Molecular Microbiology (1992) 6(7), 853–862

Molecular basis of host epithelial cell recognition by *Trichomonas vaginalis*

R. Arroyo,* J. Engbring and J. F. Alderete
Department of Microbiology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7758, USA.

Summary

Parasitism of host epithelial cells by *Trichomonas vaginalis* is a highly specific event. Four trichomonad surface proteins (adhesins) with molecular masses of 65000 daltons (65 kDa; AP65), 51 kDa (AP51), 33 kDa (AP33), and 23 kDa (AP23) mediate the interaction of *T. vaginalis* with epithelial cells. Fresh isolates, when compared with long-term-grown isolates, had greater amounts of adhesins, which corresponded with increased levels of cytoadherence. Anti-adhesin antibodies reacted by immunoblot only with the respective protein and detected, by indirect immunofluorescence, each adhesin on the parasite surface. These antibodies inhibited the binding of live parasites to epithelial cells and protected epithelial cells from contact-dependent cytotoxicity. The pretreatment of epithelial cells with a preparation of purified adhesins also blocked trichomonal cytoadherence. Moreover, HeLa cells possessed molecules which recognized and bound to adhesins on nitrocellulose blots.

Introduction

Trichomonas vaginalis is a flagellated protozoan responsible for one of the most common sexually transmitted diseases in humans. For human infection, trichomonads must overcome the mucus barrier and parasitize the vaginal epithelium (Nielsen and Nielsen, 1975; Alderete *et al.*, 1988). It is noteworthy that cytoadherence is a highly specific, receptor-ligand type of event (Alderete and Garza, 1985; Alderete *et al.*, 1988), which appears to be a prerequisite for cytopathogenicity (Hogue, 1943; Heath, 1982; Alderete and Pearlman, 1984; Krieger *et al.*, 1985; Rasmussen *et al.*, 1986). Identification and immunochemical characterization of the precise parasite molecules involved in this initial attachment phase is critical for understanding the molecular basis of host epithelial recognition by *T. vaginalis*.

Four epithelial cell-binding proteins have been implicated as the adhesins of *T. vaginalis* (Alderete and Garza, 1988; Alderete *et al.*, 1988). These earlier studies, however, did not address some concerns involving the adhesins. For example, the inhibition of cytoadherence required large amounts of anti-adhesin IgG antibody, and this antibody paradoxically failed to detect the adhesins on live parasites or in immunoblots of trichomonal lysates. Needing further clarification was the apparent unrelatedness of the four adhesins. Also, the reported variations in cytoadherence levels between fresh and long-term-grown isolates (Rasmussen *et al.*, 1986) have remained undefined, and these differences require clarification at the molecular level. Moreover, the lability of the surface adhesins to trichomonad cysteine proteinases must be examined in view of the release of proteinases into culture supernatants during *T. vaginalis* growth (Lockwood *et al.*, 1987; 1988), as these proteinases have been shown to degrade a prominent surface immunogen of the parasite (Alderete and Neale, 1989). Finally, antibody to other non-adhesin, surface proteins has been shown to affect parasite motility and inhibit cytoadherence (Krieger *et al.*, 1990), showing the need for better definition of the exact nature of trichomonal cytoadherence involving the previously implicated adhesins.

The *T. vaginalis* adhesins are four parasite surface proteins implicated previously (Alderete and Garza, 1988), and that these adhesins mediate trichomonal parasitism of vaginal epithelial cells (VECs) is demonstrated in this report. These four adhesins are present in all isolates examined and have been identified on the basis of their reproducible binding to chemically stabilized host cells. *In vitro* cultivation of fresh isolates reduced the parasite's ability to cytoadhere, and the relationship between adherence levels and the amounts of the four trichomonad adhesins was shown. Overall, data indicate that *in vivo* environmental signals, lost during *in vitro* cultivation of the parasite, influence or enhance expression of the adhesin genes. Furthermore, data show that the purified adhesin proteins were capable of preventing host parasitism, as did antibodies to each adhesin protein. Finally, epithelial cells appear to have putative receptor molecules for the adhesins. These adhesin proteins, called AP65, AP51, AP33, and AP23, fulfil the criteria for adhesins and are *T.*

Received 23 April, 1991; revised 5 September and 27 November, 1991; accepted 12 December, 1991. *For correspondence. Tel. (512) 567 3940; Fax (512) 567 6612.

