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Observation of membrane fusion on the interaction of *Trichomonas vaginalis* with human vaginal epithelial cells

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Abstract The in vitro cytopathic effect of *Trichomonas vaginalis* on epithelial cells was analyzed through the interaction of two parasite strains with freshly collected human vaginal epithelial cells (HVECs) from normal women. Videomicroscopy, light and electron microscopy (scanning and transmission), freeze-fracture, the tracer lanthanum nitrate, and the periodic acid-thiosemicarbazide-silver proteinate techniques were used to analyze regions of close contact between the HVECs and *T. vaginalis*. After 2 h of interaction, all HVECs were dead, whereas all the trichomonads were alive. Microscopic observations demonstrated that in addition to previously described regions of adhesion and interdigitations, areas of continuity between the cytoplasm of the two interacting cells were found. They were not easy to find since they correspond to focal spots placed in different depths of the section. When these regions were depicted, the plasma membranes of the *T. vaginalis* and the vaginal epithelial cells seemed to be fused.

Introduction

Trichomonas vaginalis is the causative agent of human trichomoniasis, one of the most common parasitic infections in humans. These are flagellate protozoa, which interact with the squamous epithelium lining the human urogenital cavities (Honigberg 1978). Despite its high incidence, especially among women of all world societies, little is known about the pathogenesis of trichomoniasis and many questions remain open. The parasite does not penetrate the epithelium and is therefore considered noninvasive. It adheres to the epithelial cells, often forming interdigitations (Rasmussen et al. 1986; González-Robles et al. 1995). It has been suggested that substances released by the parasite and/or through direct cell contact (Nielsen and Nielsen 1975; Krieger et al. 1985; Krieger 1990; Alderete et al. 1995; González-Robles et al. 1995) may produce epithelial cell damage. Previous studies have used epithelial monolayer models, such as MDCK, Madin-Darby Canine Kidney (cells). (González-Robles et al. 1995), HeLa cells (Alderete et al. 1995; Krieger 1990), and Chinese hamster ovary (CHO) cells (Krieger et al. 1985) to examine the cytopathic effect of *T. vaginalis*. González-Robles et al. (1995) have demonstrated that damage to MDCK cells initially occurs through adhesion and clumping of parasites, which induce changes in the plasma membrane of target cells followed by cell death. These authors also presented one form of adhesion where the formation of surface microchannels takes place. Alderete et al. (1995) claim that the specific cytoadherence is responsible for the cell cytotoxicity and that the extent of host cell killing is related to the levels of parasite cytoadherence.

In the present study we decided to visualize better the membrane-membrane contact points using techniques that allow visualization of the intimate contact at the interdigitating area at different planes of the cell. For further investigation of this point, normal human vaginal epithelial cells (HVECs) were used as target cells for two strains of *T. vaginalis* [30236 from American Type Culture Collection (ATCC) and the virulent JT strain isolated in Brazil]. The morphology of the interaction process was studied using videomicroscopy, light microscopy, scanning electron microscopy, and thin sections as analyzed by transmission electron microscopy. Freeze-fracture replicas and thin sections of cells processed after incubation with the tracer lanthanum nitrate were also used in the present study.

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Materials and methods

Cells

Trichomonads

One strain of *Trichomonas vaginalis* used in this study was obtained from ATCC (strain 30236), and the Judith strain was isolated from a patient attending the University Hospital at Ilha do Fundão, Rio de Janeiro, Brazil. The cells were grown in medium 745 (ATCC) for 24–36 h at 35 °C. The parasites were collected by low-speed centrifugation, washed three times in phosphate-buffered saline (PBS, pH 7.2), and processed using one of the following procedures.

Human vaginal epithelial cells

HVECS were collected by scraping of the vaginal surface with a disposable swab and were immediately suspended in 10 ml of warmed (35 °C) PBS. The donors were healthy young women (24 years old) working as scientific staff in our laboratory. The trypan blue viability test was performed before each experiment. The cells were collected weekly, during the 1st month, and, thereafter, in different periods during the course of 2 years.

Interaction assay

Culture tubes containing the parasites were cooled in an ice-water bath for 10 min, and the cells were pelleted by centrifugation at 1,000 *g* for 5 min. Trichomonads were counted in a Neubauer chamber and resuspended in pre-warmed (35 °C) TYM medium. Thereafter, these cells were added at a parasite/HVEC ratio of approximately 10:1 to 75-cm^3 plastic tissue-culture flasks (Falcon, USA). At the beginning, different interaction times were tested: 30 min, 1 h, 2 h, 5 h, 12 h, and 24 h. We chose 5 h for all subsequent experiments. As controls, some HVECs without trichomonad interaction were left in the culture medium for 2 or 5 h and the trypan blue viability test was performed.

