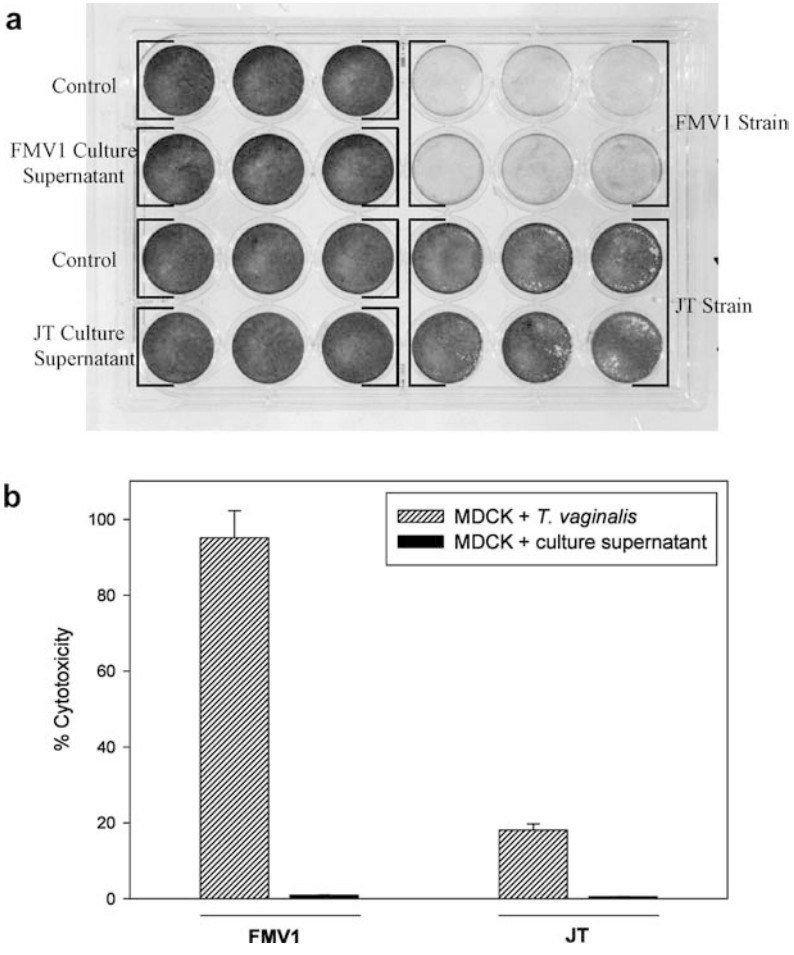

Fig. 8A, B A comparison of the cytotoxicity exerted by either supernatants or whole parasites of the FMV1 or JT strain of *T. vaginalis* on MDCK monolayers. A total of 5×10^5 parasites or their respective supernatants were added to the MDCK cells monolayers for 90 min. **A** The monolayers were stained after parasite-host cell interaction. **B** Cytotoxicity was spectrophotometrically determined by dye recovery at 660 nm. Control monolayers were not in contact with *T. vaginalis*. The values represent the means($\pm$SE) of at least three independent experiments which were performed in triplicate

similar amoeboid forms are easily observed in association with human vaginal epithelial cells (Nielsen and Nielsen 1975; Alderete and Garza 1988; Petrin et al. 1998). Fresh isolates of *T. vaginalis* also adhere and modify their morphology upon contact with laminin and fibronectin, suggesting that a common signaling mechanism may be triggered by adhesion (Crouch and Alderete 1999).

T. vaginalis binds in vivo to stratified, terminal squamous epithelial cells of the vaginal mucosa, a target different from HeLa cell monolayers (Alderete and Garza 1988; Arroyo and Alderete 1989; Arroyo et al. 1993) or other cell culture systems usually employed for in vitro studies (Arroyo et al. 1993; Silva-Filho and De Souza 1988; Krieger et al. 1985). Several studies performed on tissue samples obtained from the vaginal biopsies of patients with trichomoniasis demonstrated amoeboid *T. vaginalis* attached through interdigitations formed between the parasite and epithelial cell surfaces (Nielsen and Nielsen 1975; Petrin et al. 1998). Therefore, the interaction with human vaginal epithelial cells appears to be an important triggering factor for the transformation of the ellipsoid and spheroid trophozoites into amoeboid ones (Rasmussen et al. 1986; Honigberg and Brugerolle 1990; Arroyo et al. 1993; Alderete et al. 1995). In addition to vaginal epithelial cells, human erythrocytes and microorganisms of the vaginal flora have also been found to induce morphological changes in *T. vaginalis* (Rendon-Maldonado et al. 1998). In spite of these observations, we have shown that amoeboid forms of *T. vaginalis* (Figs. 1, 2, 4, 5) can be found in vitro and in situations distinct from previous reports (Brugerolle et al. 1974; Nielsen and Nielsen 1975; Rasmussen et al. 1986; Honigberg and Brugerolle 1990; Arroyo et al. 1993; Alderete et al. 1995; Petrin et al. 1998; Gilbert et al. 2000). Here we demonstrate that a fresh *T. vaginalis* isolate (FMV1 strain) presents amoeboid forms even after axenic subculturing for many weeks (Fig. 5) which are similar to 24 h-isolated ones (Fig. 4A, B). Such morphological diversity is strongly distinct from the homogeneity and absence of amoeboid forms of the JT strain, which has been maintained in culture for several years (Figs. 1A, 3A–C). Under axenic conditions, FMV1 parasites displayed remarkable homophilic adhesion with pronounced surface interdigitation. Interestingly, *T. vaginalis* pseudopodia were devoid of organelles and presented a fibrillar/granullar appearance. *T. vaginalis* has a cortical actin cytoskeleton (Brugerolle et al. 1996) with associated α-actinin (Bricheux et al. 1998) and coronin (Bricheux