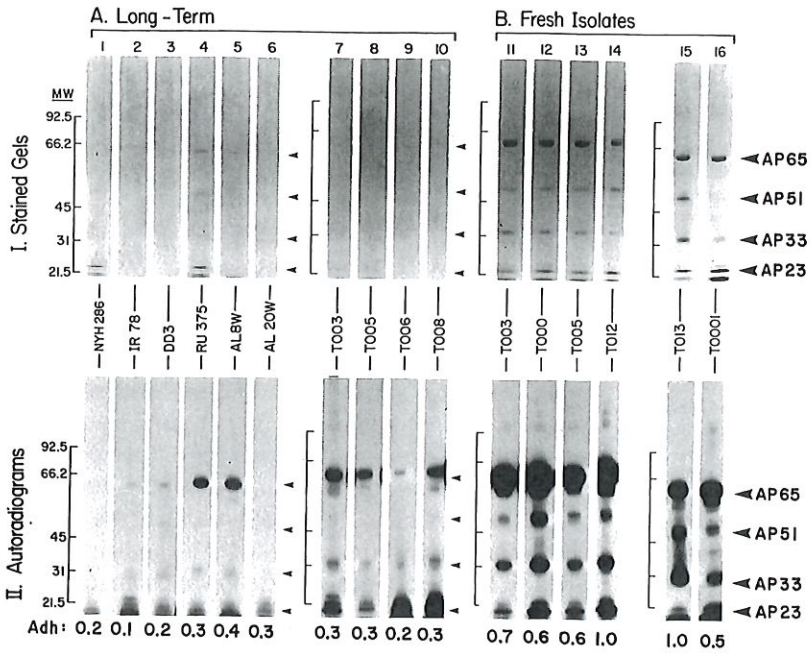

Fig. 1. The increased expression of adhesins and cytoadherence by fresh *T. vaginalis* isolates.
I. Coomassie brilliant blue-stained patterns of proteins from trichomonal detergent extract of long-term-grown (A) and fresh (B) isolates which bound to fixed HeLa epithelial cells in the ligand assay (*Experimental procedures*). Epithelial cell-binding proteins were eluted by boiling fixed cells in dissolving buffer, and adsorbed proteins were electrophoresed in 7.5% acrylamide gels. Arrows point to adhesin proteins (AP) with molecular masses of 65 kDa (AP65), 51 kDa (AP51), 33 kDa (AP33), and 23 kDa (AP23) as described in the text. Molecular size markers (MW) are indicated on the left (×1000). For comparative purposes, the same number of iodinated trichomonads of each isolate was used in the ligand assay (Alderete and Garza, 1988).
II. Autoradiograms of gels presented in part I. 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. A fresh isolate (T012) with the highest cytoadherence level was given a value of 1 for comparative purposes. This absolute value of 1 for T012 was equal to 51.0% ±5% (SD) 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.

vaginalis virulence proteins. Our work now permits a molecular understanding of a crucial event leading to host infection and pathogenesis by this protozoan parasite.

Results

Optimizing conditions for identifying the trichomonad adhesins

It was important to re-evaluate the ligand assay conditions established before for detection of the host-cell binding proteins of *T. vaginalis* (Alderete and Garza, 1988). This was necessary because of recent evidence that parasite detergent extracts contained many cysteine proteinases (Lockwood *et al.*, 1987; Neale and Alderete, 1990) that might degrade *T. vaginalis* adhesins, as was the case for surface immunogens (Alderete and Neale, 1989).

The detergent extract indeed contained proteinases, which degraded adhesins after solubilization of organisms and incubation of the extract for 60 min at various temperatures prior to the ligand assay (*Experimental procedures*). Proteolysis of the adhesins by the trichomonad proteinases was extremely rapid, occurring in <5 min at 37°C after solubilization; adhesins were rapidly degraded even at 22°C and 0°C (data not shown). The presence of a cysteine proteinase inhibitor (*N*-α-*p*-tosyl-L-lysine chloro-methyl ketone, TLCK), effective for trichomonad cysteine proteinases (Lockwood *et al.*, 1987), in the detergent extract prevented proteolysis of adhesins. This inhibitor was included in the parasite detergent extract for the remaining experiments.

Trichomonads of fresh isolates express higher amounts of adhesin proteins and correspondingly elevated levels of cytoadherence

It was essential that a relationship between the levels of cytoadherence and the amounts of adhesins be examined, since such a relationship provides for a molecular understanding of previously reported differences in cytoadherence of fresh and long-term-grown isolates (Rasmussen *et al.*, 1986).