Videomicroscopy

A 40 × 0.75 objective lens was chosen to give a good depth of field, making it possible to visualize the interaction process. The sequences of videomicroscopy images were obtained with a high-resolution videocamera (Pasecon-Pal-G system/Grundig electronic or NTSC-optronics camera) and the IBAS digital image processing system (Kontron-Zeiss) attached to a Zeiss Axioplan light microscope. The video images were subjected to a process of analog contrast enhancement and digitized, and a background-defocused image was then subtracted. Afterward a special procedure to enhance contours was used in each frame.

Light microscopy

Examination of *T. vaginalis* before and after the interaction with HVECs was carried out in a Zeiss Axiovert inverted microscope and Axioplan equipped with differential Nomarsky contrast optics.

Scanning electron microscopy

After 5 h of interaction the cells were collected by centrifugation at 1,500 *g* for 5 min and fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2) for 2 h at room temperature. After fixation they were washed three times in PBS, allowed to adhere to poly-L-lysine-coated glass coverslips, postfixed for 5 min at room temperature in 1% OsO_4 in cacodylate buffer, dehydrated in ethanol, critical-point-dried with CO_2, coated with gold, and observed in a Zeiss EM 962 electron microscope.

►

Fig. 1 Interference contrast (Nomarsky) of a human epithelial cell in contact with *Trichomonas vaginalis* after 5 min of interaction. (*T T. vaginalis*, *V* vaginal epithelial cell) × 2,000. **Fig. 2** Scanning electron micrograph (SEM) showing an early event occurring after 5 min of interaction between *T. vaginalis* trophozoites and an HVEC. The HVEC presents microvilli all over its surface and many attached parasites. The parasites adhere by their flagella and by the axostyle-containing projection (*long arrows*). (*Short arrow* Anterior flagella, *T. trophozoites*) × 4,000. **Fig. 3** Higher magnification SEM showing microchannels (*arrows*) at the surface of a *T. vaginalis* trophozoite (*T*) after 1 h of interaction. The HVEC (*V*) presents small pores on its surface (*arrowheads*). × 9,000. **Figs. 4, 5** Thin sections of the areas of cytoplasmic interdigitations after 1 h of interaction. Visible are areas of continuity (*arrows*) between the plasma membranes of the *T. vaginalis* (*T*) and the HVEC (*V*). Several bacteria can be seen in the vacuoles of the parasite (*b*). (*Arrowheads* Glycogen granules) **Fig. 4** × 36,000. **Fig. 5** × 22,500

Transmission electron microscopy

The cells were fixed overnight at room temperature in 2.5% glutaraldehyde/4% paraformaldehyde/5 m*M* $CaCl_2$ in 0.1 *M* PHEM buffer [5 m*M* $MgCl_2$, 70 m*M* KCl, 20 m*M* HEPES, 60 m*M* PIPES, and 10 m*M* ethylene glycol tetraacetic acid (EGTA), pH 7.2] (Schliwa and Blerkom 1981). Postfixation was performed in 1% OsO_4 in the same buffer plus 5 m*M* $CaCl_2$ and 0.8% potassium ferricyanide for 30 min (Hepler 1980). Thereafter the cells were washed in PBS, dehydrated in acetone, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and observed in either a Zeiss 900 or a Zeiss 902 electron microscope. When necessary, the grids containing the thin sections were tilted and the image was rotated up to 45° using the goniometer accessory of the Zeiss 902.

