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Signalling of *Trichomonas vaginalis* for amoeboid transformation and adhesin synthesis follows cytoadherence

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Summary

The cytoadherence of *Trichomonas vaginalis*, the sexually transmitted flagellated protozoan, to vaginal epithelial cells (VECs) is the key to infection. Electron microscopy revealed that *in vitro*-grown parasites having typical globular shape transformed rapidly after contact with VECs into thin, flat, amoeboid cells, maximizing the area of adhesion to the surface of VECs. Amoebic trichomonads formed filopodia and pseudopodia, which interdigitated at distinct sites on the plasma membrane of target cells. In contrast, the amoeboid transformation did not occur for *T. vaginalis* interacting with HeLa cells, the previously used *in vitro* host model cell. Initial parasitism of VECs by a single organism was followed by establishment of a monolayer of trichomonads on the host cell. Finally, parasites adhering to either VECs or HeLa cells were induced to synthesize greater amounts of the four previously described adhesins. Therefore, distinct signals after contact with either epithelial cell type leads to the morphological transformation and/or induction of adhesin synthesis by *T. vaginalis*.

Introduction

Attachment to host cells of the human vaginal mucosa by *Trichomonas vaginalis*, which leads to colonization and infection, represents a cascade of events that includes specific adherence of epithelial cells, which overcomes the flushing effects of the normal fluid secretion of the vagina. For this protozoan, which is one of the most common sexually transmitted disease agents, *in vitro* recognition and binding to epithelial cells occurs in a highly specific manner, involving trichomonad surface proteins that interact with the host cell surface through receptor–ligand events (Alderete and Garza, 1985; 1988; Arroyo *et al.*, 1992) and cysteine proteinase activity (Arroyo and Alderete, 1989). The association between this parasite and host cell is followed by contact-dependent cytopathogenicity (Alderete and Pearlman, 1984; Garcia-Tamayo *et al.*, 1978; Heath, 1981; Hogue, 1943; Krieger *et al.*, 1985; Pindak *et al.*, 1986; Rasmussen *et al.*, 1986).

Four trichomonad adhesin proteins have been found to mediate attachment to vaginal epithelial cells (VECs) (Alderete *et al.*, 1988; Arroyo *et al.*, 1992). During the course of these investigations, it was observed that *in vitro* cultivation of fresh isolates reduced the levels of cytoadherence to epithelial cells, with a concomitant reduction in the amounts of the four adhesins (Arroyo *et al.*, 1992). Adhesin gene expression was regulated by iron (Lehker *et al.*, 1991), and this environmental signal was also found to affect parasite growth rates, multiplication and immunogen expression. These and other reports (Lehker and Alderete, 1990; 1992) reinforced the notion that adaptation by the parasite to the constantly changing environment of the vagina provides signals that influence the expression of virulence.

In vivo, the trichomonads associate with stratified, terminal squamous epithelial cells of the vaginal mucosa, a target different from HeLa cell monolayers (Alderete and Garza, 1985; 1988; Arroyo and Alderete, 1989; Arroyo *et al.*, 1992; Silva Filho and de Souza, 1988) or other cell culture systems used *in vitro* (Heath, 1981; Hogue, 1943; Krieger *et al.*, 1985; Pindak *et al.*, 1986; Silva Filho and de Souza, 1988). Studies with tissue samples of vaginal biopsies from patients with human trichomoniasis revealed that *T. vaginalis* organisms on the stratified squamous epithelium were amoeboid and attached through interdigitations formed between the plasma membrane of the parasite and small projections of the epithelial cell surfaces (Nielsen and Nielsen, 1975). In other observations from experimental infections, ovoid *T. vaginalis* organisms became amoeboid after contact with tissues in liver lesions (Brugerolle *et al.*, 1974). In contrast to *in vivo* observations, *in vitro*-grown organisms in suspension cultures are typically non-amoeboid, rounded and ovoid in morphology (Honigberg, 1978; Nielsen, 1975). These observations have led to the idea that the

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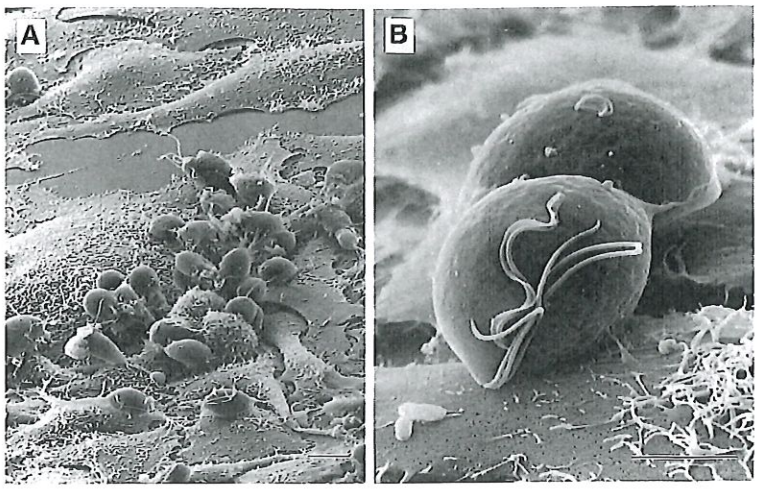

Fig. 1. Ellipsoidal morphology of *Trichomonas vaginalis* after contact with HeLa cells in monolayer cultures.
A. Scanning electron micrographs show that the majority of parasites attached to HeLa cells retain a pear-like shape; bar, 10 μm.
B. Trichomonads bound to HeLa cells have the four flagella, undulating membrane and axostyle on the free surface; bar, 5 μm.

amoeboid phenotype or the ability to transform to an amoeboid morphology may be a virulence trait.

The study of the initial attachment phase of *T. vaginalis* with VECs is clearly important for understanding the molecular basis of cytoadherence, host infection and pathogenesis. An examination was undertaken, using ultrastructural and immunobiochemical techniques, of trichomonal attachment to epithelial cells in order to determine whether signalling occurs for parasite transformation to an amoeboid morphology. This knowledge will likely contribute to our understanding of trichomonal virulence, especially as it relates to host parasitism of VECs (Honigberg, 1978). This is important, since cytoadherence has been previously examined mostly with *in vitro* cell culture systems (Alderete and Garza, 1985; Heath, 1981; Honigberg, 1978; Krieger *et al.*, 1985). Therefore, any investigations that contribute to our understanding of signalling for this and other protozoa, occurring during or immediately subsequent to infection, are clearly needed to understand the process involved in successful host parasitism. Such information might advance the knowledge base for this host–parasite relationship, from which intervention–interference strategies might be developed.

This report presents evidence for specificity in signal transduction as a function of *T. vaginalis* contact with certain host cells. The reproducible, immediate morphological transformation of highly adherent trichomonads after contact with VECs, but not HeLa epithelial cells, was demonstrated. Amoeboid trichomonads formed filopodia and pseudopodia, which interdigitated with the plasma membrane of target cells. Also noteworthy was that the formation of a layer of other parasites on the cell surface always followed the initial binding of a single trichomonad to a VEC. Parasites bound to both VECs and HeLa cells were induced to synthesize greater amounts of all adhesins, possibly ensuring for successful parasitism of the host. Overall, these observations indicate that host–parasite interactions result in generation of multiple responses to signals that follow the initial contact event. To this end, this report now includes *T. vaginalis* among a growing list of microbial pathogens that have sophisticated signal transduction systems which control expression of virulence genes (Mekalanos, 1992), such as those which encode for adhesins (Arroyo *et al.*, 1992).

