

## ***Trichomonas vaginalis* interactions with fibronectin and laminin**

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**The sexually transmitted protozoan *Trichomonas vaginalis* cytoadheres to vaginal epithelial cells and causes contact-dependent cytotoxicity which, when combined with the normal exfoliation process, leads to erosion of the epithelium, which may allow trichomonads into extracellular matrix and basement membrane sites. Therefore, the association of *T. vaginalis* with immobilized fibronectin (FN) and laminin (LM) on cover-slips was examined. Binding of live parasites to coated cover-slips was time- and parasite-density-dependent. Coincubation with an inhibitor of trichomonad cysteine proteinases resulted in an increased attachment of parasites to FN but had no effect on binding to LM, denoting that protease activity influenced optimal FN associations. Further, 20 h mid-exponential phase trichomonads placed in fresh culture medium for 3 h gave higher levels of binding to FN, suggesting that changes during growth *in vitro* to *T. vaginalis* organisms affect maximal levels of binding to FN. Extended incubation with substrates diminished the capacity of parasites to bind FN and LM. Treatment of live organisms with periodate reduced binding to LM but not FN, suggesting a role for carbohydrates. In addition, trypsinization of live parasites decreased numbers bound to both substrates. Placement of trypsinized parasites in medium for 2 h fully regenerated binding to FN but not LM. Incubation of trypsinized parasites with cycloheximide abrogated regeneration of attachment to FN, affirming a role for synthesized surface proteins in FN binding. Importantly, the *T. vaginalis* adhesin proteins that mediate cytoadherence, and iron, a factor that regulates adhesin synthesis, were not involved in FN and LM recognition. These results suggest a role for surface proteins and carbohydrates in trichomonal associations with FN and LM, respectively.**

Keywords: colonization, fibronectin, laminin, pathogenesis, *Trichomonas vaginalis*

### **INTRODUCTION**

*Trichomonas vaginalis* is a flagellated protozoan that causes the most prevalent non-viral sexually transmitted disease (STD) worldwide (World Health Organization, 1995). Trichomonosis is associated with significant morbidity for women, with vaginitis symptoms ranging from mild to severe inflammation, foul-smelling discharge, and severe irritation and discomfort (Wolner-Hanssen *et al.*, 1989; Krieger *et al.*, 1990; Soper *et al.*, 1990). Infection by the parasite can have significant

health consequences for women, including increased risk for adverse pregnancy outcome (Hardy *et al.*, 1984; Minkoff *et al.*, 1984; Cotch *et al.*, 1991; Read & Klebanoff, 1993), HIV seroconversion (Wasserheit, 1992; Laga *et al.*, 1993) and cervical cancer (Zhang & Begg, 1994).

The vaginal wall consists of a stratified squamous outer epithelium and the underlying connective tissue. The hormones of the menstrual cycle, particularly oestrogen and progesterone, control the growth and differentiation of epithelial cells and ultimately lead to the terminal differentiation and exfoliation of vaginal epithelial cells (VECs). Maintaining and supporting the epithelium is a network of macromolecules comprising the extracellular matrix (ECM) and the basement membrane (BM), such as fibronectin (FN) and laminin (LM).

**Abbreviations:** BM, basement membrane; ECM, extracellular matrix; FN, fibronectin; LM, laminin; TLCK, *N* $\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone; VECs, vaginal epithelial cells.

As with other STD-causing pathogens, persistence within the urogenital tract by *T. vaginalis* would predictably require specific binding of the parasite to host structures. As such, these organisms adhere to VECs via surface adhesins (Alderete & Garza, 1985, 1988; Alderete *et al.*, 1988; Arroyo & Alderete, 1989; Arroyo *et al.*, 1992, 1995). However, exfoliation of VECs from the vaginal epithelium in addition to trichomonal cysteine-proteinase-mediated cytotoxicity (Alderete & Pearlman, 1984) point towards parasites possibly residing at sites below the epithelial surface. This possibility may help explain the non-self-limiting nature of trichomonosis.

This report supports the existence of another mechanism by which *T. vaginalis* colonizes host tissues during trichomonosis. We hypothesized that persistence of parasites in the vagina may be due to the interaction of *T. vaginalis* with FN and LM. Early studies by us demonstrated the interaction of plasma proteins, including FN, with the *T. vaginalis* surface (Peterson & Alderete, 1982, 1984a). In addition, parasite attachment to LM has been reported (Casta e Silva Filho *et al.*, 1988; Benchimol *et al.*, 1990). That LM does not reside on the surface of VECs (unpublished observations) suggests that specific parasite binding to FN may contribute to host parasitism. In this report, we demonstrate that *T. vaginalis* interacts with both FN and LM and that these associations are mediated by different mechanisms and surface sites from the previously identified adhesins (Arroyo *et al.*, 1992). The relevance of these findings with regard to pathogenesis of trichomonosis is discussed.