Freeze-fracture

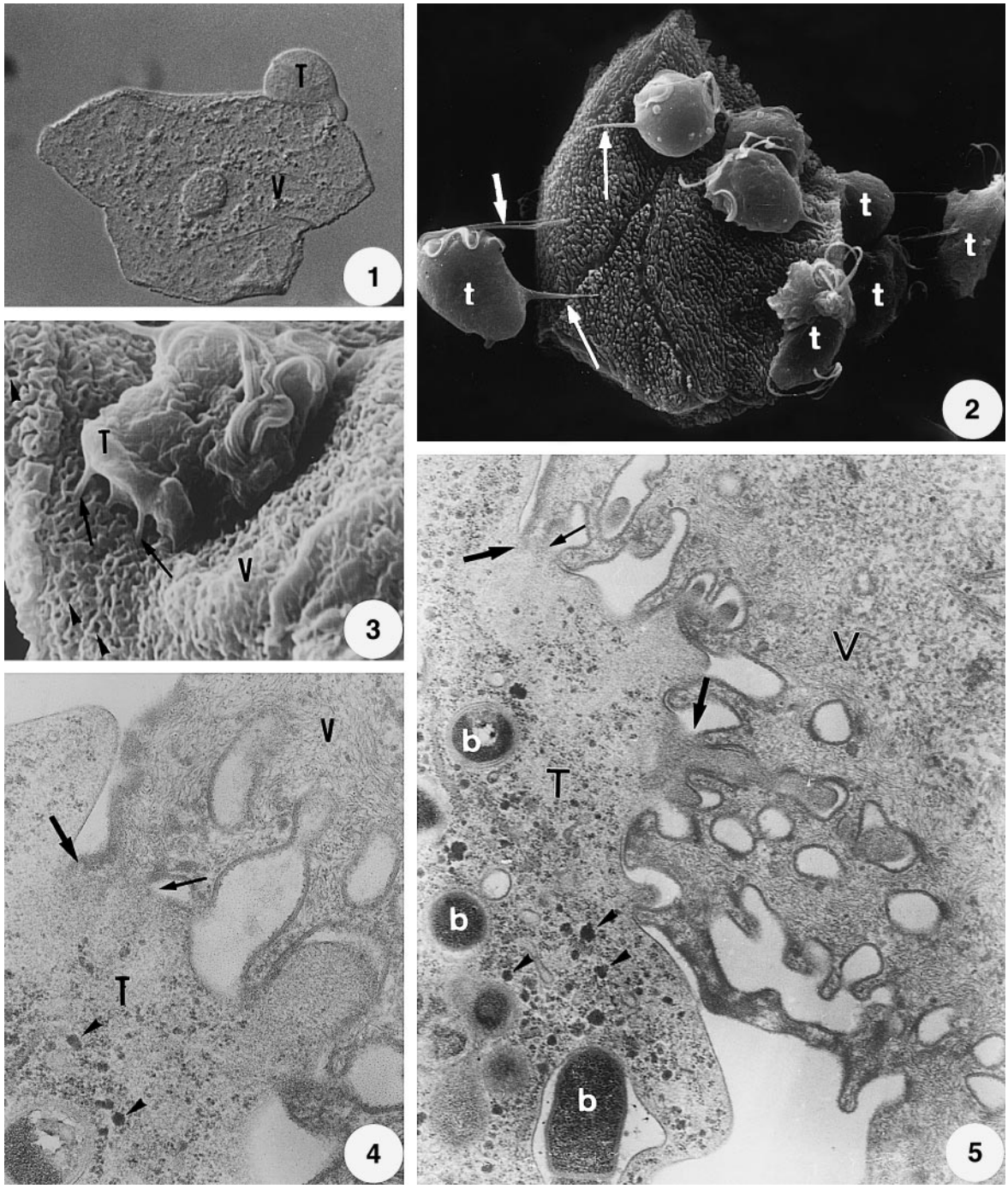
For conventional freeze-fracture, after the interaction the cells were fixed in 2.5% glutaraldehyde as described above, washed twice in PBS, and then exposed to ascending concentrations of glycerol in 0.1 *M* cacodylate buffer until a final concentration of 30% glycerol was attained. Specimens were mounted on Balzers support disks, rapidly frozen in the liquid phase of Freon 22 cooled by liquid nitrogen, and immediately transferred to liquid nitrogen. Frozen cells were freeze-fractured at −115 °C in a Balzers BAF 300 freeze-etching machine. The specimens were shadowed with platinum/carbon at 2×10^{-6} Torr after fracturing. Replicas were recovered in distilled water, cleaned with sulfuric acid and/or sodium hypochloride, mounted on 200-mesh grids, and examined in a Zeiss 902 electron microscope.

Periodic acid-thiosemicarbazide-silver proteinate technique

Ultrathin gold sections from material previously embedded in Epon were collected on gold grids and treated with 1% periodic acid for 20 min at room temperature (Thiéry 1967). After being rinsed in distilled water, grids were treated with 1% thiosemicarbazide in 10% acetic acid for 72 h at room temperature. After being rinsed sequentially with 10%, 5%, and 1% (v/v) acetic acid and with distilled water, the material was exposed to 1% silver proteinate for 30 min in a dark environment. Sections were observed unstained in a Zeiss EM 900 electron microscope. Controls were obtained by omission of the treatment with periodic acid.

Lanthanum nitrate technique

After the interaction the cells were fixed for 2 h at room temperature in 2.5% glutaraldehyde/4% paraformaldehyde in 0.16 *M* Colidine buffer (pH 7.0) containing 2% lanthanum nitrate under



constant agitation. After fixation the cells were washed twice in the same buffer with lanthanum nitrate and then once in PBS, post-fixed, and processed for transmission electron microscopy (TEM) as described above (Hayat 1975). The grids containing the thin sections were tilted and the image was rotated up to 45° using the goniometer accessory of the Zeiss 902.