Results

Contact-initiated amoeboid transformation of T. vaginalis

We begin by examining, by scanning electron microscopy, the appearance of *T. vaginalis* after contact with HeLa cells, since this host cell has been used extensively as an *in vitro* model for cytoadherence studies (Alderete and Garza, 1985; 1988; Arroyo and Alderete, 1989; Arroyo *et al.*, 1992). Figure 1A shows the typical morphology of trichomonads attached to HeLa cells in monolayer cultures. The ellipsoid morphology (Fig. 1B) described previously in observations with light and dark-field microscopy (Alderete and Garza, 1985) predominated for the adherent organisms. Similar observations were made with adherent trichomonads of T016 and T068 fresh *T. vaginalis* isolates. The integrity of both parasite and host cell was maintained under these experimental conditions, as evidenced by comparative scanning-electron micrographs of the organism and HeLa cells alone (Fig. 2A and C).

Surprisingly, as seen in Fig. 3, all parasites of highly adherent *T. vaginalis* isolate T016 in contact with VECs exhibited a rapid amoeboid morphological transformation when compared with parasites on HeLa cells (Fig. 1) or trichomonads in suspension (Fig. 2A). In <5 min, the

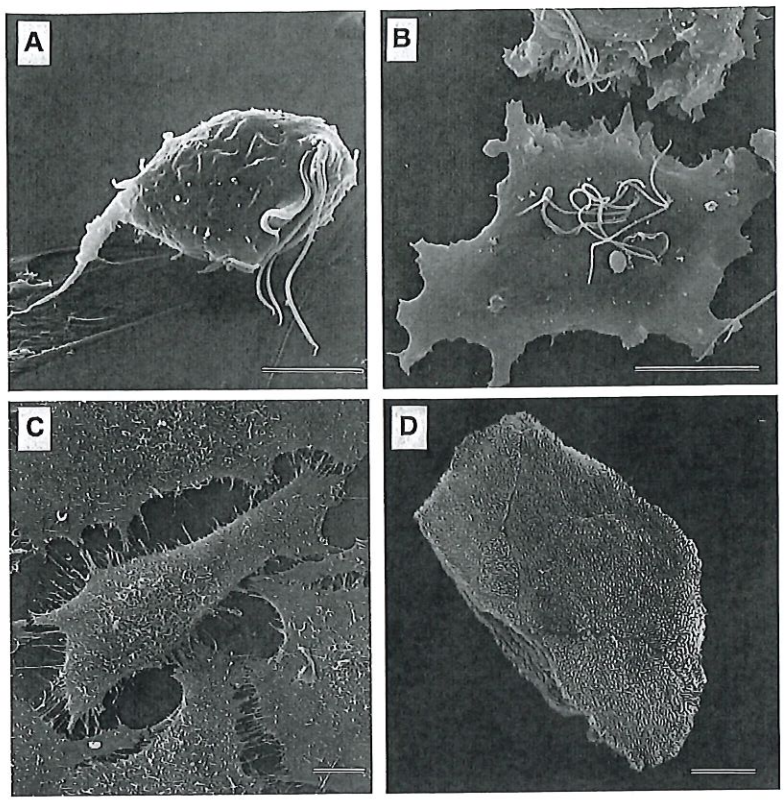

Fig. 2. Maintenance of integrity during processing and the morphological appearance of parasites and host epithelial cells before interaction.
A. *T. vaginalis* isolate T016 grown in suspension to the late logarithmic phase; bar, 5 μm.
B. Trichomonads grown in contact with plastic for at least 12 h; bar, 10 μm.
C. HeLa cells in monolayers grown in DMEM-containing serum in 25 cm^2 plastic culture flasks; bar, 10 μm.
D. Vaginal epithelial cells (VECs) obtained by swabs from healthy, uninfected women and purified before processing; bar, 10 μm, (see the *Experimental procedures*).

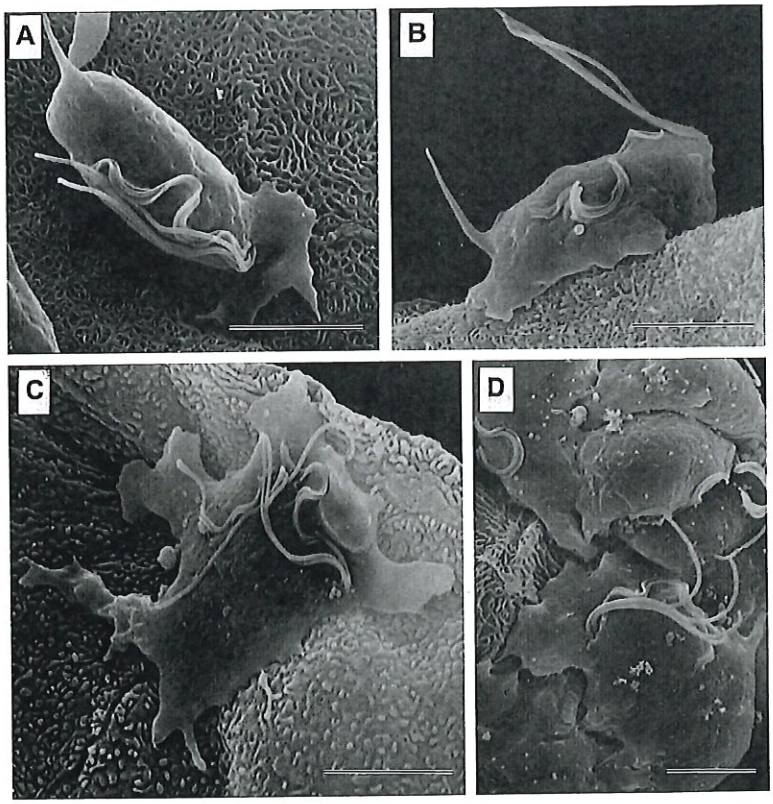

Fig. 3. Rapid amoeboid morphological transformation of trichomonads of *T. vaginalis* isolate T016 in contact with VECs. Parasites (4×10^7 ml^{-1}) were mixed with an equal volume of VECs (8×10^6 ml^{-1}).
A. At 1 min, the parasitic body changed slightly to a more elongated appearance, with numerous pseudopodia forming at the site of contact throughout the periphery of the organism.
B. At 3 min, multiple focal contact points and a continued change in appearance with short filopodia or lamellopodia were observed.
C. By 5 min, parasites underwent a complete morphological transformation, from a pear-like to a 'fried egg' appearance. The host-parasite, membrane–membrane associations became more prominent, with multiple contact points and thin lamellopodia.
D. By 20 min, VECs always had several trichomonads adjacent and seemingly anchored to each other by multiple membrane interdigitations; bar, 5 μm.

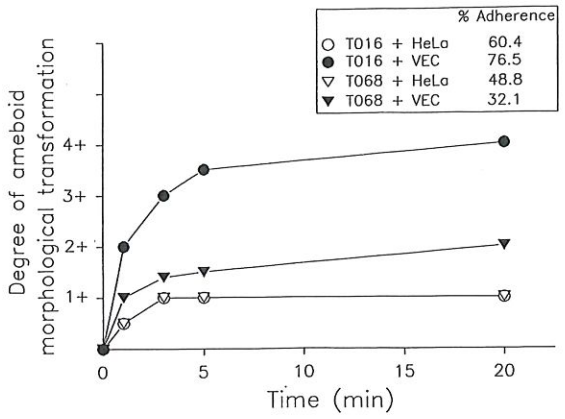

Fig. 4. Quantification of trichomonad morphological transformation after contact to either VECs or HeLa cells. An evaluation was performed of the degree of amoeboid morphological transformation, as presented on the scanning electron micrographs (of Fig. 3), from *T. vaginalis* isolates T016 and T068 and at different incubation times with VEC or HeLa cells. The experiment was repeated numerous times and the extent of transformation was recorded for multiple samples at each experiment and for each time-point. A degree of transformation was arbitrarily assigned according to the proportion of amoeboid/ellipsoid parasite shape in the majority of parasites corresponding to the specific time-point of the host–parasite interaction presented in Fig. 3. A 1+ value corresponds to a parasite beginning to form pseudopods (Fig. 3A) while a 2+ value means that half of the parasite body is amoeboid (Fig. 3B). A 3+ value indicates the parasite body has an amoeboid morphology with the axostyle still apparent and the upper part of the organism still ellipsoid. A 4+ value represents a completely amoeboid trichomonad (Fig. 3C and D).