Figure 1 presents Coomassie brilliant blue-stained patterns (part I) and autoradiograms (part II) for visualization of adhesins in the comparative analysis between fresh isolates and long-term-grown isolates. All isolates possessed the four iodinated proteins, although a more prolonged exposure of X-ray film was required on autoradiograms of long-term-grown trichomonads (part II). Stained gel patterns for fresh isolates (Fig. 1B, lanes 11–16) had correspondingly higher protein band intensities than did the stained gels of long-term-grown isolates (Fig.

1A, lanes 1–10). The cytoadherence levels were reduced two- to fivefold for all of the long-term-grown isolates (lanes 1–10), when referenced with the value of 1 given to a fresh isolate with the highest cytoadherence level (lane 14). Also shown in this figure, the daily passage of fresh isolates for more than four weeks resulted in a decrease in the amount of adhesins on stained gels and band intensities on autoradiograms (lanes 7–10). These parasites also gave lower cytoadherence values, similar to those seen for the other long-term-grown, laboratory isolates. At no time over a one-year period of *in vitro* cultivation did long-term cultures show a reversal towards higher amounts of adhesins, as evidenced by protein band intensities in stained gels or autoradiograms, nor did they exhibit any elevation in levels of cytoadherence. The higher amounts of adhesins and levels of cytoadherence and subsequent loss after *in vitro* culture were obtained for numerous other fresh isolates that were examined. It should be noted that band intensities in autoradiograms have been quantified by densitometric scanning and do in fact relate to the relative cytoadherence levels as previously observed (Alderete and Garza, 1988).

The four epithelial cell-binding proteins specifically mediate cytoadherence

Because of their higher amounts of adhesins, fresh isolates were used for all remaining experiments. The surface localization of the trichomonad adhesins was first reaffirmed by indirect immunofluorescence. Antibodies in the IgG fraction of individual anti-adhesin serum reacted with the surface of live organisms (Fig. 2). Each anti-adhesin IgG antibody gave strong fluorescence in contrast to the absence of reactivity with IgG of each individual prebleed, control rabbit serum.

Immunoblots were then performed on the four adhesins using antibodies from the same IgG fractions (Fig. 3A). Each IgG reacted only with the corresponding adhesin, showing the specificity of the antibodies and establishing the distinctness of each adhesin. IgG to AP23 (Fig. 3A, lane 4) failed to react by immunoblot, presumably because of the denaturing conditions used, although this antibody was reactive with live parasites by immunofluorescence, as seen above. Prebleed, control serum from all rabbits, either singly or as a mixture, did not react with any adhesin protein on duplicate blots (Fig. 3A, lane C).

Antibodies purified from the immunoblots of adhesins AP65, AP51 and AP33 inhibited *T. vaginalis* attachment to HeLa cells in a concentration-dependent manner, obtaining maximum inhibition of approximately 55% with 8 μg ml^{-1} of each antibody. A mixture of antibodies did not give any greater level of inhibition (Fig. 3B). Controls, as described below and used in duplicate samples under identical conditions, did not reduce parasite cytoadherence. Because anti-AP23 antibody could not be purified from immunoblots, the IgG fraction of anti-AP23 serum employed for immunofluorescence was used for this inhibition experiment. This anti-AP23 IgG also inhibited cytoadherence to the same extent, although the amount of IgG required was greater. None of the anti-adhesin antibodies caused agglutination of parasites at the 8 μg ml^{-1} concentration, which gave maximal inhibition of cytoadherence.

Finally, antibodies to other trichomonad surface proteins, such as P270 (Alderete *et al.*, 1986b; Alderete