Results

The viability test by trypan blue exclusion showed that 12% of HVECs and 100% of trichomonads were alive at the beginning of the interaction process. After 2 h, all HVECs had died, whereas all trichomonads were alive. Although the trichomonads adhered to both live (Fig. 2) and dead cells (Figs. 3, 13) the present experiments were mainly performed on living HVECs. The whole sequence of events of interaction of the trichomonads with the HVECs was recorded on video (not shown). From the initial 5 min until 5 h after the beginning of the interaction it was possible to observe parasites adhering to HVECs at different positions as shown by light and scanning electron microscopy (Figs. 1, 2). They

appeared to be attached laterally to the HVECs by their flagellae and, in many cases, by the axostyle-containing projection (Figs. 2, 13). We did not observe any difference in attachment pattern between the two strains used in this study.

Following interaction, several parasites with a pear-like form changed to an amoeboid appearance (Figs. 3–7, 11). Zero, one, two, or a dozen trichomonads were found to be attached to one HVEC (Figs. 2, 11). The adhesion was strong enough to withstand subsequent steps of centrifugation. In most instances a hit-and-run effect was observed by videomicroscopy. This approach also provided evidence of the asynchronism of the lytic effect.

Observation by scanning electron microscopy (SEM) showed microvilli on the surface of living HVECs at the beginning of the interaction (Figs. 2, 11). No microvilli was seen, but small holes with a diameter of 0.1 μm were observed at the surface of HVECs after 5 h of interaction (Figs. 3, 13). Figure 3 demonstrates the presence of conspicuous microchannels. Some trichomonads were in tight lateral contact to HVECs by their flagellum-free surface (Figs. 1–3, 11), whereas others were rounded or pear-shaped with less extension of the contact area (Figs. 2, 11, 13).

By SEM and TEM it was possible to see both trichomonads and HVECs in good conditions of preservation after 5 min of interaction (Fig. 2), showing interdigitations at the contact areas (Figs. 4–6). Just below the plasma membrane of the interacting cells a web of microfilaments was observed and the cell coat became more prominent, especially in the HVECs (Figs. 4–6). Glycogen granules and several endocytic vesicles containing bacteria at different steps of degradation were seen (Fig. 5). Endocytic activity was seen as demonstrated in Fig. 6, in which a trichomonad is simultaneously interacting with an HVEC and ingesting a bacterium-like microorganism. This figure also shows fibrils connecting the trichomonad and the microorganism and the presence of a network of filaments in the cytoplasm of *Trichomonas vaginalis*. These fibrils were not seen between the HVEC and the bacterium-like microorganism (Fig. 6)