Levels of cytoadherence were determined as the per cent of c.p.m. associated with monolayers of fixed HeLa cells after 30 min, as has previously been described (Alderete and Garza, 1985; Arroyo and Alderete, 1989; Arroyo *et al.*, 1992). Chemical stabilization of HeLa cells for the adherence assay was required because of the rapid, total destruction of live HeLa cell monolayers within 30 min of incubation with isolate T068. For VEC adherence, the numbers indicate the per cent of VECs in suspension with attached parasites, also reported before by us (Alderete *et al.*, 1988). The standard deviation of each adherence value varied no more than 5% (see insert).

organisms exhibited a change from a typical pear-like appearance (with a rough surface, four anterior flagella, a lateral undulating membrane and posterior axostyle) to that of a flat, smooth-surfaced organism (Fig, 3C). Initially after binding to VECs, the cell body of *T. vaginalis* becomes elongated (Fig. 3A), with pseudopods forming at the site of contact throughout the periphery of the organism. Multiple focal contact points, seen as short filopodia or lamellopodia (Fig. 3B), were also evident. The host–parasite, membrane–membrane associations became more prominent with numerous contact points and thin lamellopodia (Fig. 3C). Interestingly, within 20 min, single organisms on VECs were followed by other trichomonads, which were always found adjacent to each other with multiple membrane interdigitations (Fig. 3D). It is important to mention that initial colonization of HeLa cells was also accompanied by colonization by more parasites (data not shown). As a control, the amoeboid appearance of *T. vaginalis* on a plastic surface, a feature that has been reported previously (Cappuccinelli *et al.*, 1975; Silva Filho *et al.*, 1987), required an incubation period of >12 h under the same experimental conditions (Fig. 2B), and in artificial surfaces, the amoeboid morphology was not a property of all trichomonads. Figure 2D shows as a control, the integrity and appearance of a VEC alone.

The highly reproducible nature of this morphologic transformation by these two isolates in contact with VECs allowed us to quantify the difference at various time-points after incubation and to compare both host cell types. As shown in Fig. 4, VECs, but not HeLa cells, promoted to a greater extent the transformation of T016 trichomonads to the amoeboid morphology. The parasites of isolate T016, like trichomonads of other isolates, synthesize elevated amounts of adhesins and higher levels of cytoadherence (Arroyo *et al.*, 1992) than T068 trichomonads, which express lesser amounts of adhesins and lower values of cytoadherence (Fig. 4, insert). These data show the relationship between elevated amounts of surface-expressed adhesins and signalling for rapid morphological transformation.

The trichomonad–VEC associations were visualized by transmission electron microscopy. As seen in Fig. 5 (parts A and C), a clearly demarcated ectoplasmic region constituted mainly by microfilaments was seen at the regions of attachment to the host cell. Similar microfilament components were detected at the interlocking regions of adjacent parasites (Fig. 5A), as previously indicated (Nielsen and Nielsen, 1975). A higher magnification of a scanning electron micrograph of the attachment site (Fig. 5B) revealed that the flagella and undulating membrane remain at the free surface, as has been previously suggested (Alderete and Garza, 1985; Nielsen, 1975; Nielsen and Nielsen, 1975), whereas the axostyle was no longer apparent after the change to the amoeboid morphology (Fig. 3C).

Signalling for enhanced adhesin synthesis follows trichomonal contact with epithelial cells

Attempts were then made to examine the role of adhesins in promoting the transformation after parasite recognition and binding to the vaginal cells. Immunofluorescence was performed on semi-thin cryosections labelled with antibody to each of the four adhesins (Arroyo *et al.*, 1992) or with monoclonal antibodies (mAbs) to AP65 or AP33. As a control, no reaction was ever observed using IgG from each of the prebleed sera of the respective anti-adhesin antisera or with an unrelated hybridoma supernatant (Fig. 6A). Fluorescence of *T. vaginalis* organisms with the anti-adhesin IgG antibodies alone was not strong (Fig. 6B),

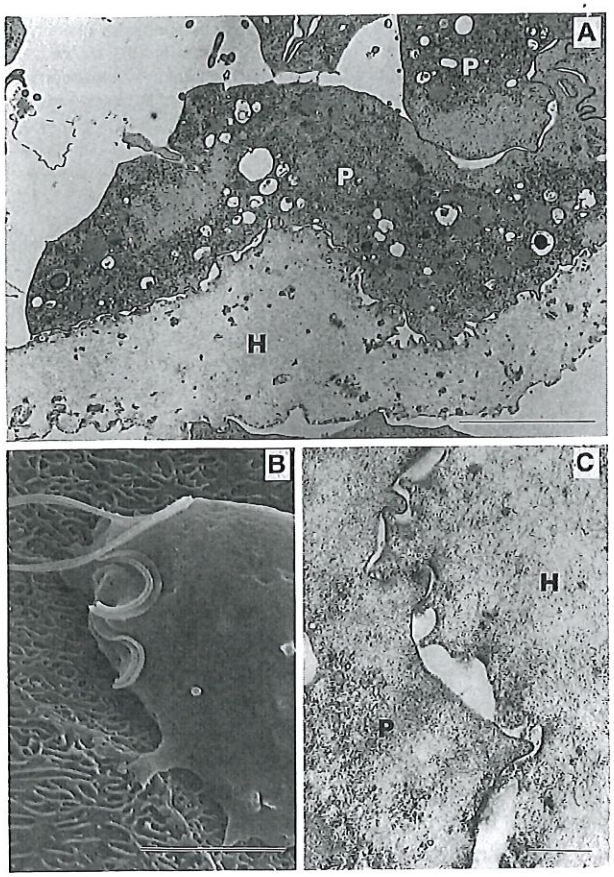

Fig. 5. Contact-focal points in trichomonad–VEC associations prepared for transmission (A and C) and scanning (B) EM. Parasites formed numerous cytoplasmic projections interdigitating with the microvilli of the VEC membrane. Adjacent trichomonads had extensive membrane–membrane interdigitations (A). A clearly demarcated exoplasmic region constituted mainly by microfilaments was seen at the region of contact between the host cell and parasite and between parasites alone (A and C). Bars in A and B, 5 μm in C, 0.5 μm. P and H refer to parasite and host respectively.

although clearly more visible than the negative control. Surprisingly, parasites in contact with VECs revealed a strikingly enhanced and generalized cytoplasmic fluorescence when reacted with polyclonal, anti-AP65 adhesin IgG (Fig. 6C), a reaction which was reaffirmed by using mAb to the AP65 adhesin (Fig. 6D) or mAb to the AP33 adhesin (data not shown). These results indicated that, like the morphological transformation, parasite binding to VECs also signalled for increased synthesis of the AP65 and AP33 adhesins.

