The Mechanisms and Molecules Involved in Cytoadherence and Pathogenesis of *Trichomonas vaginalis*

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All urogenital mucosal pathogens must overcome host factors and responses in order to colonize the mucosa and establish infection. These include the extensive mucus layer, nutrient-limiting conditions, antibody responses and the constant fluid flow of the vagina. In this article, John Alderete, Michael Lehker and Rossana Arroyo review the recent work describing the specificity by which the protozoan, Trichomonas vaginalis, *adheres to the vaginal epithelial cells via four surface proteins. They discuss three distinct signals that may be received by the parasite upon recognition and binding to these cells, illustrating the type of adaptive responses evolved in the establishment and maintenance of infection.*

Cytoadherence, one of the early steps in the infectious process, is essential for colonization and persistence of the pathogen[1]. It is not surprising, therefore, that the mechanisms of cytoadherence have been studied intensively (at a biochemical, genetic and molecular level) for numerous prokaryotic and eukaryotic pathogens, and that criteria have been established to show specificity in the recognition and binding of the pathogen to host target sites[1]. In the simplest form, cytoadherence involves the interaction between molecules on the surface of the microbe (adhesin) with specific molecules on the surface of the host cell (receptor).

Trichomonas vaginalis, a protozoan parasite, is one of the best-known sexually transmitted disease (STD) infectious agents worldwide. Trichomoniasis is the most common, clinically recognized STD in the USA and, perhaps, the world. Although theoretically possible, there are no recent data to support a non-sexual transmission of this parasite. An exact number for reports of vaginitis caused by the parasite is unknown, although between three million and ten million new cases of trichomoniasis per year have been suggested. Women infected with *T. vaginalis* probably exhibit an enhanced (sixfold) susceptibility to HIV infection (M. Laga, N. Nzila and A.T. Monoka, abstract*) and an adverse pregnancy outcome[2–4], manifested by preterm rupture of membrane, preterm delivery and low-birth-weight infants. Despite the high human morbidity, especially among women of all world societies, this protozoan remains one of the most poorly studied with respect to virulence properties and pathogenesis.

Nonetheless, it may be possible to discuss the idea of future vaccine development from an understanding of the mechanism and molecules involved in specific cytoadherence of *T. vaginalis* to the epithelial cells of the vagina. Parasite attachment to host cells is a prerequisite for the establishment of infection, as the organisms must overcome the constant secretions of the vagina. In fact, a clinical manifestation of trichomoniasis is the foul-smelling discharge[6], which would flush out a parasite that was incapable of anchoring itself to the vaginal epithelium. In addition, the parasite must survive in an adverse host environment, which is nutrient limiting for optimal growth and multiplication[6,7] and which contains specific anti-trichomonal immunoglobulin[8,9] as well as numerous, soluble trichomonad proteinases[9]. Also, the site of infection for *T. vaginalis* is under constant hormonal influence during the progression of the menstrual cycle[10].

Cytoadherence, Cytotoxicity and Adhesins

Dark field microscopy of cover slips with HeLa cells in monolayer cultures incubated with suspensions of *T. vaginalis* have reaffirmed numerous early reports[11–14] of the interaction of parasites to the host cells. Especially intriguing is the polarity of trichomonads adhering to HeLa cells, occurring mostly at sites opposite the undulating membrane[15]. A series of experimental findings (summarized in Box 1) has begun the dissection of the mechanism(s) by which this protozoan cytoadheres[15–21].