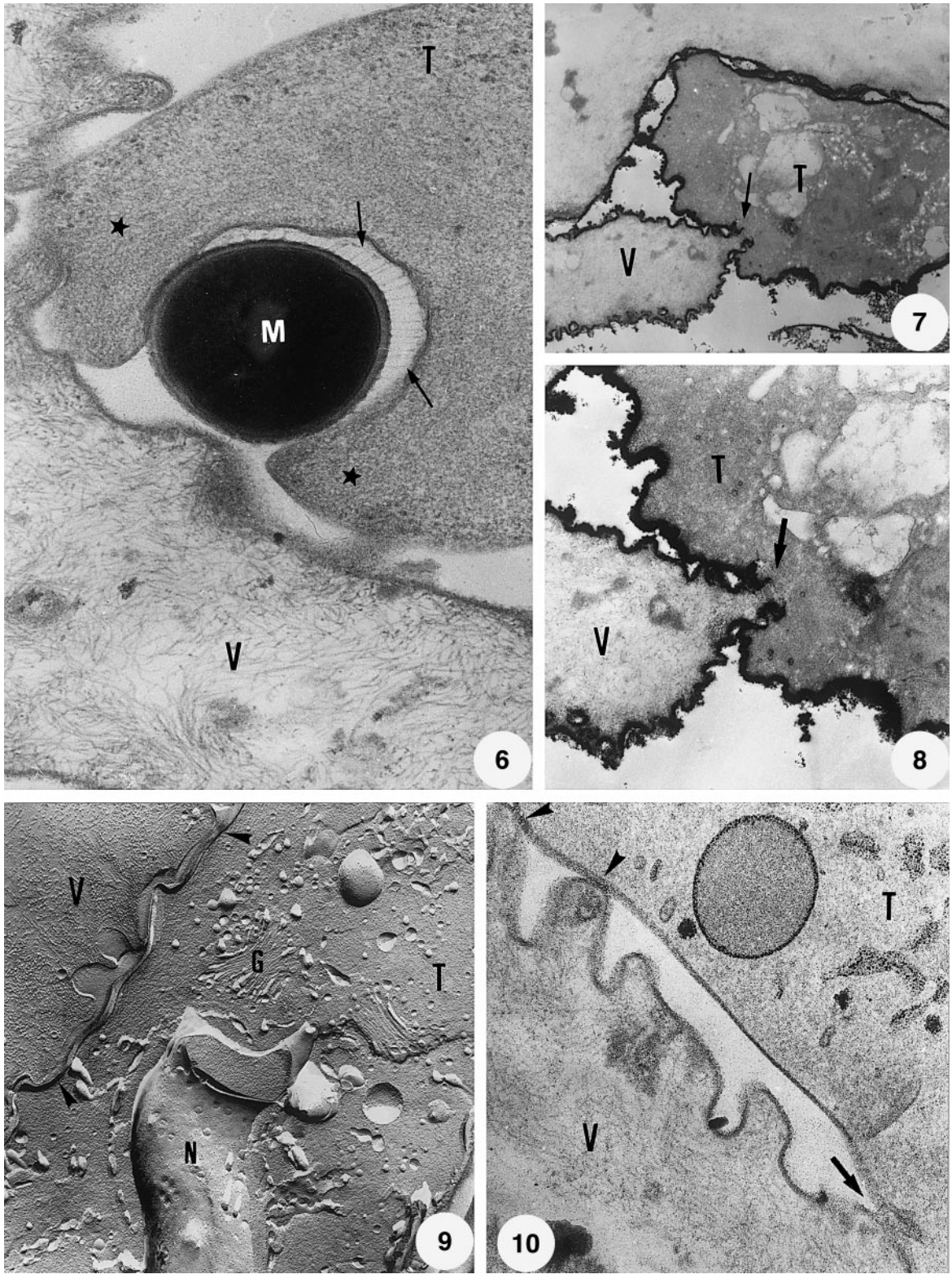
After 1 h of interaction, areas of contact appeared as regions of continuity between HVECs and *T. vaginalis* as shown by TEM (Figs. 4, 5), lanthanum nitrate (Figs. 7, 8), and freeze-fracture techniques (Fig. 12). In one case the HVEC was less electron-dense than the trichomonad cell (Figs. 7, 8). After fixation in the presence of lanthanum nitrate a heavy labeling of the cell coat of the two cells was observed, except at the region of close contact, where continuity between the two cells was evident. This continuity was observed even when the section was tilted 45° with the goniometer (Figs. 7, 8). Using the Thiéry technique, we also observed areas of close proximity of the cell coats (Fig. 10). Examination of freeze-fracture replicas showed large surfaces of inner or outer membrane halves and intracytoplastic structures (Fig. 9) in both *T. vaginalis* and HVECs. By freeze-fracture it was possible to detect some cytoplasmic vesicles very close to the contact area of interaction (Figs. 9, 12). Some replicas showed areas of continuity between the plasma membrane of *T. vaginalis* and that of HVECs (Fig. 12). In some experiments the cytoplasm of trichomonads appeared normal, whereas the HVEC showed signs of degeneration (Figs. 7–9) and depletion of microvilli (Figs. 3, 13). After 2 h of interaction, all HVECs were dead. Pores at their surfaces were easily observed, whereas the trichomonads appeared normal (Figs. 3, 13). There was no difference between the two strains used in the present study.

Fig. 6 Transmission electron micrograph of an interaction area, showing a microorganism (*M*) in the process of endocytosis. Symmetrically arranged fibrils (*arrows*) are disposed between the microorganism wall and the trichomonad (*T*) membrane. No fibril is present in the opposite region at the area between the microorganism and the HVEC. The cytoplasmic region of the trichomonad presents a network of microfilaments (*stars*). No organelle is visible in this area. The cytoplasm of the HVEC (*V*) shows filaments that are more concentrated at the attachment region. × 42,000. **Figs. 7, 8** Contact region between *T. vaginalis* (*T*) and an epithelial cell (*V*) after 1 h of interaction, showing continuity of the plasma membranes of the two cells (*arrows*) after the use of the tracer lanthanum nitrate. **Fig. 8** Higher magnification. **Fig. 7** × 6,000. **Fig. 8** ×10,000. **Fig. 9** Freeze-fracture of the region of interaction between a trichomonad (*T*) and a vaginal epithelial cell (*V*) after 5 h of interaction. The trophozoite presents a normal intracellular aspect, showing the Golgi complex (*G*), the nucleus with nuclear pores (*N*), and vesicles close to the surface, whereas the HVEC shows an empty aspect. The *arrowheads* point to areas of attachment. × 12,000. **Fig. 10** High magnification of an unstained ultrathin section subjected to the periodic acid-thiosemicarbazide-silver proteinate technique for detection of carbohydrates, showing a region of interaction. Reaction product is visible on the cell surfaces and in the vesicles and tubules in the trichomonad cell (*T*). The *arrowheads* show areas of adhesion and the *arrow* points to the membrane, which presents the first sign of fusion. × 30,000