This prompted us to ask whether this enhanced fluorescence occurred for all adhesins and whether signalling was specific only for VECs. Interestingly, both cell types gave enhanced levels of fluorescence (Fig. 7), and again, fluorescence intensities for all adhesins were elevated when compared with trichomonads alone (Fig. 6B). Variations in fluorescence intensities were also observed among the trichomonads in contact with VECs. Especially noteworthy was that parasites that appeared in maximum contact with the host cell because of their amoeboid morphology gave the brightest fluorescence. In parallel with the enhancement of indirect immunofluorescence of *T. vaginalis* in contact with epithelial cells (Fig. 6C), experiments with immunogold-labelled antibody preparations confirmed the uniform distribution of the adhesins throughout the parasite (our unpublished observations). The number of gold particles observed with all anti-adhesin antibodies with parasites alone were less than those seen for organisms in contact with epithelial cells.

As an important control, the reactivities of the individual anti-adhesin antibodies with the respective adhesin proteins were demonstrated by immunoblot of total trichomonad proteins (Fig. 8A). Identical reactions were obtained when the anti-adhesin sera were reacted with blots of proteins from the ligand assay, as seen previously (Arroyo *et al.*, 1992). The absence of immunoblot reactivity of the AP23 adhesin (lane 4) has been reported previously (Arroyo *et al.*, 1992) and likely is due to the lability of this protein under the denaturing conditions of electrophoresis and blotting.

Finally, because the four adhesins have been found to be co-ordinately regulated (Lehker *et al.*, 1991), a finding consistent with the enhanced synthesis of all adhesins (Fig. 6), it was important to show a relationship between the elevated adhesin synthesis and enhanced cytoadherence. To accomplish this, we measured the extent of inhibition by anti-adhesin antibodies of control, untreated organisms and compared this with that of cycloheximide-treated parasites incapable of increased adhesin synthesis. Fig. 8B illustrates that the extent of inhibition achieved by anti-adhesin antibodies was greater using trichomonads treated with cycloheximide (Fig. 8C). These data suggest that synthesis of adhesins resulting from contact with host cells (Figs 6 and 7) promoted elevated cytoadherence, as antibody was more effective at blocking attachment of parasites incapable of adhesin synthesis.

Discussion

This study was initiated by observations that our laboratory and others have made on a pronounced amoeboid morphology among trichomonads of *T. vaginalis* fresh isolates (Arroyo *et al.*, 1992; Brugerolle *et al.*, 1974; Garcia-Tamayo *et al.*, 1978; Heath, 1981; Honigberg, 1978; Nielsen and Nielsen, 1975; Ovcinnikov *et al.*, 1975). Inferences that the morphological phenotypes (ovoid, spherical and amoeboid) of this protozoan may be related to virulence (Honigberg, 1978) and our recent findings that this organism responds to environmental conditions, especially in cytoadherence (Lehker and Alderete, 1990) and regulation of adhesin genes (Lehker *et al.*, 1991),

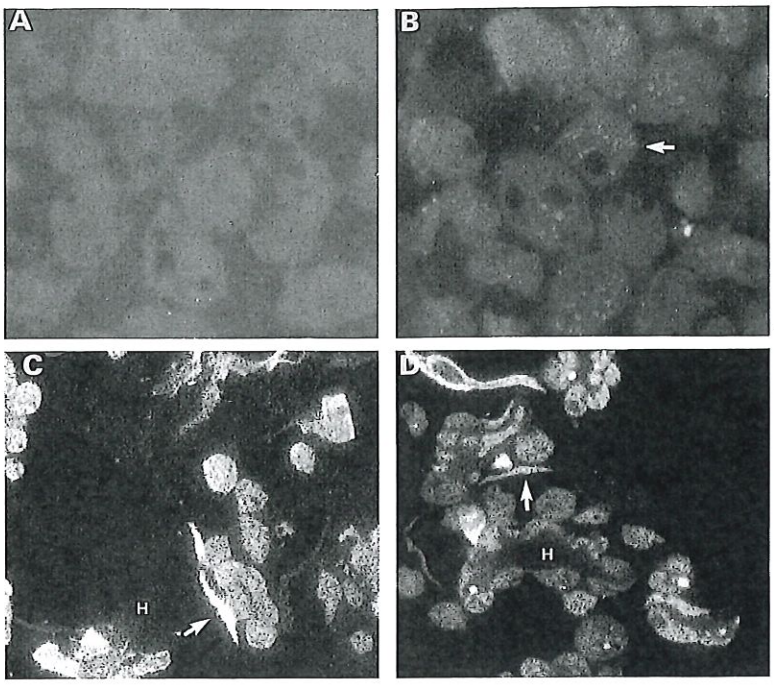

Fig. 6. Enhanced immunofluorescence intensity follows trichomonal contact with VEC. Visualization of adhesins by indirect immunofluorescence on semi-thin cryosections (1 μm thickness) of trichomonads alone in suspension (A and B) or at 30 min after contact with VECs (C, and D). IgG of polyclonal antiserum to AP65 (B and C) and IgG from mAb F11 (anti-AP65) (D) was compared with the reaction of IgG from pre-bleed sera of the respective anti-adhesin antisera (A), or undiluted, unrelated hybridoma supernatant(s) used as negative controls (Alderete *et al.*, 1986). As a positive control, IgG from serum to total proteins of isolate T016 was used (not shown). All antibodies were diluted accordingly to obtain a final concentration of 0.24 μg ml^{-1} IgG. Preparation were viewed with a fluorescence microscope at a magnification of ×1000 (A and B) and ×400 (C and D). H, host cells. Arrows in B indicate the fluorescence within the parasite. Arrows in C and D show the brighter fluorescence reactivity of the amoeboid trichomonads.

prompted us to investigate whether intercellular communication occurred during recognition and binding by the parasite to host cells.

Several noteworthy findings were made during the course of these investigations. It is clear that the parasite–host cell interactions, mediated by the trichomonad adhesins, result in signalling to the *T. vaginalis* attached to VECs to undergo a rapid transformation to an amoeboid morphology. The specificity in signalling was evident, since VECs, but not HeLa cells, initiated the transformation process. A consistent and reproducible finding was that VECs initially parasitized by a single organism ultimately had numerous, adjacent trichomonads, with extensive membrane interdigitations between the parasite membranes. Finally, a signal distinct from that involved in morphologic transformation appeared to be involved in stimulating parasites to synthesize, to a greater extent, four adhesin proteins (Arroyo *et al.*, 1992).

The two fresh isolates chosen for this study had levels of cytoadherence and amounts of adhesins greater than long-term-grown isolates, as recently demonstrated by us (Arroyo *et al.*, 1992). Nevertheless, both isolates possessed intrinsically different cytoadherence levels (Fig. 4, insert) that were also consistent with earlier results (Arroyo *et al.*, 1992). While these two isolates contain organisms capable of efficient host parasitism, additional variability among isolates was demonstrated on the basis of the extent to which parasites of a fresh isolate can undergo amoeboid transformation. These results reinforce the suggestions that trichomonads that undergo morphological transformation may have enhanced virulence capabilities (Honigberg, 1978; Kulda *et al.*, 1986) and may help explain the variability that has been reported in the literature regarding the pathogenicity levels among *T. vaginalis* isolates (Honigberg, 1978).