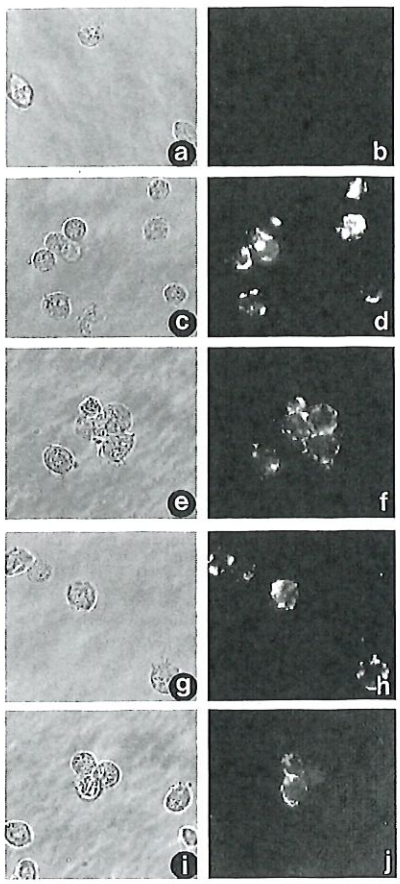

Fig. 2. Localization by indirect immunofluorescence of the adhesins on the surface of live organisms of the fresh trichomonal isolate, *T. vaginalis* T015.
Left panels: phase-contrast photomicrographs of live trichomonads reacted with antibodies in the IgG fractions of prebleed, control rabbit serum (panel a) and individual antiserum to adhesin protein AP65 (panel c), AP51 (panel e), AP33 (panel g), and AP23 (panel i). Trypan blue in the reaction mixture showed the absence of any complement-independent, antibody-mediated killing of organisms. The reactivity of each individual antiserum with blotted adhesins was as presented in Fig. 3 (part A).
Right panels: photomicrographs of corresponding indirect immunofluorescence of fluoresceine isothiocyanate-conjugated anti-rabbit Ig added to organisms after incubation with the IgG fractions of prebleed, control serum (panel b) or respective anti-adhesin serum as indicated above (panels d, f, h and j).

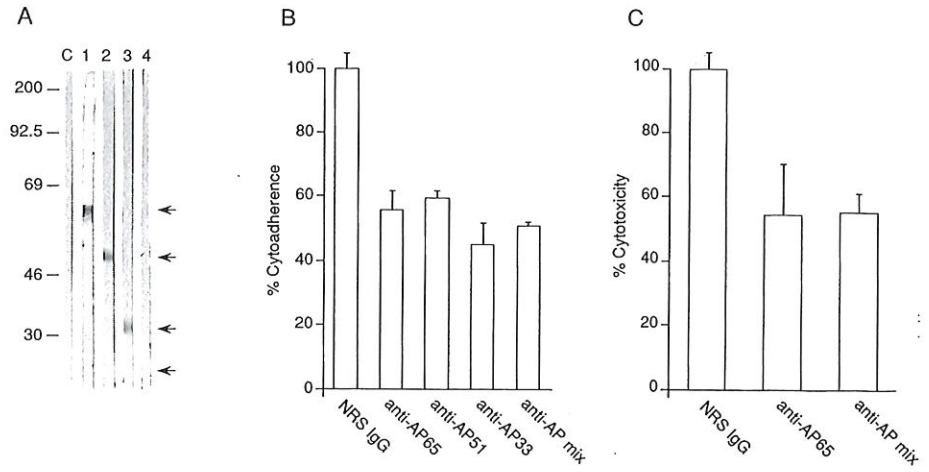

Fig. 3. Immunoblot reactivity (part A) of each of the anti-adhesin serum antibodies and the ability of these antibodies to inhibit cytoadherence (part B) and cytotoxicity (part C) of HeLa cells by the fresh *T. vaginalis* isolate, T015.
A. The four adhesins isolated from a ligand assay were immunoblotted onto nitrocellulose and probed with each IgG fraction of anti-adhesin serum. Control IgG from sera of each of the four rabbits used to generate each anti-adhesin antibody was pooled (lane C) and compared with the reactions of IgG to AP65 (lane 1), AP51 (lane 2), AP33 (lane 3), and AP23 (lane 4). Only IgG to AP23 did not react with its corresponding adhesin, in spite of the positive immunofluorescence reaction seen in Fig. 2.
B. Antibodies affinity-purified from preparative immunoblots of each adhesin (part A) AP65, AP51, and AP33 or a mixture of all purified antibodies (anti-AP-mix) were used for inhibiting cytoadherence by *T. vaginalis* T015. For this assay, IgG from prebleed, control serum of all rabbits or a mock control from antibody to other surface proteins eluted from blots handled similarly failed to give any inhibition of cytoadherence. The assay was performed over a 30-minute time period at 37°C in the presence of 8 μg ml^{-1} affinity-purified antibodies. The IgG fraction of anti-AP23 serum, which was immunoreactive with live organisms by immunofluorescence (Fig. 2), also produced inhibition of cytoadherence (data not shown). Each value is the mean of triplicate experiments with triplicate samples, and bars indicate the standard deviation.
C. Affinity-purified anti-adhesin antibody to AP65 or a mixture of all four antibodies (AP-mix) as shown for part B above were used to show inhibition of cytotoxicity by *T. vaginalis* T015. For this assay, 8 μg ml^{-1} antibody was used. The affinity-purified anti-adhesin antibodies to AP51, AP33, or the IgG fraction of anti-AP23 serum also gave similar inhibition of cytotoxicity (data not shown). IgG from prebleed control serum of all rabbits or a mock control as in part B failed to give any inhibition of cytotoxicity. Each value is the mean of triplicate experiments with triplicate samples, and bars indicate the standard deviation.