Cytoadherence by trichomonads is dependent on time, temperature and pH[15 17], as well as on cytoskeletal perturbations. That microtubule and microfilament inhibitors adversely affected cytoadherence[15] is consistent with more-recent work[21] on the change in morphology and recruitment of adhesins necessary for trichomonal attachment (see below). Proteinase treatment of live organisms abolishes parasite attachment to host cells[15], indicating the involvement of specific trichomonad surface proteins. When allowed to synthesize and re-express surface proteins, trypsin-treated trichomonads readily re-acquired the adherence phenotype. Inhibition of the regeneration of adherence of trypsinized organisms was accomplished by preventing protein synthesis with cycloheximide[15]. In addition, sugars and lectins added to parasite–host cell mixtures and glycosidase and periodate treatment of live organisms do not interfere with cytoadherence[15]. Competition experiments in adherence assays between radiolabeled trichomonads of homologous versus heterologous isolates show that only the homologous trichomonads give stoichiometric competition, indicating quantitative variations of surface components on the different isolates[15] (confirmed later[20]).

That specific cytoadherence is a property of *T. vaginalis* has been further reaffirmed by the reported contact-dependent nature of host cytotoxicity[22–24]. The extent of host cell killing is directly related to the levels of parasite cytoadherence[15]. Examination of the property of cytoadherence using HeLa cells as the *in vitro* experimental model[15,16] has been followed by using swab-derived vaginal epithelial cells (VECs), the *in vivo* targets of trichomonads[17]. Similar, if not identical, findings have been obtained for both cell types[20,21], but not for cells of non-urogenital origin[15].

Trichomonad adhesins have been identified using a ligand assay in which detergent extracts of radiolabeled *T. vaginalis* proteins are incubated with glutaraldehyde-stabilized host cells[16,19,20]. Why use this technique? First, live trichomonads bind to glutaraldehyde-fixed HeLa cells and fixed VECs, indicating the preservation of putative host

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Box 1. Summary of Findings from the Cytoadherence Assay[a]

- Trichomonal parasitism of HeLa and vaginal epithelial cells is time, temperature and pH dependent
- Cytoadherence is energy dependent
- Perturbers of cytoskeleton and microfilaments affect cytoadherence
- Host cell recognition is specific (epithelial cells are better targets than fibroblast cells)
- Saturation binding kinetics of HeLa cells suggest that the number of receptors is limited
- Radiolabeled trichomonads with unlabeled organisms of the same isolate compete stoichiometrically
- Competition of cytoadherence varies when using heterologous isolates, indicating different amounts of surface adhesins among isolates; non-pathogenic *T. tenax* does not compete with attachment of *T. vaginalis* organisms
- Putative adhesins and receptors are proteinaceous; sugars and lectins and treatment of trichomonads with neuraminidase and periodate does not abolish cytoadherence
- Live organisms treated with trypsin do not attach, and regeneration of surface proteins restores cytoadherence; protein synthesis inhibition of trypsin-treated parasites prevents regeneration of cytoadherence
- Glutaraldehyde fixation of host cells still allows for parasite recognition and binding; fixation of trichomonads abolishes attachment of live HeLa cells and vaginal epithelial cells
- Trichomonad cysteine proteinase activity is required for cytoadherence; action of proteinase is on the parasite surface
- Maximal levels of cytoadherence and elevated synthesis of adhesins occur with parasites grown under iron-replete conditions
- Anti-adhesin antibody detected, by indirect immunofluorescence, the adhesins on trichomonal surfaces and blocked cytoadherence by live parasites
- Purified adhesins inhibited parasite attachment to HeLa cells in a concentration-dependent fashion

[a] Data from Refs 15–21.

receptors in fixed host cells needed for the ligand assay. Second, it has been used in the identification of *Treponema pallidum* fibronectin-binding adhesins[25] and the *Mycoplasma pneumoniae* adhesin[26].

Four trichomonad surface proteins are selectively enriched and avidly bind to fixed HeLa cells[16,20] and VECs[20] (Fig. 1a). It is remarkable that four proteins were identified as putative adhesins in the initial experiments[16], since optimal conditions for adhesin detection by the ligand assay requires an understanding of other biological parameters: the selective solubilization of the adhesins with detergents[16,20]; the highly sensitive nature of the adhesins to trichomonad cysteine proteinases released during solubilization[20]; knowledge of downregulation of adhesin synthesis by long-term grown trichomonads[19,20]; and the discovery of iron-regulated adhesin gene expression[19]. These all are important in the identification of the four adhesins by the ligand assay.