## METHODS

**Culture and growth of *T. vaginalis*.** Fresh clinical isolates of *T. vaginalis* were axenized and passaged daily in normal medium of trypticase/yeast extract/maltose (TYM) supplemented with 5% heat-inactivated horse serum (Diamond, 1957; Peterson & Alderete, 1982, 1984b). All isolates except for SA94-175 have been previously used by us in a variety of assays that display virulence properties, such as that involving the adhesins and cytoadherence (Arroyo *et al.*, 1992, 1995) and expression of cysteine proteinases that degrade ECM/BM proteins (Provenzano & Alderete, 1995; Provenzano *et al.*, 1997). The isolates were not passaged in batch culture longer than 1 month, since long-term-grown trichomonads show decreased expression of some virulence properties (Lehker *et al.*, 1991). High- and low-iron-grown trichomonads were cultivated in TYM-serum medium containing 50  $\mu\text{M}$  2,2-dipyridyl (2,2-DP) for 24 h at 37 °C starting with  $10^5$  parasites  $\text{ml}^{-1}$  (Lehker *et al.*, 1991). Organisms were centrifuged at 670 *g* and suspended in TYM-serum containing 75  $\mu\text{M}$  2,2-DP for incubation for an additional 24 h. These low-iron parasites were then harvested by centrifugation at 670 *g*, seeded into individual flasks of medium supplemented with either 75  $\mu\text{M}$  2,2-DP (low iron) or 250  $\mu\text{M}$  ferrous ammonium sulfate (high iron), and cultivated for 20 h. Final cultures were processed as described below for the binding assay.

**ECM components and coating of cover-slips.** FN was purified from human plasma, obtained from the UTHSCSA hospital blood bank, by gelatin-Sepharose affinity chromatography according to the manufacturer's instructions (Pharmacia

Biotech). Briefly, human plasma was diluted 1:1 (v/v) in PBS prior to chromatography. Purified FN, at concentrations of 0.5–1 mg  $\text{ml}^{-1}$ , was dialysed for 48 h with three changes of PBS. Protein concentration was determined using bicinchoninic acid (BCA; Pierce Chemical). Entactin-free mouse LM was purchased from Collaborative Research (Becton Dickinson Labs). Cover-slips (12 mm diam.; Bellco Glass) were coated with 1  $\mu\text{g}$  FN or LM by spreading a 100  $\mu\text{l}$  volume of the protein solution in PBS over the entire surface, as before (Peterson *et al.*, 1983; Thomas *et al.*, 1985). Cover-slips were then air-dried overnight at room temperature and washed by immersion in PBS before placing individual cover-slips in 24-well plates. The uniform coating of cover-slips with each protein was confirmed by indirect immunofluorescence using specific antisera to each protein.

**Parasite-binding assay.** Parasites at  $10^5 \text{ ml}^{-1}$  were grown in normal medium containing 5–10 mCi [ $^3\text{H}$ ]thymidine  $\text{ml}^{-1}$  (185 MBq; Dupont, NEN Research Products). Efficient radiolabelling of parasites and the extent of radiolabel for each density of parasites used (specific activity) was monitored throughout, as before (Alderete & Pearlman, 1984; Alderete & Garza, 1985, 1988; Arroyo *et al.*, 1992).

Trichomonads used in all binding assays were from the mid-exponential phase of growth ( $\sim 20$  h) at  $10^6 \text{ ml}^{-1}$  (Peterson & Alderete, 1982). Except for the experiment presented in Fig. 2, radiolabelled parasites were then centrifuged and suspended in the same volume of medium without radioisotope for an additional 2 h at 37 °C before use in binding assays. Trichomonads were then harvested and washed twice in minimal binding (MB) buffer (120 mM NaCl, 1.3 mM KCl, 0.9 mM  $\text{NaH}_2\text{PO}_4$ , 5.5 mM glucose and 26 mM  $\text{NaHCO}_3$ , pH 5.0) by centrifuging at 670 *g* for 5 min. Parasites were enumerated using a Neubauer haemocytometer (Alderete & Pearlman, 1984; Alderete & Garza, 1985; Peterson & Alderete, 1982). Different numbers of organisms from  $5 \times 10^5$  to  $5 \times 10^6$  parasites in 1 ml MB buffer supplemented with 400  $\mu\text{M}$  *N* $\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma) were added to individual wells containing the protein-coated cover-slips. These were incubated for 30 min at 37 °C or as stated for each experiment. Cover-slips were washed by immersing several times in 37 °C pre-warmed MB buffer. Bound radioactivity remaining on the cover-slips was measured by scintillation spectroscopy. The number of c.p.m. on cover-slips reflected the number of parasites bound to FN or LM and was confirmed by light microscopy and enumeration of organisms in individual fields.