Discussion

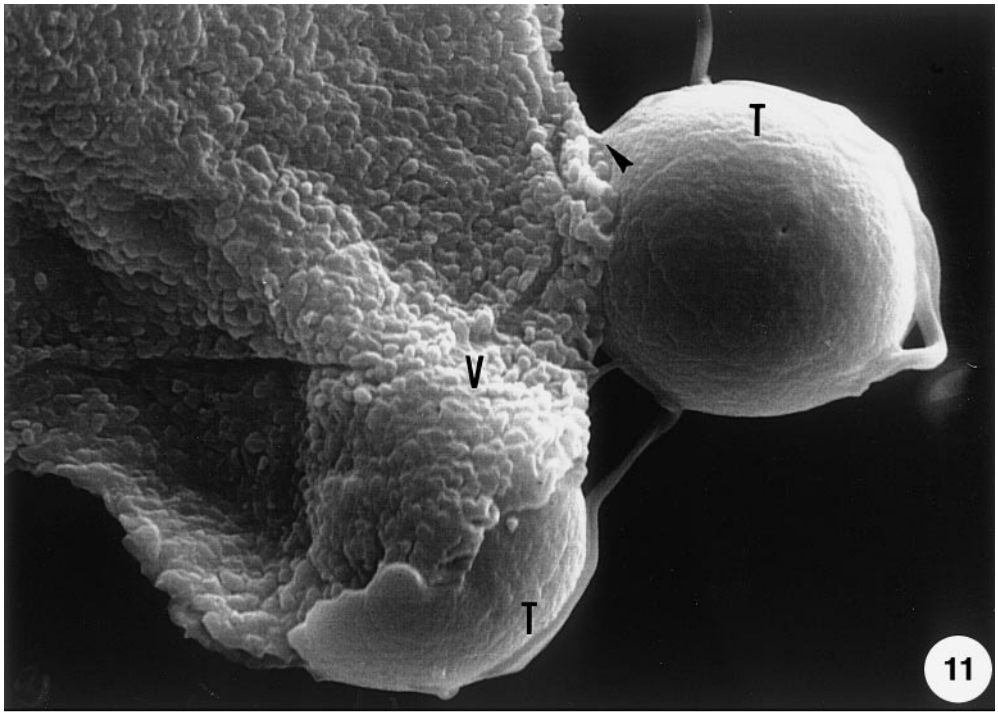
Extensive studies had previously been performed to clarify basic aspects of interaction between *Trichomonas vaginalis* and cells from the host (Brugerolle et al. 1974; Nielsen and Nielsen 1975; Heath 1981; Alderete and Pearlman 1984; Krieger et al. 1985; Rasmussen et al. 1986; Silva Filho and De Souza 1988; Krieger 1990; González-Robles et al. 1995). However, the mechanism responsible for the cytopathic effect had not yet been clarified.

Cytoadherence, one of the early steps in the infectious process, has been shown to be essential for the colonization and persistence of the parasite and has therefore been studied intensively (Alderete et al. 1995; González-Robles et al. 1995). It has been shown that cytoadherence involves the interaction of adhesins on the surface of the parasite with specific molecules of the host cell (Alderete and Garza 1988). Previous reports have shown that cytoskeletal elements are involved in the process of adhesion (Krieger et al. 1985; Silva Filho and De Souza



1988) and that proteinase treatment of living organisms abolishes parasite attachment to the host cell (Alderete et al. 1995). On the other hand, it has been shown that *T. vaginalis* kills target cells by direct contact without phagocytosis (Krieger et al. 1985).

Morphology studies have shown that during interaction the trichomonads acquire an amoeboid form (Rasmussen et al. 1986; González-Robles et al. 1995). The presence of interdigitations in the contact area and the reduced intercellular space in contact areas are also