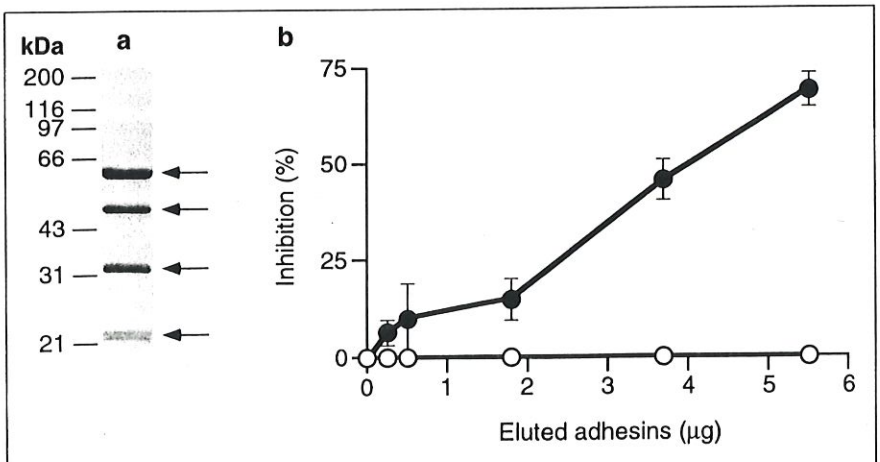

Fig. 1. Pretreatment of epithelial cells with purified trichomonad adhesins blocked cytoadherence by *Trichomonas vaginalis*[20]. A fraction (a) of the adhesin preparation used for the competition assay in part b was re-electrophoresed to show the absence of any degradation of the adhesins, demonstrating that the inhibition was due to unmodified proteins. Arrows on the right of the gel point to the adhesins, and molecular mass markers are indicated on the left in kDa. Adhesins from a ligand assay (closed circles) (b) were dialysed and equilibrated in incubation medium before being added to monolayer cultures of fixed HeLa cells. After incubation for 18 h at 4°C, live parasites were added. Each point is the mean of the per cent reduction of cytoadherence compared to the control (open circles) (mock, whole normal serum or bovine serum albumin) of duplicate experiments with triplicate samples. Bars represent standard deviations. (Reproduced, with permission, from Ref. 20.)

Antibodies to the four individual adhesins detect the proteins on the surface of live organisms and inhibit cytoadherence[20]. The availability of the antibody makes it possible to demonstrate that the adhesins are not immunocrossreactive[20], indicating their encoding by distinct genes. Purified adhesins also inhibit, in a concentration-dependent fashion, parasite attachment to host cells[20] (Fig. 1b). The quantities of the four adhesin proteins being synthesized and surface-expressed correlate with levels of cytoadherence[20] (Fig. 2).

The presence of surface expressed adhesins is not sufficient for cytoadherence. In experiments using cysteine proteinase inhibitors, the additional requirement for cysteine proteinase activity for *T. vaginalis* recognition and binding to host-cell surfaces is strongly suggested[18]. Surprisingly, the site of proteinase action was the parasite surface, not the host-cell surface. It should also be noted that, under the experimental conditions used, proteinase processing of the adhesins was not detected. The exact function of the proteinase(s) remains unknown, although an unmasking function has been proposed[18]. The adhesins on the parasite surface are protected by proteins, from the family of cysteine proteinases being elaborated during normal growth[27,28]. Expression of adhesin functionality, therefore, requires unmasking of the adhesins by degrading the protective proteins.