Two types of experiments were performed to demonstrate that trichomonads degrade FN immobilized on cover-slips. In one case, 30  $\mu\text{g}$  FN was coated onto the cover-slips and interacted with  $3 \times 10^6$  organisms suspended in 400  $\mu\text{l}$  of the normal, complex TYM medium without serum. Wells were incubated at 37 °C prior to collection of samples at the indicated time points. The samples were then processed as described below. Cover-slips were also coated with 10  $\mu\text{g}$  FN and incubated with  $10^6$  parasites per ml MB buffer with or without TLCK added to each well. At different time points, supernatant was collected and centrifuged at 13000 *g* for removal of organisms. The trichomonads and supernatant were prepared for SDS-PAGE as described below.

**Periodate and trypsin treatment of live trichomonads.** Washed, pelleted radiolabelled parasites ( $2 \times 10^7$ ) were suspended in 10 ml 1 mM periodate prepared in PBS, as before (Alderete & Garza, 1985), under conditions that do not adversely affect trichomonal motility and viability. Trichomonads were then incubated on ice for 30 min before washing

twice in PBS prior to suspending in MB buffer for use in the binding assay, as described above. In a competition assay, the MB buffer was supplemented with 250 mM maltose, glucose, sucrose or *N*-acetylgalactosamine (Alderete & Garza, 1985; Warton & Honigberg, 1980, 1983). Trichomonads were suspended in the modified MB immediately prior to incubation with coated cover-slips at 37 °C for 30 min. MB buffer without sugars was used as a control.

Prior to protease treatment,  $2 \times 10^7$  washed radiolabelled organisms from batch culture were incubated for an additional 2 h in fresh TYM-serum. Parasites were pelleted by centrifugation at 670 g, suspended in 10 ml MB buffer containing 30 mg trypsin (Sigma) and incubated for 30 min at 37 °C. The reaction was then terminated by the addition of equal amounts of trypsin inhibitor (Sigma). The cells were washed and suspended in MB buffer at the appropriate density for binding assays. In addition, two aliquots of trypsinized cells were placed in TYM-serum medium for an additional 2 h with and without 10 nM cycloheximide, a protein synthesis inhibitor (Alderete & Garza, 1985). Parasites were monitored throughout to ensure that experimental conditions did not affect viability and motility.

**Immunofluorescence.** FN- and LM-coated cover-slips were placed in individual wells of a 24-well plate with 200  $\mu$ l PBS containing 5  $\mu$ l of either rabbit anti-FN or anti-LM serum, respectively. Normal rabbit serum (NRS) served as a negative control on cover-slips handled similarly. Cover-slips were incubated on a shaker for 30 min at 4 °C before being washed in cold PBS. Then, 250  $\mu$ l cold PBS containing 5  $\mu$ l goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (Sigma) was added. Cover-slips were incubated in the dark for 30 min at 4 °C followed by washing with cold PBS and placing onto glass slides for observation by epifluorescence.

**Antibody pretreatment of trichomonads.** IgG antibody was purified from prebleed NRS and anti-adhesin sera, reported and used previously by us (Arroyo *et al.*, 1992). Parasites were suspended in MB buffer at  $2 \times 10^6$  ml<sup>-1</sup> in the presence of 400  $\mu$ M TLCK to inhibit cysteine proteinases known to degrade immunoglobulins (Provenzano & Alderete, 1995). One microgram, 5  $\mu$ g and 50  $\mu$ g of each of the four anti-adhesin IgGs were pooled prior to mixing with live trichomonads. As a control, 50  $\mu$ g NRS IgG was used. Trichomonads were pretreated with IgG for 10 min at 37 °C prior to addition of the parasite-antibody mixture to wells containing coated cover-slips.

**Electrophoresis and immunoblotting.** Total protein preparations of parasites, grown either in high- or low-iron TYM-serum medium, were prepared as described before (Lehker & Alderete, 1992). Briefly,  $2 \times 10^7$  cells were washed and suspended in 1 ml cold PBS-TCA (10%, v/v), and total proteins were precipitated overnight at 4 °C. The protein pellet was collected by microcentrifugation at 4 °C and washed three times with cold PBS. Proteins were dissolved by suspension and boiling in electrophoresis dissolving buffer (Laemmli, 1970). Standard SDS-PAGE was performed using 4% stacking and 7% separating acrylamide gels (Laemmli, 1970). The proteins and molecular size markers (Amersham) were visualized by staining of gels with Coomassie brilliant blue R (Laemmli, 1970).

To visualize FN, proteins from supernatants or cell pellets were blotted onto nitrocellulose after SDS-PAGE using a standard protocol (Towbin *et al.*, 1979; Lehker & Alderete, 1992). Membranes were incubated in TNT (10 mM Tris/HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20) buffer with 5% non-fat milk as a blocking agent. Nitrocellulose blots were

then incubated overnight at 4 °C in goat anti-FN IgG (1:400), followed by washing in TNT buffer, and incubated for 1 h at room temperature in rabbit anti-goat secondary antibody conjugated to alkaline phosphatase (1:4000) in TNT buffer with 3% milk. Blots were finally washed three times in TNT for development (Sambrook *et al.*, 1989).