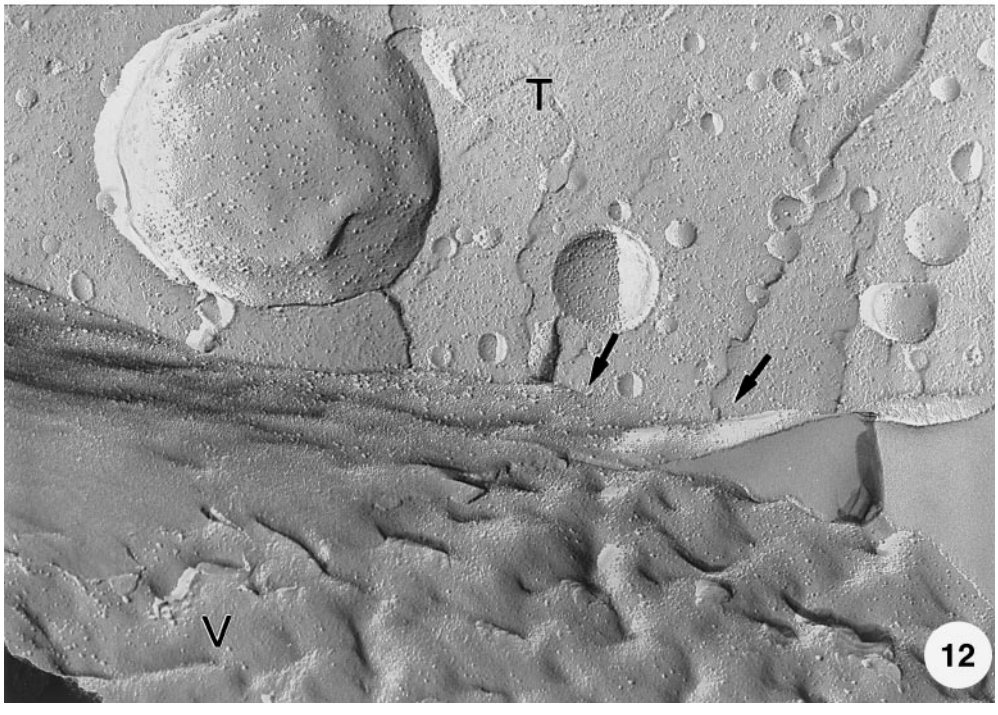


Fig. 11 SEM of two trichomonad cells (*T*) adhering to a normal HVEC (*V*). One of the trichomonads presents a rounded shape, whereas the other has adopted a flat and amoeboid configuration. The *arrowhead* points to a region of attachment. × 15,000. **Fig. 12** Freeze-fracture of the region of surface contact between a trichomonad (*T*) and an HVEC (*V*). In the trichomonad the fracture plane has reached the cytoplasm and part of the plasma membrane (*arrows*), whereas in the HVEC the fracture has exposed only the plasma membrane. The continuity between the two plasma membranes is clearly visible. × 53,000

well shown (Brugerolle et al. 1974; Nielsen and Nielsen 1975). On the other hand, microchannels through which microvilli and cytoplasmic fragments of epithelial cells have been internalized have been suggested to exist (González-Robles et al. 1995).

Honigberg (1978) and Arroyo et al. (1993) have suggested that a sign of virulence could be the capacity for transformation to an amoeboid shape. In the present study we found cells of the same strain presenting rounded and amoeboid shapes at the same time of interaction with HVEC. On the other hand, it has been shown that only living parasites are cytotoxic and host cells have shown irreversible damage caused by

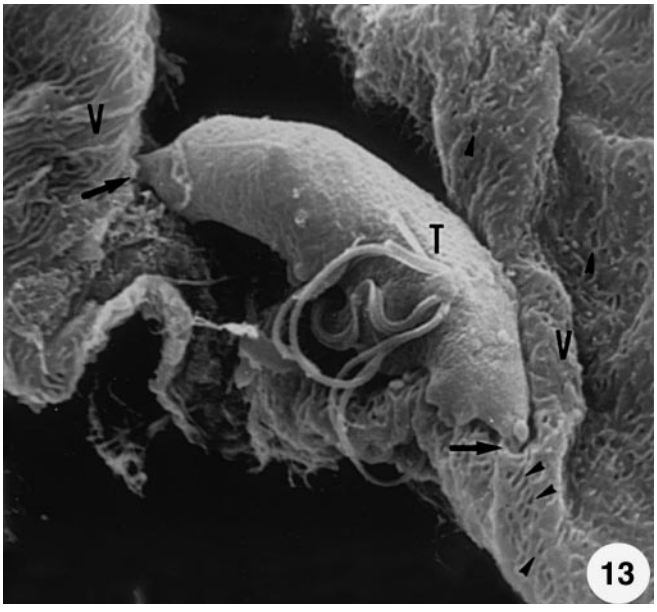


Fig. 13 SEM obtained after 5 h of interaction between an HVEC (*V*) and *T. vaginalis* (*T*). The interaction with the host cell occurs through both extremities of the parasite (*arrows*). No microvilli is visible on the surface of the epithelial cell, but many small holes can be seen (*arrowheads*). The HVEC looks dead. × 10,000

trichomonads (Alderete and Pearlman 1984). Also, Rasmussen et al. (1986) showed that *T. vaginalis* organisms with amoeboid morphology contained a dense network of microfilaments in the part of the trichomonads that was in contact with an epithelial cell. Recently, Brugerolle et al. (1996) presented evidence that the ectoplasmic microfibrillar layer along the adhesion zone of amoeboid *T. vaginalis* adhering to coverslips was mainly composed of actin microfilaments.

Electrophysiological methods have been used to establish transepithelial resistance in Madin-Darby Canine Kidney (MDCK) cells following contact with *T. vaginalis* (González-Robles et al. 1995). It was demonstrated that resistance dropped rapidly after the addition of live trichomonads, indicating that parasites induced a leak in the plasma membrane of the host cell, resulting in cell death. Trophozoites of *Entamoeba histolytica* are known to produce membrane damage (Martínez-Palomo et al. 1985). Also, evidence has been presented of functional pore formation on red cell membranes, leading to hemolysis by *T. vaginalis*. It was shown that hemolysis is a contact- and temperature-dependent phenomenon (Fiori et al. 1993).