Environmental Regulation of Adhesin Synthesis

The target cells, VECs, mature under hormonal influence during the progression of the menstrual cycle[9]. Perhaps maturation of the target host cell presents different receptors during the menstrual cycle[10]. As such, a one adhesin–one receptor interaction may not allow the parasite to colonize the vagina throughout the menstrual cycle

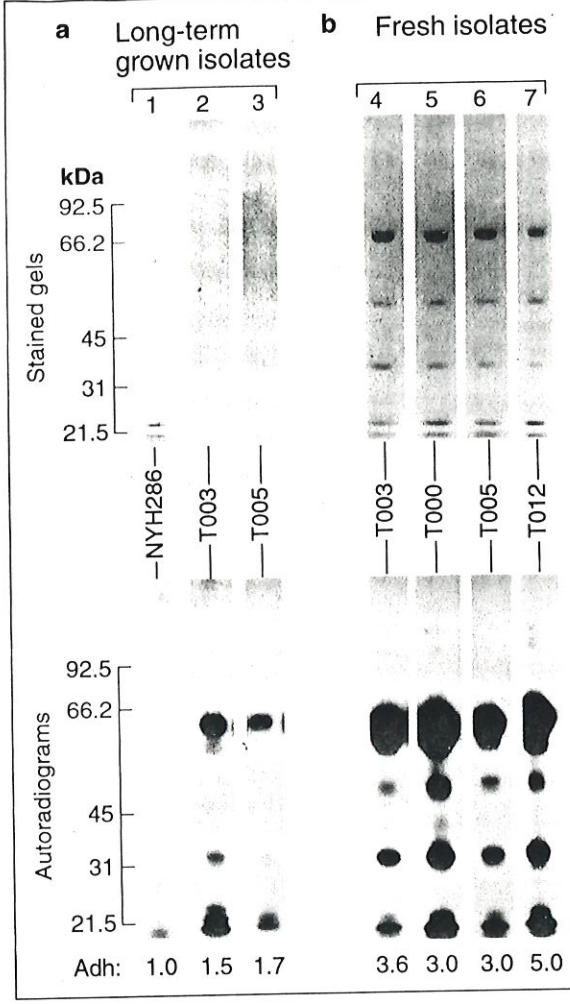

Fig. 2. The increased expression of adhesins and cytoadherence by fresh *Trichomonas vaginalis* isolates. Coomassie brilliant blue-stained patterns (top) of proteins from trichomonal detergent extract of long-term grown (a) and fresh (b) isolates which bound to fixed HeLa epithelial cells in the ligand assay. Adhesins were eluted by boiling fixed cells in dissolving buffer, and adsorbed proteins were electrophoresed in 7.5% acrylamide gels. Adhesin proteins (AP) with molecular masses of 65 kDa (AP65), 51 kDa (AP51), 33 kDa (AP33), and 23 kDa (AP23) are as described before[20]. Molecular size markers are indicated on the left in kDa. For comparative purposes, the same number of iodinated trichomonads of each isolate was used in the ligand assay[16]. Autoradiograms of gels are shown (bottom). It is noteworthy that the intensities of proteins in stained bands and in autoradiograms were highly reproducible for the fresh isolates for a period of ≤4 weeks. Adherence (Adh) values under each autoradiogram pattern are relative cytoadherence levels. The common laboratory isolate (NYH 286) used in early studies[16,25] was given a value of one for comparative purposes. This absolute value of one for NYH 286 was equal to 10% of trichomonads from the total parasite population adhering to a monolayer of HeLa cells. Levels of cytoadherence were obtained at the same time that the ligand assay was performed and were from triplicate samples repeated at least twice. (Reproduced, with permission, from Ref. 20.)

In this case, the presence of multiple adhesins may be advantageous to a parasite existing in an ever-changing host environment. It also follows that such a complex interaction between trichomonads and target cells must be controlled. Regulation of expression of the adherence mechanism may even be a prerequisite for survival of the parasite within the host. Is the expression of trichomonal cytoadherence regulated and, if so, how is this regulation accomplished? What are the signals that lead to regulation of cytoadherence?