**Statistical analysis.** Unless otherwise stated, each experiment was performed at least three times, and each representative experiment included quadruplicate samples. Except where noted differently, percentage binding of parasites to substrate on cover-slips is c.p.m. bound divided by total c.p.m. added per well and multiplied by 100. As all the data have a normal distribution, the Student's *t* test was performed where indicated.

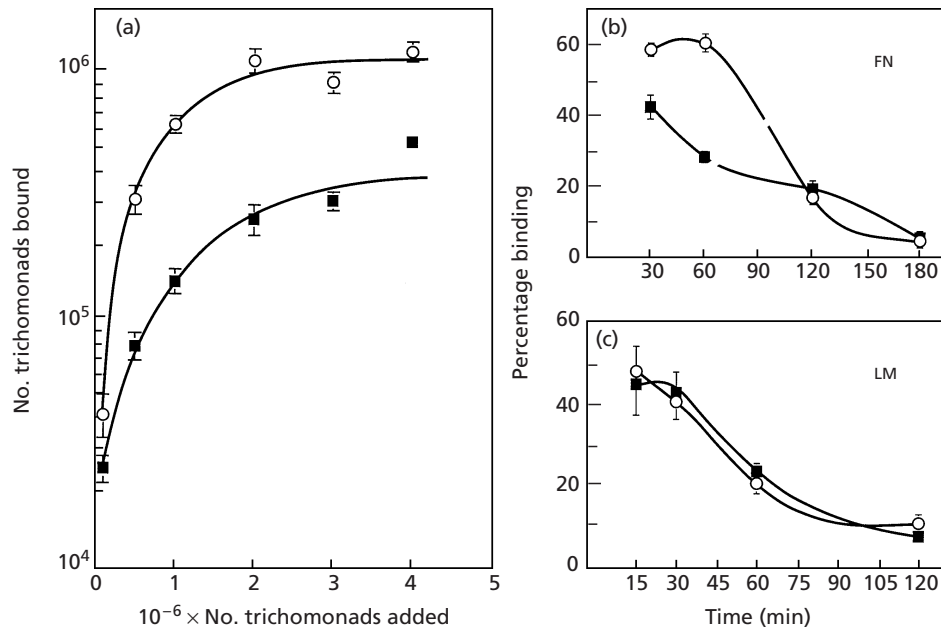
## RESULTS

### Optimization of parasite binding to FN and LM

Because FN and LM immobilized on cover-slips has been used previously by us to study their interaction with microbial pathogens (Peterson *et al.*, 1983; Thomas *et al.*, 1985), we first determined the optimal amount of FN and LM immobilized on cover-slips for association with  $10^6$  radiolabelled trichomonads. Amounts ranging from 0.25 to 20  $\mu$ g were coated onto the cover-slips. Maximal numbers of organisms binding to both FN and LM surfaces was within the 1  $\mu$ g range. This amount of protein on cover-slips was then employed for subsequent experiments. Two million trichomonads ml<sup>-1</sup> gave maximal numbers bound for both FN and LM cover-slips (Fig. 1a). Fig. 1(b) shows that using  $10^6$  trichomonads in the assay gave the highest numbers of organisms bound on FN by 30 min, and levels were maintained up to 60 min. In contrast, Fig. 1(c) illustrates that highest binding levels to LM occurred by 15 min and remained maximal for 30 min. A decrease in numbers of organisms was seen during extended incubations with both substrates. Addition to the interaction buffer of TLCK, a cysteine proteinase inhibitor, resulted in higher numbers of organisms bound to FN, but not to LM. These preliminary results suggest that the numerous trichomonad proteinases synthesized (Neale & Alderete, 1990; Provenzano & Alderete, 1995) and released (Lockwood *et al.*, 1988) may adversely affect maximal levels of parasites associating with the immobilized FN.

### Growth in medium or extended incubation in binding buffer affects the levels of *T. vaginalis* organisms binding to FN-coated cover-slips

Extended incubations resulting in loss of binding to immobilized substrates as seen above in Fig. 1(b, c) could be due to alteration of the parasite surface and/or degradation of the FN on cover-slips. To test for these possibilities, mid-exponential phase organisms were harvested and divided into three groups. Group one parasites were suspended in MB buffer and interacted with the FN cover-slips for 30 min and 180 min at 37 °C. Groups two and three were trichomonads suspended in MB buffer or TYM growth medium, respectively, at the

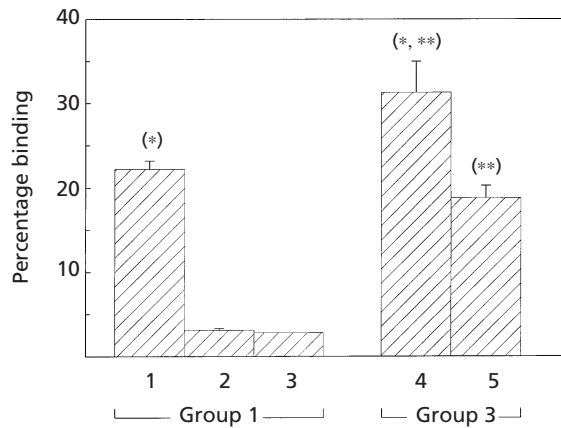


**Fig. 1.** Representative experiments showing optimization of the *in vitro* binding assay. (a) Increasing densities of parasites, suspended in MB buffer containing 400  $\mu$ M TLCK, were added to each well with coated cover-slips. Cells were incubated for 30 min at 37 °C as described in Methods.  $\circ$ , FN;  $\blacksquare$ , LM. (b, c) Trichomonads ( $10^6$ ) were incubated for up to 3 h at 37 °C on either FN- or LM-coated cover-slips with or without a cysteine proteinase inhibitor (400  $\mu$ M) added to the buffer.  $\circ$ , +TLCK;  $\blacksquare$ , -TLCK.