The change in shape, from ellipsoid to amoeboid, possibly conferring an enhanced binding of the parasite to the VEC seemed to occur simultaneously with the interdigitating membrane–membrane interactions, which were readily visualized in the adherent amoeboid trichomonads. The enhanced synthesis of adhesins seen following contact and occurring during the changes in trichomonal morphology possibly suggest a need for mobilization of newly-synthesized adhesins to distant contact sites on the host cell being recognized by the spreading parasite. A possible reason to adopt the amoeboid morphology may be the need to maximize the cell–cell contact required for a more stable and efficient parasitism. Although spreading of *T. vaginalis* occurs only on VECs, increased adhesin synthesis also occurs when parasites come in contact with HeLa cells. This might suggest that the association itself, between the adhesins and their putative receptor(s) (Arroyo *et al.*, 1992), provides the signal for synthesis. This idea is testable following the identification of the receptor molecule(s) on the host cells, and such cell surface constituents must be common to VECs, HeLa cells and other epithelial cells which induce increases in amounts of adhesins. Future understanding of the channeling, mobilization and recruitment of the adhesins to the interdigitating regions where the parasite–host cell-binding occurs will be of considerable interest.

The specificity imparted by host cell types, like those

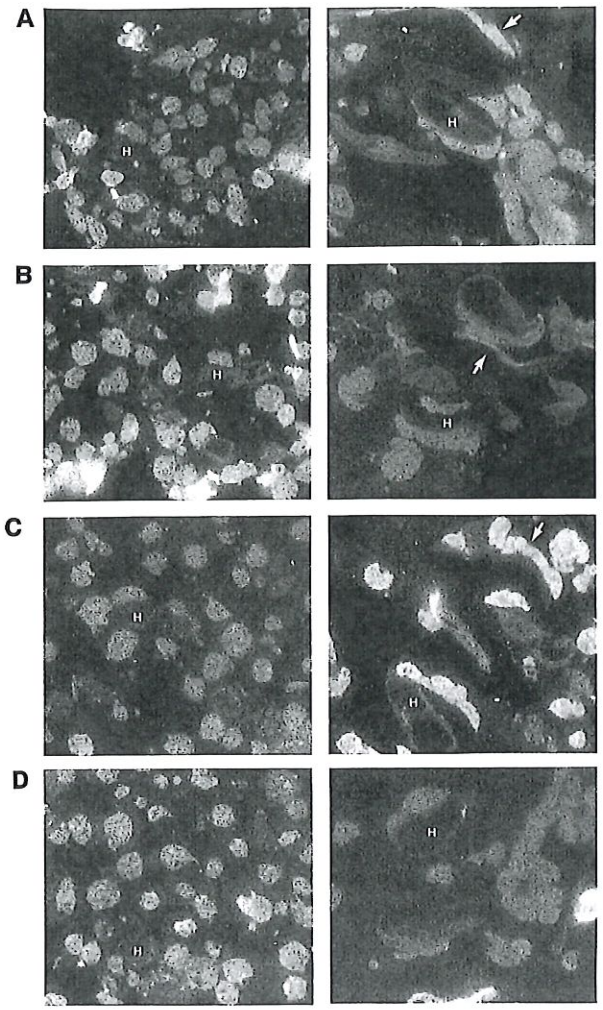

Fig. 7. Immunofluorescence showing increases of all four adhesins after contact with VECs and HeLa cells. Cryosections of parasites bound to HeLa cells in suspension (left) or VECs (right), after incubation for 30 min at 37°C, were processed for immunofluorescence as described in the legend for Fig. 6 and preparations were viewed at a magnification of ×400. Individual IgG of anti-AP65 serum (A), anti-AP51 serum (B), anti-AP33 serum (C), and anti-AP23 serum (D) (all at 0.24 μg ml^{-1} final concentration) were used for fluorescence. The reactivity of each individual antiserum with blotted adhesins are presented in Fig. 8 and are as previously reported (Arroyo *et al.*, 1992). IgG from normal rabbit serum (NRS) was unreactive as seen in Fig. 6A. H represents either HeLa cells (left) or VECs (right); arrows point to trichomonads with a flat amoeboid morphology, which always presented with the brightest fluorescence.

seen here between HeLa cells, a transformed cervical epithelial cell (Gey *et al.*, 1952), and VECs, is especially significant. Although adherence to HeLa and VECs occurs through the same adhesins (Arroyo *et al.*, 1992), that VECs only are capable of signalling for amoeboid transformation may provide a basis for characterizing the particular signal(s). The specificity afforded by VECs may reside with the ability of the host cell to mobilize or sequester the adhesin receptors. For example, the vaginal squamous cells may be unable to recruit receptors to the contact area. Alternatively, a low density of receptors on VECs may initiate an amoeboid transformation response by the parasite to increase receptor–adhesin contact sites. In this regard, HeLa cells may possess both a higher density of receptors and an ability to mobilize receptors to the contact area (Gingell and Owens, 1992; Singer, 1992), leading to absence of signalling for transformation. It is easy to envision how such a transformation, which leads to better adherence, would be beneficial to a mucosal parasite, in danger of being lost by the normal secretions of the vagina. Clearly, identification of the host cell receptor, the existence of which was recently indicated experimentally (Arroyo *et al.*, 1992), will allow for testing of this hypothesis.

Adhesin-mediated *T. vaginalis* attachment to both epithelial cell types is sufficient for stimulation of adhesin synthesis. Recently, up-regulation by iron of adhesin gene expression was demonstrated (Lehker *et al.*, 1991). Therefore, of particular interest is whether iron is involved in the explosive increase in adhesin synthesis. The short time-interval required for adhesin gene transcription (Lehker *et al.*, 1991) might indicate that iron from iron-containing or iron-binding proteins of the parasitized host cell is an unlikely source to mediate this new synthesis of adhesins, although this must be tested experimentally. It may be that parasite contact with both host cell types leads to immediate utilization of the existing trichomonal cytoplasmatic iron pools for enhancement of adhesin synthesis. This idea is consistent with the results showing the presence of pools of iron within trichomonads (Lehker and Alderete, 1990; 1992). Thus, the concept that signalling by contact mediates consumption of internal iron pools is attractive.

The finding that contact produces a dramatic increase in adhesin production explains why there is no total abolition of cytoadherence by the specific, polyclonal anti-adhesin antibodies — a finding shown here (Fig. 8B) and in earlier reports (Alderete and Garza 1988; Arroyo *et al.*, 1992). The recruitment and binding of newly synthesized adhesins, along with internal pools of existing adhesins mobilized to the surface after contact, as shown before (Lehker *et al.*, 1991), may represent a mechanism by which antibody recognition is rendered ineffective in neutralizing the biofunctionality of these molecules — something that has also been reported (Alderete and Garza, 1988; Arroyo *et al.*, 1992). An alternative and intriguing possibility is that the morphological transformation, occurring on the host cell surface, allows for expression of the adhesins at the membrane–membrane juncture, a site inaccessible to antibody recognition of adhesins.