When parasites have been grown in chemostat cultures[29] (to simulate *in vivo*-like conditions), it has been clear that environmental signals have an impact on the expression of trichomonad surface protein repertoires[30]. In the light of what is known about the effect of iron on microbial pathogenesis[31], we have examined the conditions of iron-limiting versus iron-replete growth on trichomonal cytoadherence and adhesin synthesis[19]. Low-iron parasites express a basal level of adhesins. Amounts of adhesins, but not of most other cellular proteins, can be elevated several-fold upon stimulation of low-iron trichomonads with iron. Importantly, the increase in amounts of adhesins is due to newly synthesized adhesins, as measured by incorporation of [^{35}S]methionine. When low-iron organisms are exposed to iron in the presence of transcriptional inhibitors, synthesis of adhesins cannot be demonstrated. This result, suggesting that iron is specific in regulating the expression of adhesin genes at the transcriptional level, has more recently been demonstrated by Northern analysis using cDNAs encoding the adhesins (unpublished observations). It is likely that modulation of adhesin synthesis occurs *in vivo*, given that lactoferrin is a key iron source for *T. vaginalis*[19] and that lactoferrin concentrations in vaginal fluids undergo dramatic variations during the menstrual cycle[32]. A simple growth assay has revealed that iron is obtained from a variety of sources, including lactoferrin, ferritin, hemoglobin and cytochrome[30].

Isolates passaged daily for longer than three weeks in a complex medium seem to lose the responsiveness to iron. Figure 2 illustrates the downregulation in adhesin synthesis observed for long-term cultures when compared to fresh isolates, and although all isolates are able to respond to iron-replete conditions, only fresh isolates yield greater amounts of adhesins and higher levels of cytoadherence[19].

Signaling for Amoeboid Transformation, Adhesin Synthesis and Recruitment of Other Trichomonads

Three signals transduced during or immediately following recognition and binding of *T. vaginalis* with host cells have been identified[21]. The reproducible and dramatic morphological transformation (Fig. 3) is a function of host cell type. Trichomonads change from the typical *in vitro* ellipsoid shape always seen on HeLa cells (Fig. 3a) to a flattened amoeboid form with extensive pseudopods forming at the site of contact throughout the periphery of the organisms (Fig. 3b). The host-cell–parasite, membrane–membrane associations are prominent, exhibiting many contact points and thin lamellopodia and filopodia on the transformed organisms (Fig. 3b, c). The morphological transformation occurs within five minutes of attachment and is proportional to the level of cytoadherence and the amount of adhesins presented by individual