same density and incubated for 180 min at 37 °C prior to addition to the FN-coated cover-slips. Group one organisms were harvested after the 180 min incubation, washed, and placed onto newly coated cover-slips. The cover-slips from the 180 min group one parasites were also washed prior to addition of the group three freshly harvested, radiolabelled parasites. As presented in Fig. 2 and consistent with the above results, fresh 20 h mid-exponential phase parasites bound to newly coated cover-slips by 30 min (column 1) followed by decreased binding by 180 min (column 2). The 180 min organisms remained viable and motile but were unable to associate with newly coated FN cover-slips (column 3). Group two parasites placed in MB buffer alone also gave low levels of binding to FN cover-slips, as seen in column 3. These results suggest that extended incubation in the MB buffer leads to alteration of the parasite, resulting in an inability to attach to the FN-coated cover-slips. It is noteworthy that the 180 min parasites, although unable to bind to FN cover-slips (column 3), had associated FN, as detected by immunoblot analysis of total trichomonad proteins probed with goat anti-FN serum (data not shown). These data reinforced 30 min as the optimal binding period. Moreover, group three trichomonads attached at higher levels (column 4) compared to organisms directly from 20 h mid-exponential phase of growth (column 1). Binding levels were higher with new (column 4) versus used (column 5) cover-slips, suggesting strongly that FN is modified during extended incubation with live organisms. The results indicate that experiments evaluating optimal interactions between *T. vaginalis* organisms and ECM components require that

mid-exponential phase organisms be placed in fresh medium prior to use in binding assays.

During our optimization of the binding assay and since addition of TLCK to the interaction buffer affected binding to FN only (Fig. 1b, c), we tested whether trichomonads degraded FN present on the cover-slips. To this end, cover-slips coated with 30  $\mu$ g FN were incubated with parasites as described in Methods. As shown in Fig. 3(a), supernatants after interaction of cover-slips with trichomonads contained soluble FN that was degraded in a time-dependent manner. We also examined for FN degradation under the optimal experimental conditions employed for measuring binding to this substrate (Methods). As seen in Fig. 3(b), supernatants contained degraded FN released from the coated cover-slips, and this degradation was inhibited by TLCK. Analysis by immunoblot of total proteins from washed parasites recovered from supernatants detected FN bound to trichomonads (data not shown), possibly leading to the decrease in numbers bound to cover-slips as shown in Fig. 2. These results show the ability of *T. vaginalis* organisms to actively release and degrade immobilized FN substrate. Although this aspect of parasite-substrate interactions was not examined for LM, it is likely that similar events also occur, as we have shown previously that trichomonads degrade BM (Provenzano & Alderete, 1995), of which LM is a component. Collectively, the data suggest that a combination of modifications occurring on trichomonads and target substrate affects binding levels of parasites at extended periods.