Also noteworthy was the apparent recruitment of other trichomonads to the VEC surface following parasitism by one or a few organisms. Previous investigators have described cytotoxicity of cells in monolayer cultures as

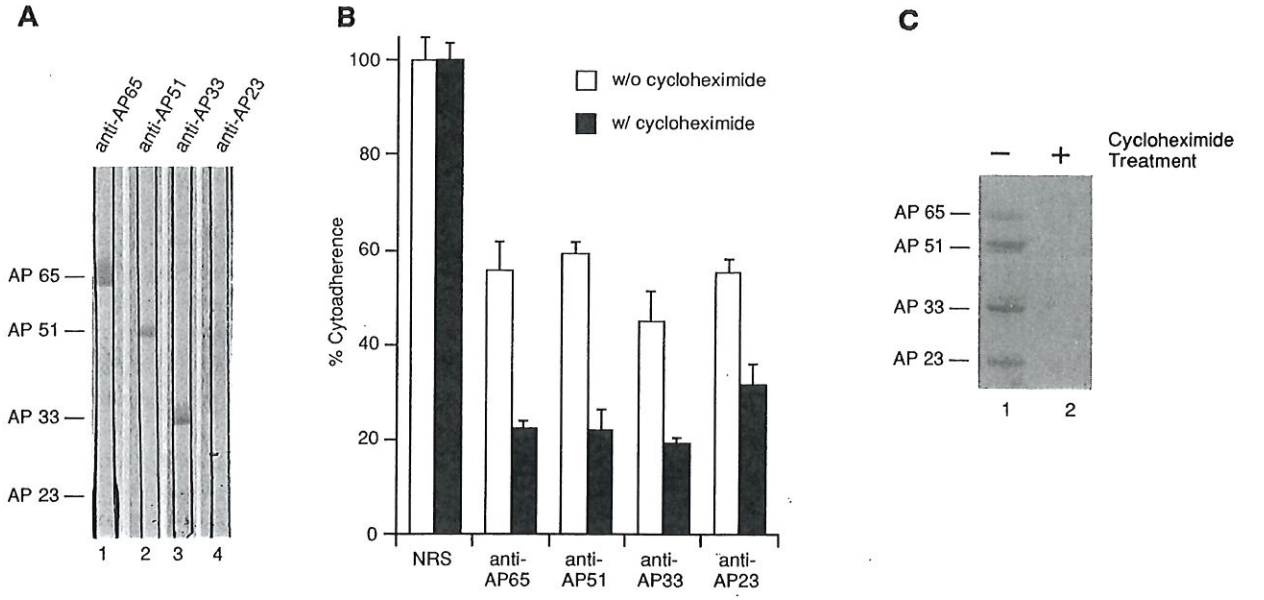

Fig. 8. Elevated synthesis of all adhesins results in an enhanced ability to cytoadhere.
A. Reactivity to adhesins of each anti-adhesin serum to AP65 (lane 1), AP51 (lane 2), AP33 (lane 3) and AP23 (lane 4) was tested by immunoblot using nitrocellulose-containing total detergent extract of isolate T016 (Arroyo *et al.*, 1992; Lehker *et al.*, 1991). Nitrocellulose paper was incubated with individual anti-adhesin IgG antibody (final concentration of 0.48 μg ml^{-1}) for 18 h at 4°C, washed four times with PBS–0.05% Tween 20, then incubated with goat anti-rabbit Ig conjugated to horseradish peroxidase (Bio-Rad Laboratories) diluted 1:2000 (in PBS–0.05% Tween 20–5% non-fat dry milk) for 2 h at 25°C. After washing three times with PBS–0.05% Tween 20 blots were treated with 4-chloro-1-napthol (2 mg ml^{-1}) prepared in PBS–20% methanol containing 0.015% H_2O_2. mAbs F11 (anti-AP65) and F5 (anti-AP33) gave identical reactions as those seen for antiserum IgG antibody to AP65 (lane 1) and to AP33 (lane 3) adhesins respectively. NRS (as used in Fig. 6 for fluorescence) was used as a negative control (not shown) and has been reported to give no immunocrossreactions with any of the adhesins (Arroyo *et al.*, 1992). Lines indicate the position of the adhesins.
B. Effect of anti-adhesin antibodies on cytoadherence. ^{3}H-labelled parasites from isolate T016 grown for 18 h in a TYM-serum medium were washed and suspended for treatment with cycloheximide (20 μg ml^{-1}) to a density of 1×10^6 parasites ml^{-1} in TYM without serum. After 4 h at 37°, cytoadherence levels were measured in the presence of 8 μg ml^{-1} of each anti-adhesin antibody IgG as before (Arroyo *et al.*, 1992). Comparative cytoadherence inhibition was obtained for cycloheximide-treated versus untreated parasites. IgG from pre-bleed control serum of all rabbits or a mock control from antibody to another unrelated surface protein (at 8 μg ml^{-1} concentration) (Arroyo *et al.*, 1992) failed to give any inhibition of cytoadherence. Each value is the mean of three experiments each with triplicate samples, and bars indicate the standard deviation.
C. Inhibition of adhesin synthesis among cycloheximide-treated parasites. Unlabelled parasites were treated first with cyclohexamide for 15 min at 37°C before addition of ^{35}S-label (2 mCi per 4×10^7 parasites). The ligand assay (see the *Experimental procedures*) for detection of adhesins was then performed, and fluorography of HeLa cell-bound adhesins (Arroyo *et al.*, 1992) of untreated (1) versus treated (2) parasites were compared. Lines indicate the position of the adhesins.

resulting from congregation of parasites at defined foci, rather than a uniform killing of the cells in monolayer culture (Alderete and Pearlman, 1984; Nielsen and Nielsen, 1975; Pindak *et al.*, 1986; Rasmussen *et al.*, 1986). Whether soluble factors are being generated by the parasites by signalling after contact with host cells is unknown. This host–parasite relationship may be complicated further by the possibility of inter-parasite communication as a result of the extensive interdigitation occurring among adjacent organisms (Figs 3D and 5A), an observation and suggestion previously reported by Kulda *et al.* (1986). Any exchange of genetic information occurring on the host cell surface by adherent trichomonads might contribute greatly to our understanding of the extensive heterogeneity of trichomonal populations and responsiveness to environmental conditions (Lehker and Alderete, 1990; 1992; Lehker *et al.*, 1991).

It has been known that inhibitors of microfilament and microtubule assembly affect the ability of the parasite to adhere to certain cell types (Alderete and Garza, 1985; Krieger *et al.*, 1985) and polystyrene substrates (Cappuccinelli *et al.*, 1975; Silva Filho *et al.*, 1987). Also, fixed host cells are suitable targets for parasitism by live *T. vaginalis*; however, fixed trichomonads are unable to attach to epithelial cells (Alderete and Garza, 1985; 1988). Finally, a recent report showed that mAbs to surface, non-adhesin proteins of *T. vaginalis* inhibited cytoadherence (Krieger *et al.*, 1990). These earlier studies and the requirement for live organisms in cytoadherence studies can now be understood on the basis of morphologic transformation and on the synthesis and placement of adhesins to the parasite surface.

The possible intercellular communication resulting from adhesion between parasite and host cell and between adjacent organisms is the subject of intense interest in cell biology (Gingell and Owens, 1992; Singer, 1992; Young *et*

al., 1992). It has been established that ligand–receptor interactions between cells lead to recruitment/synthesis of additional molecules to enhance intercellular adhesion (Gingell and Owens, 1992; Singer, 1992), which in turn results in morphological changes in the cell surfaces that are in contact (Singer, 1992). Our work now shows that, for this sexually transmitted protozoan, a complex integration of cytoadherence with subsequent signalling events occurs, supporting the view that adherence and intercellular communication are correlated phenomena. Although it has been established previously that host cell attachment occurs via four adhesins, this study extends our knowledge by showing that intercellular communication results from the initial host receptor–adhesin associations. Clearly, as suggested by others (Mekalanos, 1992), elucidation of environmental signals, which control expression of virulence genes, is essential to understanding the strategies used by microorganisms to become successful pathogens.

Experimental procedures

Parasites, target cells, and cytoadherence assay

Two fresh *T. vaginalis* isolates, T016 and T068, were used as described recently (Arroyo *et al.*, 1992). Parasites were passaged daily for ≤2 weeks in trypticase–yeast extract–maltose (TYM) medium supplemented with 10% heat-inactivated horse serum (Diamond, 1957). Only trichomonads from the late logarithmic-phase of growth were used and, under these conditions, maintained their respective levels of cytoadherence to HeLa cells and VEC, as shown in Fig. 4. These isolates were chosen because they are representative of fresh isolates that have been examined on the basis of cytoadherence properties (Arroyo *et al.*, 1992). Intrinsic differences in levels of cytoadherence between isolate T016 and isolate T068 are especially noteworthy when considering the ability of parasites to undergo morphological amoeboid transformation, as described in this report.