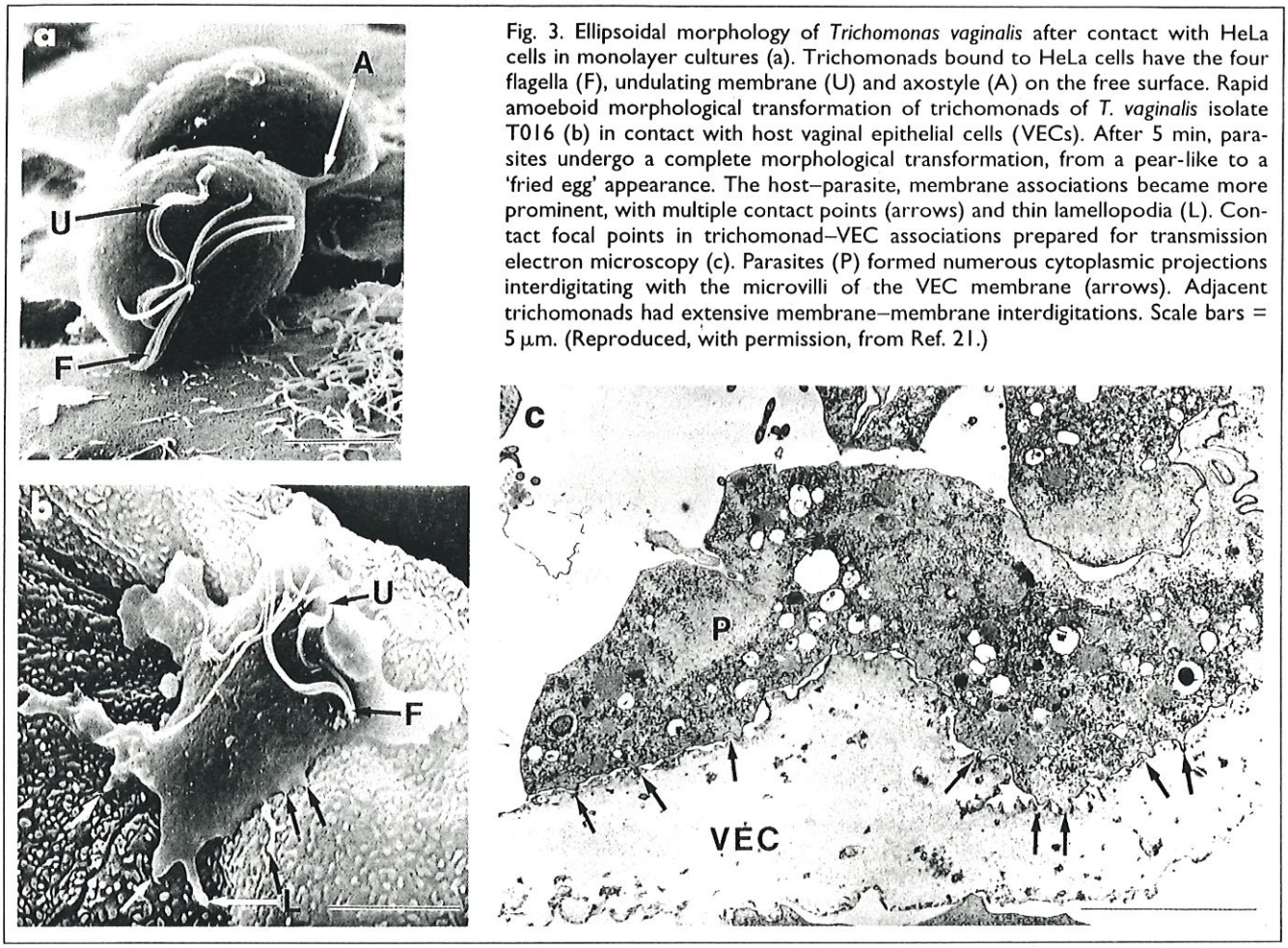

Fig. 3. Ellipsoidal morphology of *Trichomonas vaginalis* after contact with HeLa cells in monolayer cultures (a). Trichomonads bound to HeLa cells have the four flagella (F), undulating membrane (U) and axostyle (A) on the free surface. Rapid amoeboid morphological transformation of trichomonads of *T. vaginalis* isolate T016 (b) in contact with host vaginal epithelial cells (VECs). After 5 min, parasites undergo a complete morphological transformation, from a pear-like to a 'fried egg' appearance. The host–parasite, membrane associations became more prominent, with multiple contact points (arrows) and thin lamellopodia (L). Contact focal points in trichomonad–VEC associations prepared for transmission electron microscopy (c). Parasites (P) formed numerous cytoplasmic projections interdigitating with the microvilli of the VEC membrane (arrows). Adjacent trichomonads had extensive membrane–membrane interdigitations. Scale bars = 5 μm. (Reproduced, with permission, from Ref. 21.)

isolates[21]. The VECs, and not HeLa cells, induce the transformation processes.

A signal distinct from that mediating a change in shape appears to involve a stimulation of parasites after attachment to synthesize, to a greater extent than that of trichomonads alone, the adhesin proteins (Figs 1, 2). These results were obtained by fluorescence experiments with anti-adhesin IgG antibodies[21] and *T. vaginalis* organisms that were either in suspension or to those adherent to HeLa cells or VECs. Adherent trichomonads exhibit strikingly enhanced and generalized fluorescence, compared with poor staining by the organisms in suspension, indicating that binding to host cells signal for increased synthesis of adhesins.