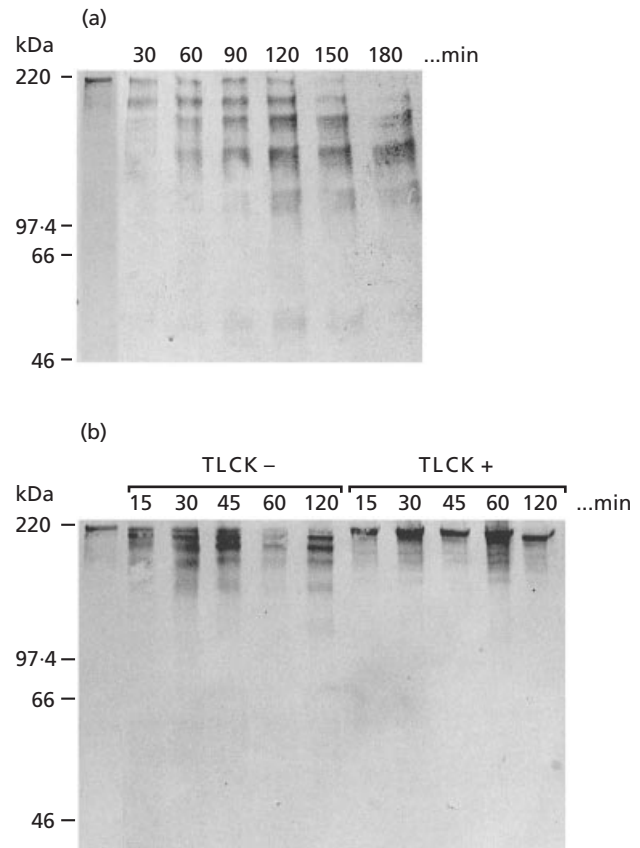


**Fig. 2.** Representative experiment showing that growth and extended incubations influence numbers of organisms bound to FN cover-slips. For this experiment, radiolabelled trichomonads were derived directly from the 20 h mid-exponential phase of growth (group one). Columns 1 and 2 show the levels of parasite ( $10^6$ ) binding at 30 and 180 min incubation, respectively. Parasites at 180 min incubation (column 2) were then washed in MB buffer and placed with new protein-coated cover-slips (column 3). Note the absence of any increased association with these cover-slips. Trichomonads from the mid-exponential phase of growth were placed in MB buffer for 3 h prior to incubation with FN cover-slips (group two). These group two parasites gave levels of binding equal to those seen in column 3. Organisms at the mid-exponential phase of growth were incubated in fresh TYM medium for an additional 3 h (group three). Parasites were then washed and added to newly coated (column 4) versus used (column 5) cover-slips derived from prior incubation with trichomonads for 3 h (column 2). The Student's *t* test was performed when comparing column 1 versus column 4 (single asterisk) and column 4 versus column 5 (double asterisk). Both were found to be significantly different ( $P < 0.05$ , for both).

### Trichomonad surface sites mediate binding to FN- and LM-coated cover-slips

We next attempted to define the nature of the trichomonad surface structures involved in the interaction with FN and LM. As shown in Fig. 4(a), 1 mM periodate treatment of trichomonads under conditions that do not adversely affect motility and viability (Alderete & Garza, 1985) resulted in a 55% decrease in binding to LM but had no effect on parasite association with FN, suggesting that carbohydrates on the organisms were involved in LM binding. We further performed binding experiments in the presence of 250 mM of various mono- and disaccharides (Alderete & Garza, 1985), and no inhibition of parasite binding to LM was observed, reinforcing that complex carbohydrate structures might be involved.

As seen in Fig. 4(b), trypsinization of live parasites resulted in 85% and 70% decreased binding of organisms to FN and LM, respectively. Interestingly, placement of treated parasites in medium for 2 h resulted in full regeneration of binding to FN but only partial restoration of association with LM. Finally, incubation of trypsinized parasites in growth medium in the

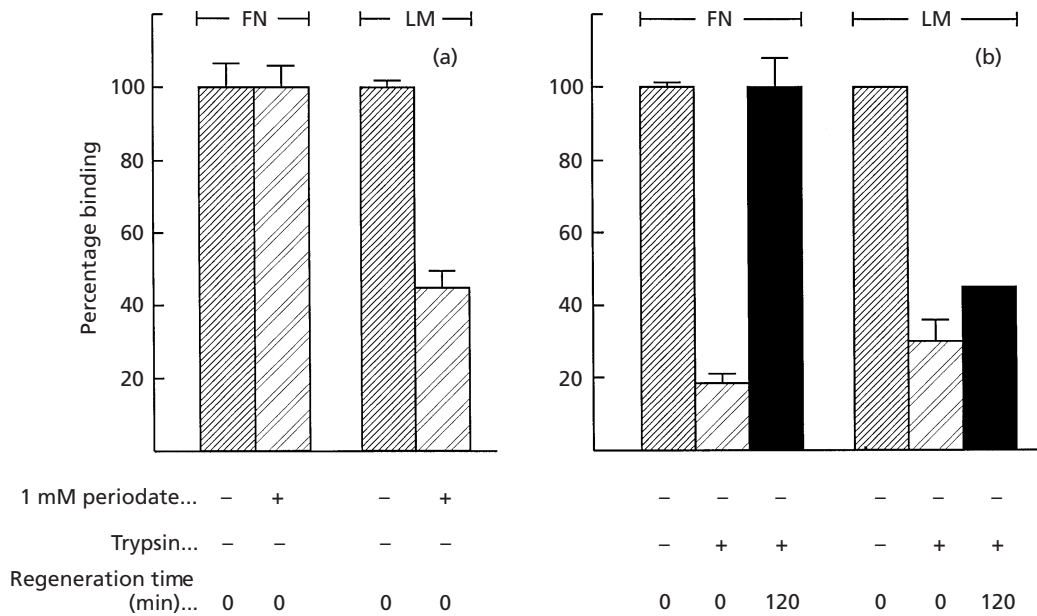


**Fig. 3.** Representative experiments showing degradation of immobilized FN by trichomonad cysteine proteinases. (a) Immunoblot detection of FN and FN fragments showing degradation of 30  $\mu$ g immobilized FN recovered from supernatant after incubation with trichomonads in TYM alone, as described in Methods. (b) Immunoblot detection of FN (10  $\mu$ g) released from cover-slips by  $10^6$  parasites in the presence or absence of 400  $\mu$ M TLCK at 37 °C for 3 h.

presence of cycloheximide, a protein synthesis inhibitor, completely prevented the regeneration of binding to FN (data not shown), confirming the requirement for synthesis and surface placement of proteins on the *T. vaginalis* surface. These data strongly suggest that recognition and binding of *T. vaginalis* to FN and LM involve different molecules and mechanisms.