Although many studies have been carried out, there is no information on the basic mechanism of membrane damage produced by *T. vaginalis* in host cells. In the present study we used fresh epithelial cells from vaginal swabs obtained from healthy women. We did not use the purification method for vaginal epithelial cells as previously suggested by Alderete et al. (1988), since in vivo the trichomonads are in contact with extensive mucus layer, vaginal fluids, and bacteria. After 2 h of interaction, no living HVEC was found, whereas all trichomonads survived and remained in adherence to HVECs. The viability of the host cells was revealed by the use of trypan blue and by the observation of small holes using SEM. No microvillus was observed at the host cell surface after 2 h of interaction.

Previous studies have shown that *T. vaginalis* adheres by the side opposite the active undulating membrane (Alderete and Garza 1985). We found many trichomonads attached in this way but also many adhering in variable positions as shown in Fig. 2. Nielsen and Nielsen (1975) had suggested that the space between the surface membranes of the trichomonads and the epithelium was occasionally partly obliterated. We used the Thiéry technique to visualize better the glycocalix of the two cells and to identify zones of focal contact between them. Rasmussen et al. (1986) used ruthenium red and showed staining of the whole cell surface, implying that there was no tight junction contact between closely apposed cell membranes. However, the authors presented a very low-magnification picture and showed only one focal plane from a thin section of cells. The junctional area could be located in another level of the section. On the other hand, tight junctions are belt junctions. We observed that in this case the junctional areas should be spot-like junctions.

Previous studies have shown that *T. vaginalis* is capable of ingesting mycoplasma and, more rarely, bacteria (Nielsen and Nielsen 1975), although the ingestion of *Neisseria gonorrhoeae* has been reported (Francioli et al. 1983). In the present study we frequently found ingested bacteria in trichomonads. We also observed that *T. vaginalis* could simultaneously attach to an epithelial cell and ingest a microorganism. Microfibrils were seen only in the phagocytosis area between the trichomonad plasma membrane and the microorganism cell wall. These fibrils were symmetrically disposed and were not present in the adhesion area or in the region facing the epithelial cell. We believe that the good fixation achieved in this experiment also enabled the preservation of adhesive structures involved in the microorganism-trichomonad association. On the other hand, we observed only images of tentative phagocytosis of epithelial cells by trichomonads.

Previous studies performed by Krieger et al. (1985) suggest that *T. vaginalis* exerts a cytolethal effect only on direct contact with a target cell. The absence of cytotoxicity in the presence of fixed trichomonads or fractions of culture filtrate strongly implicates contact-dependent mechanisms (Alderete and Pearlman 1984). The difficulty in finding the intimate contact zone indicative of how the pathogenicity occurs may be due to the observation that ultrathin sections do not show the three-dimensional appearance of the contact area and represent only a very small fraction of the cell profile (about 1/100). A thin section is about 70 nm thick, whereas the thickness of the cell is 7–8 μm. Scrutiny of hundreds of thin sections was necessary before one region could be found in which the continuity took place.

On the other hand, use of the freeze-fracture technique allowed us to find larger areas of contact, providing the advantage of exposing inner and/or outer half-membranes in which membrane specializations could be detected. We made the same observations found by other authors using SEM and thin sections: regions of attachment, interdigitations, and an intercellular space of 2–10 nm (Nielsen and Nielsen 1975; González-Robles et al. 1995). By the freeze-fracture technique, areas of membrane fusion were revealed in the present study.

The conspicuous microchannels shown in Fig. 3 had also been observed previously (González-Robles et al. 1995). In addition to their role in adherence to target cells, their possible function as a potential pathogenicity factor seems very likely.

Studies on cell junctions and on cell surface coats have frequently used heavy metals such as lanthanum hydroxide before and during fixation (Shea 1971; Peracchia and Dulhunty 1976). It was demonstrated that this material penetrated between the cell membranes as a good tracer. For determination of the space between the plasma membranes of host and parasite cells, incubation with lanthanum nitrate was performed after the interaction. The lanthanum appeared to cover the entire surface of all cells, except in some regions in which evident cytoplasmic continuity between cells was seen. In this case the section was tilted using the goniometer to see whether the assumed continuity could be confirmed. It seemed that the points of contact between cell membranes represent focal regions of membrane fusion.

On the basis of freeze-fracture studies and the use of tracers such as lanthanum nitrate, we suggest that fusion of the plasma membrane of *T. vaginalis* with that of the host cell takes place, inducing leakage and host cell death. In conclusion, we suggest that close proximity followed first by parasite attachment to the host cell and later by membrane fusion may be the mechanism that leads to the observed cell damage, cytopathic effect, and cell lysis.

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