et al. 2000) in the pseudopodia. The pseudopod-mediated homophilic adhesion may lead to the phagocytosis of dead parasites. Microbial cannibalism has been observed in different protozoa, but this is, to our knowledge, the first report for *T. vaginalis*. It remains to be determined whether cytotoxic mechanisms, such as perforin or hydrolase release, take place among living parasites under intraspecific competition. This kind of mechanism might control the *T. vaginalis* population, preventing a very high parasite load and invasive symptomatic infections. In addition, while the material was being prepared for light microscopy examination, the FMV1 strain rapidly adhered to glass surfaces, changing into the amoeboid form in 5–10 min (Fig. 1A), but such modifications were not observed in the JT strain (Fig. 1B). Spherical forms that appear to have invaginated flagella and axostyle (Figs. 3A, C), were observed in the 24 h-cultured JT strain even without temperature or nutrient modification. Several authors have identified such forms in other trichomonads and suggest that these represent pseudocysts, characterized as sessile spherical forms with no true cystic wall (Honigberg and Brugerolle 1990; Granger et al. 2000). This uncommon phenomenon occurs when the parasites are submitted to temperature or chemical stress. However, *T. vaginalis* pseudocyst formation has not been reported before, particularly under normal culture conditions.

Epithelial and non-epithelial cells have been used in studies assessing the *T. vaginalis*–host cell interaction. The epithelial cell lineages used in such studies include WISH (Martinotti et al. 1986), HeLa (Alderete and Pearlman 1984; Pindak et al. 1986; Escario et al. 1995), MDCK (Silva Filho and De Souza 1988) and human primary vaginal epithelial cultures (Rasmussen et al. 1986; Arroyo et al. 1993; Gilbert et al. 2000). However, *T. vaginalis* fails to adhere to, or disrupt, bovine vaginal epithelial cells and human vaginal fibroblasts (Gilbert et al. 2000). The *T. vaginalis* interaction with human vaginal epithelium is cell type- and species-specific. Such data are in contrast with many reports that demonstrate the adherence and cytotoxicity of different strains and isolates of *T. vaginalis* on diverse mammalian cells (Heath 1981; Alderete and Pearlman 1984; Pindak et al. 1986; Silva Filho and De Souza 1988; Escario et al. 1995). In the present study we demonstrated that the fresh *T. vaginalis* isolate (FMV1 strain), as well as a long-term cultured strain (JT), are able to adhere and induce cytopathic effects on a MDCK cell monolayer, an epithelial (non-vaginal) canine cell, with different lysis capabilities (Figs. 7, 8). The importance of *T. vaginalis* adhesion to the host cell for its cytopathic effect has been extensively demonstrated (Alderete and Pearlman 1984; Arroyo et al. 1993: Alderete et al. 1995; Petrin et al. 1998; Gilbert et al. 2000). Nevertheless, several authors have reported that epithelial cell cytopathogenicity can be exerted even without cell adhesion, as the parasite releases and/or secretes several soluble toxic molecules into the extracellular milieu (Silva Filho et al.1986; Silva Filho and De Souza 1988; Garber et al. 1989). We previously noticed that *T. vaginalis* releases acid phosphatase into the culture medium (Jesus et al. 2002) and that the secreted acid phosphatase activity is able to promote macrophage invasion by *Leishmania amazonensis* (Vannier-Santos et al. 1995). Furthermore, acid phosphatase secreted by *Entamoeba histolytica* can detach HeLa cells in vitro (Anaya-Ruiz et al. 2003). In the present study, no such effect was detected and cytotoxicity was solely contact-dependent (Figs. 7, 8) corroborating previous reports (Garcia-Tamayo et al. 1978; Alderete and Pearlman 1984; Krieger et al. 1985; Arroyo et al. 1993; Fiori et al. 1997; Gilbert et al. 2000).

T. vaginalis pathogenicity has always been a matter of controversy. The problem arises, in part, because natural infections by this parasite are known to occur only in humans and because the associated disease ranges from the absence of symptoms to quite severe pathology (Petrin et al. 1998). However, in vivo experimental inoculation has demonstrated that both pathogenic and non-pathogenic *T. vaginalis* isolates can cause lesions (Honigberg 1961). Studies using strains with different degrees of virulence have shown similar results (Honigberg et al. 1966; Kulda et al. 1970; Farris and Honigberg 1970; Krieger et al. 1983; Kuczynska et al. 1984). Several authors have tried to relate *T. vaginalis* pathogenicity for humans with its cytopathogenicity to epithelial cell cultures. Long-term cultured strains and those obtained from asymptomatic patients present lower cytopathic potential after 24 h interaction with human vaginal epithelial cells than strains isolated from symptomatic patients (Gilbert et al. 2000), as the relationship between in vitro cytotoxicity and cytoadherence does not reflect the in vivo behavior of *T. vaginalis* (Escario et al. 1995). This is further reinforced by the findings of this study. The FMV1 strain was obtained from a patient who remained asymptomatic even without treatment, and a sample, obtained after 7 months, was negative for *T. vaginalis*.

The low in vivo virulence of the FMV1 strain could be related to host characteristics such as immune response and vaginal flora, as well as parasite properties such as the secretion of cytolytic factors or cannibalism (Matuschka and Bannert 1987), which could control a population of the potentially pathogenic forms. It could lead to lower parasite loads and consequently produce asymptomatic cases, which would favor transmission in the absence of dyspareunia. The present study suggests that highly polymorphic and fresh adherent isolates may display high virulence in vitro but not in vivo.

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