### *T. vaginalis* interaction with FN and LM is distinct from cytoadherence

Because synthesis of adhesins and levels of cytoadherence are up-regulated by iron (Arroyo *et al.*, 1993; Lehker *et al.*, 1991), we further compared levels of binding of high- versus low-iron-grown parasites onto FN- or LM-coated cover-slips. As a control, the iron status of trichomonads was confirmed by electrophoretic analysis of differentially expressed proteins (Peterson & Alderete, 1984b; Lehker & Alderete, 1992). Binding to FN or LM was unaffected by the iron status of the organism (data not shown). Not unexpectedly,



**Fig. 4.** Representative experiments showing that treatment of live parasites affects recognition and binding to FN- and LM-coated cover-slips. (a) Trichomonads ( $2 \times 10^7$ ) were treated with 1 mM periodate as described in Methods prior to measuring numbers of organisms bound to FN- and LM-coated cover-slips after 30 min at 37 °C. (b) Live organisms were trypsinized and tested for binding to FN and LM. In this experiment and for optimal associations, parasites were harvested by centrifugation at mid-exponential phase, resuspended in fresh TYM-serum medium, and incubated at 37 °C for 2 h. Trichomonads were then washed in MB buffer and incubated with 30 mg trypsin for 30 min at 37 °C. The reaction was stopped by addition of equal amounts of trypsin inhibitor, and the organisms were prepared for binding. An aliquot of trypsinized trichomonads was also placed in growth medium for 2 h at 37 °C and monitored for regeneration of binding to FN and LM on cover-slips as described in Methods. All untreated controls were handled identically and were represented as 100% organisms bound. Percentage binding of treated parasites represents numbers bound relative to untreated controls.

incubation of parasites with a mixture of antibodies to the *T. vaginalis* adhesins (Arroyo *et al.*, 1992, 1995) had no effect on levels of organisms bound to FN-coated cover-slips. These results strongly indicate that the mode of *T. vaginalis* association with ECM proteins is distinct from cytoadherence.

#### All *T. vaginalis* isolates tested interact with the immobilized substrates

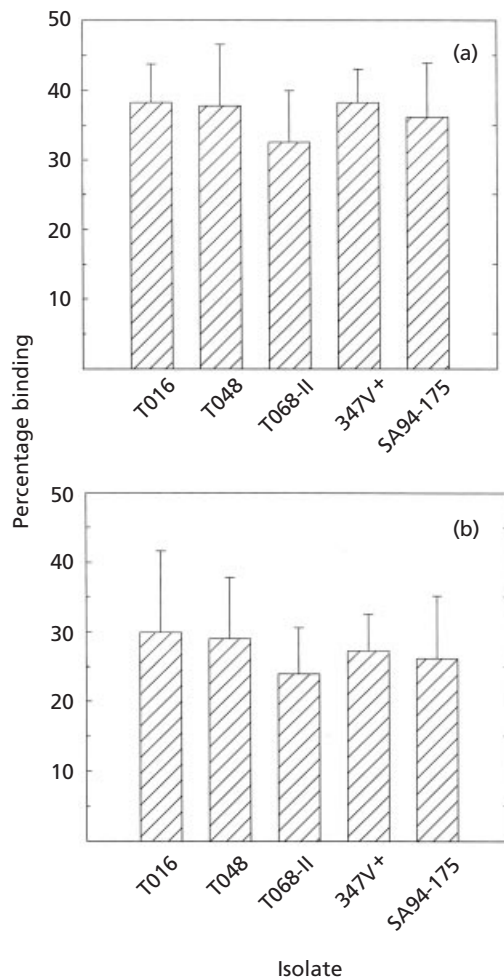
Finally, we felt it important to verify that other fresh clinical isolates of *T. vaginalis* bound comparably to FN and LM on cover-slips. Fig. 5 illustrates the results of several binding assays for isolate T016 and four additional trichomonal isolates. Data represent the mean of four separate experiments, and although binding for each isolate between each of the four experiments varied, statistical analysis shows that levels of binding to either FN or LM were not significantly different between isolates (Student's *t* test,  $P > 0.05$ ,  $n = 4$ ).

#### DISCUSSION

The non-self-limiting nature of mucosal infection by *T. vaginalis* may be the result of parasite infiltration into the ECM and BM space. The combination of parasite-mediated contact-dependent and independent cytotoxicity (Alderete & Pearlman, 1984; Krieger *et al.*,

1985) and exfoliation of the VECs suggests that despite the specific nature of trichomonal cytoadherence to VECs (Alderete & Garza, 1985, 1988; Alderete *et al.*, 1988; Lehker *et al.*, 1991; Arroyo *et al.*, 1992, 1995), alternative pathogenic mechanisms are required for long-term infection. Focal sites of erosion in the vaginal epithelium result from the presence of numerous tissue-degrading proteinases in vaginal secretions of women with trichomonosis (Alderete *et al.*, 1991). It seems, therefore, possible that trichomonads are capable of reaching the ECM and BM, and associate with FN and LM.