The change in shape from ellipsoid to amoeboid possibly confers an enhanced binding of the parasite to the VEC, simultaneous with the interdigitating membrane–membrane contact points (Fig. 3c). The enhanced synthesis of adhesins following contact, and occurring during the change in shape, suggests a need to mobilize the newly synthesized adhesins to distant contact sites on the host cell being recognized by the spreading parasite. This idea is consistent with the early report on the adverse effect of parasite treatment with microtubule and microfilament inhibitors on adherence[15]. Although the precise signals being recognized by the organisms are unknown, it is conceivable that receptor density, being different between HeLa and VECs, induces the morphological transformation to maximize the parasite–host cell contact region. In addition, the association between the adhesins and receptors itself may be sufficient to stimulate adhesin synthesis, and this may explain why both HeLa cells and VECs provide the signal for synthesis. These possibilities will be testable once identification of the receptor, the existence of which has been indicated experimentally[21], has been accomplished.

The VECs initially parasitized by a single organism, ultimately have numerous adjacent trichomonads[21]. A form of recruitment of other parasites to the site of infection may imply that a signal, perhaps a chemoattractant generated by the organism, is responsible. This observation is not unlike that reported earlier[11–14,23,24] on the congregation of parasites at defined foci in cell-culture monolayers. The formation of a monolayer of *T. vaginalis* on VECs (and at sites of infection on the vaginal wall) may be complicated further by the possibility of interparasite communication that results from the extensive membrane–membrane interdigitation occurring among the adjacent organisms[21]. Perhaps some form of genetic exchange may be occurring, especially since isolates have been defined on the basis of the presence or absence of a double-stranded RNA virus[33], which was recently found to be multi-segmented[34]. Such speculation is currently without experimental validation.

Questions and Conclusions

What is the relationship of the adhesins to each other? Although synthesis and expression of the four adhesins are coordinately regulated, the juxtaposition of one adhesin to the other three is unknown. Does only one protein have the receptor-binding epitope, for example, while the other three represent important structural or support molecules? All four proteins are bona fide adhesins and therefore we must know whether the same receptor or unique receptors on host-cell surfaces are involved. What are the distinct signals that promote the dramatic morphological transformation in addition to inducing almost immediate upregulation of adhesin synthesis? Does

the increase in adhesin expression represent directed utilization of internal iron pools, known to exist within trichomonads[19], or is a different regulatory network involved for induction of gene expression following cytoadherence? Generating high-titered antibody to the purified trichomonad adhesins has been difficult, suggesting that the proteins are immunorecessive. Clearly, understanding the structure–function properties of these biofunctional proteins requires the cloning and sequencing of the adhesin genes.

Overall, the cytoadherence mechanism(s) of *T. vaginalis* is much more complex than initially thought. Regulation of adhesin expression by environmental signals appears to be a necessity for survival of this parasite in an otherwise hostile environment. Iron regulation of adhesin expression may then be viewed as a mechanism that allows the parasite to adapt to changes in the vagina during the menstrual cycle. Amounts of lactoferrin (an important iron source for this parasite[7,19,30]) are elevated after the post-menstrual phase, and steadily decline until menstruation. The availability of lactoferrin mirrors the changes in hormonal levels and the maturation stages of epithelial cells at different times of the menstrual phase. Thus, iron availability from lactoferrin may be the environmental signal that triggers trichomonads to modulate amounts of adhesins. Given the high requirement for iron by this organism[6,7], low-iron trichomonads with downregulated expression of adhesins may be free to migrate toward sites that are richer in iron. The end result would be the persistence of this protozoan within the vagina, despite the flushing action of mucosal secretions, desquamation of the mucosal epithelium and overall nutrient limitation.

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