Details on the use of HeLa cells in monolayer culture as the *in vitro* model cells for cytoadherence studies have been described previously (Alderete and Garza, 1985; Arroyo and Alderete, 1989; Arroyo *et al.*, 1992). Briefly, HeLa cells were obtained from the American Type Culture Collection (ATCC) and grown as monolayers to confluency (~4×10^6 total cells) in 25 cm^2 flasks or in individual wells (~8×10^4 HeLa cells) of 96-well plates in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum in a 7.5% CO_2 atmosphere. Human VECs were obtained from vaginal swabs of healthy, uninfected women and purified as described elsewhere (Alderete *et al.*, 1988).

Attachment of *T. vaginalis* to HeLa cells or VECs was accomplished by well-established procedures (Alderete and Garza, 1985; Alderete *et al.*, 1988; Arroyo and Alderete, 1989; Arroyo *et al.*, 1992). Unlabelled parasites were used to determine levels of adherence to VECs and whenever fluorescence and electron microscopy (EM) experiments were performed. Levels of adherence to HeLa cells were monitored for each isolate by determining the amount of radioactivity remaining on HeLa cell monolayers incubated with ^{3}H-labelled organisms (Alderete and Garza, 1985; Arroyo and Alderete, 1989).

For EM experiments, 2×10^7 organisms in 5 ml of a medium mixture of DMEM:TYM (2:1, vol/vol) without serum were added to 25 cm^2 flasks of confluent HeLa cell monolayers (Alderete and Garza, 1985). For VECs, 4×10^6 cells in 500 μl of TYM were mixed with 2×10^7 parasites in 500 μl of TYM (Alderete *et al.*, 1988). At different time-points, the mixtures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and stored at 4°C until processed for EM. For transmission EM, fixed samples were washed with cacodylate buffer, followed by fixation for 1 h in 1% OsO_4. Samples were dehydrated in ethanol and embedded in Epon. Ultra-thin sections stained with uranyl acetate and lead citrate were examined in a JEOL 100SX electron microscope at 60 kV. For scanning EM, optimally fixed samples were critical-point dried using a Sandry apparatus (Tousimis Research Corp.). Samples were lightly coated with gold (approximately 30 nm thick) and examined with a JEOL 35-C scanning electron microscope set at 10 kV. For use in immunofluorescence microscopy, cells were first fixed for 2 h at 25°C in PBS containing 0.5% glutaraldehyde and 2.5% paraformaldehyde. Specimen preparation for cryosectioning was performed using standard techniques (Tokuyasu, 1973).

Antibodies and indirect immunofluorescence

The IgG fraction of each antiserum generated to each of the four adhesin proteins and the use of antibodies in cytoadherence inhibition and immunoblot experiments has been recently described in detail (Arroyo *et al.*, 1992). Also, IgG to each anti-adhesin serum was used for indirect immunofluorescence on 1 μm frozen sections of parasites or target cells with attached organisms. Antibody from prebleed serum of all rabbits was prepared similarly, and used singly or mixed as a negative control for all experiments. The IgG fraction from antiserum generated to total proteins of *T. vaginalis* isolate T016 was also used as a positive control in immunofluorescence and immunoblot assays.

The generation of mAbs to trichomonad proteins has been described in detail (Alderete *et al.*, 1986). For mAbs to adhesins, 3–6-week-old BALB/c mice were immunized with a mixture of the four adhesin proteins (Arroyo *et al.*, 1992), which were purified by elution from fixed HeLa cells as described in detail previously (Arroyo *et al.*, 1992). Briefly, the adhesin proteins were obtained from a ligand assay (described below) and separated by SDS–PAGE in 7.5% acrylamide gels. Mice were first immunized subcutaneously with 100 μg of total protein in the adhesin mixture in 100 μl of PBS, pH 7.4, which was mixed with 100 μl of complete Freund's adjuvant. Mice were then boosted every 3 weeks with five additional doses of 50 μg total protein in the adhesin mixture in incomplete Freund's adjuvant. Animals were tested for serum Ab to trichomonad adhesins by enzyme immunoassay (EIA) (Alderete *et al.*, 1986) before killing and hybridoma production (Alderete *et al.*, 1986). The fusion was carried out 4 d after the last injection, with the mouse myeloma clone P3/NS1/1-Ag4-1 (NS-1) (ATCC) following established procedures (Alderete *et al.*, 1986). Hybridoma culture supernatants were first screened by EIA using the eluted adhesins, and positive hybridomas were tested further by immunoblot analysis (Alderete *et al.*, 1986; Arroyo *et al.*, 1992). Six of 31 hybridoma supernatants immunoreactive with adhesins were then analysed by immunoblot for reactivity to trichomonad adhesin proteins (Arroyo *et al.*, 1992). Hybridoma cells were then

subcloned twice by limiting dilution. mAbs F5 (anti-AP33) and F11 (anti-AP65), both IgG_1, were used in the immunofluorescence assay (below). The mAbs were purified and concentrated from ascites by ammonium sulphate precipitation.

One micrometre cryosections of parasites or target cells with attached organisms in 2.3 M sucrose were washed six times with phosphate buffered saline (PBS) and stained with 0.0025% Evans blue dye for 30 min at 25°C prior to washing an additional four times with PBS. Specific IgG antibody from anti-adhesins antisera or each mAb diluted in PBS–1% bovine serum albumin (BSA) was added to the cryosections and incubated for 18 h at 4°C. Sections were then blocked for 5 min at 25°C with 3% normal goat serum followed by incubation for 30 min at 37°C with a 1:5000 dilution of goat anti-rabbit Ig coupled with biotin (Pierce). Finally, the cryosections were incubated for 1 h at 25°C with a 1:10 dilution of streptavidin–fluorescein (Pierce). When the mAb was used for fluorescence experiments, cryosections were incubated with a 1:80 dilution of fluorescein-isothiocyanate-labelled goat anti-mouse IgG (Pierce). After washing to remove excess fluorochrome, sections were mounted in 66% glycerol and studied with epifluorescence microscopy. All dilutions were made in PBS unless specified.

The ligand assay and inhibition of trichomonal adhesin synthesis by cycloheximide treatment

A ligand assay, described previously (Alderete and Garza, 1988; Arroyo *et al.*, 1992), was used to analyse the trichomonad adhesins under different conditions. Briefly, a total detergent extract from 2×10^7 ^{35}S-labelled parasites were incubated for 18 h at 4°C with 1×10^6 glutaraldehyde-fixed HeLa cells before washing of cells to remove non-specific binding. The same HeLa cell-binding proteins have been shown to be recognized by VECs (Arroyo *et al.*, 1992). Trichomonad proteins bound to the fixed cells were eluted by boiling in electrophoresis buffer (Laemmli, 1970) for 3 min. Radiolabelled adhesins were first separated by SDS–PAGE using 7.5% polyacrylamide gels for analysis by fluorography.