There are many reports in the literature on the specific nature of associations between microbial pathogens and ECM/BM membrane components (Westerlund & Korhonen, 1993; Patti *et al.*, 1994; Patti & Hook, 1994). *Candida albicans*, an opportunistic yeast, attaches to ECM and BM molecules, and it is presumed that the interaction with immobilized FN and other ECM molecules enhances the establishment and maintenance of infection at sites outside its normal niche (Calderone & Scheld, 1987; Klotz, 1994). Other mucosal pathogens, such as the streptococci, adhere to immobilized FN in a time- and dose-dependent manner (Kuusela *et al.*, 1985; van der Flier *et al.*, 1995). It may not be surprising, therefore, that *T. vaginalis*, a mucosal pathogen of the vaginal tract, utilizes components of the ECM and BM,



**Fig. 5.** Comparable binding of fresh clinical isolates of *T. vaginalis* to FN- and LM-coated cover-slips. Binding assays to FN (a) and LM (b) were performed as described in Methods. The percentage binding represents the mean and standard deviation of numbers of organisms bound relative to numbers added. The Student's *t* test shows that binding to either FN or LM was not significantly different between isolates ( $P > 0.05$ ,  $n = 4$ ).

such as FN and LM, to establish colonization and cause persistent infection.

In this report, we demonstrate and characterize the nature of *T. vaginalis* binding to immobilized FN and LM. Trichomonad surface proteins appear to be involved in both FN and LM associations, as evidenced by the effect of protease treatment on live organisms (Fig. 4). This was supported by the ability to regenerate binding to FN upon placement of trypsinized cells back into culture. The absence of similar regeneration for the trichomonad association with LM may not be surprising, as the data indicate a role for surface proteins as well as complex carbohydrate structures on *T. vaginalis* in this interaction. It is important to note, however, that, while a protein moiety is involved in FN binding, the structure may still be in the form of a glycoprotein.

Molecular characterization of these structures awaits future experimentation.

The adhesin proteins mediating cytoadherence of *T. vaginalis* to VECs (Arroyo *et al.*, 1992) were not involved in recognition and binding to FN and LM. Also, iron, an important modulator of expression of the adhesin genes (Lehker *et al.*, 1991), played no role in parasite associations with either FN- or LM-coated cover-slips. These results suggest that variations in the concentration of lactoferrin, a known *in vivo* iron source for the parasite (Peterson & Alderete, 1984b; Lehker & Alderete, 1992), would not affect the ability of *T. vaginalis* to colonize host tissues. We have described the morphological transformation that occurs upon cytoadherence to VECs of *T. vaginalis* organisms (Arroyo *et al.*, 1993). A change from the culture ellipsoid form to an amoeboid morphology was also seen on the immobilized FN and LM surfaces within the 30 min time point. These observations indicate the existence of signals similar to the ones observed during cytoadherence.

Under our experimental conditions, the binding assays did not allow all parasites to associate with the protein-coated cover-slips. Experiments were performed in which organisms that remained unbound after a 20 min incubation with immobilized FN were placed onto new FN-coated surfaces. In this manner, all trichomonads were found to attach to FN. These data suggest that the observed variations (Fig. 5) are probably due to the constraints within the assay system. It would also appear that trichomonads are capable of simultaneously adhering to VECs and attaching to ECM and BM regardless of the iron status at the site of infection.

We show that immobilized FN can be degraded by the parasite cysteine proteinases (Fig. 3). These results are consistent with earlier reports showing the ability of trichomonad cysteine proteinases, produced *in vivo* (Alderete *et al.*, 1991), to degrade FN and ECM components as determined by substrate gel analyses (Provenzano & Alderete, 1995). Degradation of FN at the site of infection may be significant. Studies with mammalian cells have shown that particular FN fragments can elicit various responses, such as changes in morphology (Chen & Culp, 1998), attachment or detachment of cells from surfaces (Saiki *et al.*, 1991; Fukai *et al.*, 1997), and even apoptosis (Fukai *et al.*, 1998). It is conceivable that, during infection, parasite degradation of ECM components could lead to unforeseen aspects of cytopathology (Draper *et al.*, 1995) and/or host cell and parasite responses.

These data further highlight the complexity of this host-parasite relationship. These studies will ultimately help in understanding the persistence of parasites in an environment that undergoes dramatic changes over the course of the menstrual cycle. Furthermore, this report emphasizes the need to continue the characterization of the mechanisms and molecules involved in the *T. vaginalis* interaction with host cells and tissue components, including FN and LM, to fully clarify the non-self-limiting nature of infection.

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