The inhibition of adhesin synthesis by cycloheximide treatment of parasites has been demonstrated previously (Alderete and Garza, 1985; Lehker *et al.*, 1991). For these experiments, either ^3H-labelled or unlabelled parasites grown for 18 h in TYM-serum were washed three times with PBS and suspended in TYM medium without serum to a density of 1×10^6 ml^{-1}. Cycloheximide (Sigma Chemical Co.) was added to a final concentration of 20 μg ml^{-1} and parasites incubated for 2 to 4 h at 37°C (Lehker *et al.*, 1991). Cycloheximide-treated organisms were also analysed by the ligand assay and in this case, unlabelled parasites processed as above were incubated with cycloheximide for 15 min at 37°C before metabolic labelling with Tran ^{35}S-label (>1000 Ci mmol^{-1}; >37 TBq mmol^{-1}; ICN Biomedicals Inc.; 2 mCi per 4×10^7 trichomonads).

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References

Alderete, J.F., and Garza, G.E. (1985) Specific nature of *Trichomonas vaginalis* parasitism of host cell surfaces. *Infect Immun* **50:** 701–708.

Alderete, J.F., and Garza, G.E. (1988) Identification and properties of *Trichomonas vaginalis* proteins involved in cytadherence. *Infect Immun* **56:** 28–33.

Alderete, J.F., and Pearlman, E. (1984) Pathogenic *Trichomonas vaginalis* cytotoxicity to cell culture monolayers. *Brit J Vener Dis* **60:** 99–105.

Alderete, J.F., Suprun-Brown, L., and Kasmala, L. (1986) Monoclonal antibody to a major surface glycoprotein immunogen differentiates isolates and subpopulations of *Trichomonas vaginalis*. *Infect Immun* **52:** 70–75.

Alderete, J.F., Demeš, P., Gombošová, A., Valent, M., Fabušová, M., Janoška, A., Štefanović, J., and Arroyo, R. (1988) Specific parasitism of purified vaginal epithelial cells by *Trichomonas vaginalis*. *Infect Immun* **56:** 2558–2562.

Arroyo, R., and Alderete, J.F. (1989) *Trichomonas vaginalis* surface proteinase activity is necessary for parasite adherence to epithelial cells. *Infect Immun* **56:** 2991–2997.

Arroyo, R., Engbring, J., and Alderete, J.F. (1992) Molecular basis of host epithelial cell recognition by *Trichomonas vaginalis*. *Mol Microbiol* **6:** 853–862.

Brugerolle, G., Govert, J.G., and Savel, J. (1974) Etude ultrastructural des lésions viscérales provoquées par l'injection intrapéritonéale de *Trichomonas vaginalis* chez la souris. *Ann Parasitol (Paris)* **49:** 301–308.

Cappuccinelli, P., Cagliani, I., and Cavalo, O. (1975) Involvement of a surface Concanavalin A-binding protein in the adhesion of *Trichomonas vaginalis* to substrates. *Experimentia* **31:** 1157–1159.

Diamond, L.A. (1957) The establishment of various trichomonads of animals and man in axenic cultures. *J Parasitol* **43:** 488–490.

García-Tamayo, J., Nuñez-Montiel, J.T., and DeGarcia, H.P. (1978) An electron microscopic investigation on the pathogenesis of human vaginal trichomoniasis. *Acta Cytol* **22:** 447–455.

Gey, G.O., Coffman, W.D., and Kubicek, M.T (1952) Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res* **12:** 264–265.

Gingell, D., and Owens, N. (1992) How do cells sense and respond to adhesive contacts? Diffusion-trapping of laterally mobile membrane proteins at maturing adhesions may initiate signals leading to local cytoskeletal assembly response and lamella formation. *J Cell Science* **101:** 255–266.

Heath, J.P. (1981) Behaviour and pathogenicity of *Trichomonas vaginalis* in epithelial cell cultures. A study by light and scanning electron microscopy. *Brit J Vener Dis* **57:** 106–117.

Hogue, M.J. (1943) The effect of *Trichomonas vaginalis* on tissue culture cells. *Am J Hyg* **37:** 142–152.

Honigberg, B.M. (1978) Trichomonads of importance in human medicine. In *Parasitic Protozoa*, vol. 2. Kreier, J.P. (ed). New York: Academic Press, Inc., pp. 276–454.

Krieger, J.N., Ravdin, J.I., and Rein, M.F. (1985) Contact-dependent cytopathogenic mechanisms of *Trichomonas vaginalis*. *Infect Immun* **50:** 778–786.

Krieger, J.N., Torian, B.E., Hom, J., and Tam, M.R. (1990) Inhibition of *Trichomonas vaginalis* motility by monoclonal antibodies is associated with reduced adherence to HeLa cell monolayers. *Infect Immun* **58:** 1634–1639.

Kulda, J., Nohýnková, E., and Ludvik, J. (1986) Basic structure and function of the trichomonad cell. In *Acta Universitatis Carolinae-Biologica*. vol. 30 (3–4). Kulda, J., and Čerkasov, J., (eds). Prague: Charles University, pp. 181–198.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227:** 680–685.

Lehker, M.W., and Alderete, J.F. (1990) Properties of *Trichomonas vaginalis* grown under chemostat conditions. *Genitourin Med* **66:** 193–199.

Lehker, M.W., and Alderete, J.F. (1992) Iron regulates growth of *Trichomonas vaginalis* and the expression of immunogenic trichomonad proteins. *Mol Microbiol* **6:** 123–132.

Lehker, M.W., Arroyo, R., and Alderete, J.F. (1991) The regulation by iron of the synthesis of adhesins and cytoadherence levels in the protozoan *Trichomonas vaginalis*. *J Exp Med* **174:** 311–318.

Mekalanos, J.J. (1992) Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol* **174:** 1–7.

Nielsen, M.H. (1975) The ultrastructure of *Trichomonas vaginalis* Donné before and after transfer from vaginal secretion to Diamonds medium. *Acta Path Microbiol Scand B* **83:** 581–589.

Nielsen, M.H., and Nielsen, R. (1975) Electron microscopy of *Trichomonas vaginalis* Donné: interaction with vaginal epithelium in human trichomoniasis. *Acta Path Microbiol Scand B* **83:** 305–320.

Ovcinnikov, N.M., Delektorskij, V.V., and Turanova, E.N. (1975) Further studies of *Trichomonas vaginalis* with transmission and scanning electron microscopy. *Brit J Vener Dis* **51:** 357–375.

Pindak, F.F., Gardner, W.A., and Mora de Pindak, M. (1986) Growth and cytopathogenicity of *Trichomonas vaginalis* in tissue culture. *J Clin Microbiol* **23:** 672–678.

Rasmussen, S.E., Nielsen, M.H., Lind, I., and Rhodes, J.M. (1986) Morphological studies of the cytotoxicity of *Trichomonas vaginalis* to normal human vaginal epithelial cells in vitro. *Genitourin Med* **62:** 240–246.

Silva Filho, F.C., and de Souza, W. (1988) The interaction of *Trichomonas vaginalis* and *Trichomonas foetus* with epithelial cells *in vitro*. *Cell Struc Func* **13:** 301–310.

Silva Filho, F.C., Elias, C.A., and de Souza, W. (1987) Role of divalent cations, pH, cytoskeleton components and surface charge on the adhesion of *Trichomonas vaginalis* to a polystyrene substrate. *Mem Inst Oswaldo Cruz* **82:** 379–384.

Singer, S.J. (1992) Intercellular communication and cell-cell adhesion. *Science* **255:** 1671–1677.

Tokuyasu, K.T. (1973) A technique for ultramicrotomy of cell suspension and tissues. *J Cell Biol* **57:** 551–565.

Young, V.C., Falkow, S., and Schoolnik, G. (1992) The invasin protein of *Yersinia enterocolitica*: Internalization of invasin-bearing bacteria by eukaryotic cells is associated with reorganization of the cytoskeleton. *J Cell Biol* **116:** 197–207.