and Neale, 1989), P230 (Alderete *et al.*, 1987; 1991), and P65 (Alderete *et al.*, 1987; Alderete and Neale, 1989) were used as negative controls in cytoadherence or cytotoxicity inhibition experiments. These control antibodies did not affect trichomonal adherence or cellular destruction under the same experimental conditions. These data negate the possibility that non-specific perturbation of the parasite membrane was responsible for the inhibition observed by the anti-adhesin antibodies. These data reaffirm the notion that each adhesin protein is essential and involved in cytoadherence, as previously indicated (Alderete and Garza, 1988).

Anti-adhesin antibodies inhibited cytotoxicity by ≈50% (Fig. 3C), a value consistent with that achieved for decreased cytoadherence, and protected HeLa cell monolayers from total trichomonal destruction, reinforcing earlier reports (Alderete and Pearlman, 1984; Krieger *et al.*, 1985) on the requirement of contact for parasite-mediated cytotoxicity of host cells.

The four adhesins themselves inhibit host-cell parasitism

It was essential that we test that the adhesins themselves blocked the parasitism of host epithelial cells by trichomonads. A pooled preparation of the four purified adhesins was incubated with host cells before addition of parasites. A concentration-dependent inhibition of cytoadherence was observed, as seen in Fig. 4. Bovine serum albumin and even whole serum used in duplicate samples under identical conditions did not reduce parasite attachment to the HeLa cells. In addition, other immunoaffinity-purified surface proteins, like those described earlier, as well as the mock control, which was electroelution buffer processed identically as for the purified adhesins, failed to inhibit cytoadherence. Duplicate samples of the adhesins used in these experiments were analysed simultaneously by electrophoresis, and the stained gel patterns (Fig. 4B) provided evidence for the stability and absence of degradation of the adhesins under these experimental conditions.

HeLa epithelial cells and vaginal epithelial cells (VECs) bind the same adhesins

A ligand assay was performed using VECs to show that adhesins involved in HeLa cell recognition and binding

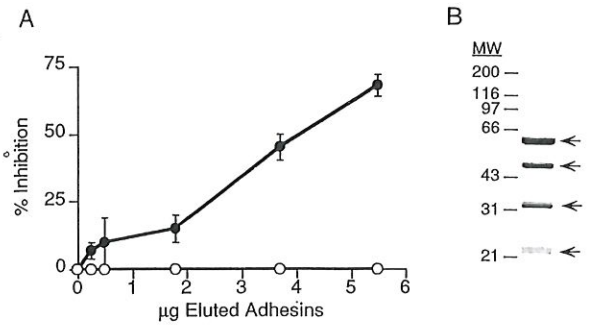

Fig. 4. Pretreatment of epithelial cells with purified trichomonal adhesins blocked cytoadherence by *T. vaginalis* T015.
A. Adhesins (●) from a ligand assay were dialysed and equilibrated in incubation medium before being added to monolayer cultures of fixed HeLa cells (*Experimental procedures*). After incubation for 18h at 4°C, live parasites were added. Each point is the mean of the per cent reduction of cytoadherence compared to the control (○) (mock, whole normal serum or bovine serum albumin) of duplicate experiments with triplicate samples. Bars represent standard deviations.
B. A fraction of the adhesin preparation used for the competition assay in part A 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 (MW) are indicated on the left in kDa.

(Fig. 5) were identical for VEC parasitism. Proteins from the trichomonal detergent extract, which bound to each cell type, were electrophoresed and immunoblotted with the anti-adhesin antibodies. The adhesins associated with HeLa cells and VECs presented the same electrophoretic mobilities as visualized by Coomassie brilliant blue staining, consistent with the data in Fig. 1B (part I). The pooled IgG from each anti-adhesin serum detected the comigrating adhesins which bound to HeLa cells (lane 3) and VECs (lane 4), showing the immuno-cross-reactivity between trichomonad proteins binding to both cell types. IgGs from prebleed animals were used as negative controls in the ligand blots and were unreactive with HeLa and VEC-binding proteins (lanes 1 and 2), respectively. As expected, the AP23 was not recognized in immunoblot, although the protein band was present based on the stained gel pattern. These data confirm that the adhesins which recognize and bind to HeLa cells and VECs are identical and show the relevance of HeLa cells as an *in vitro* experimental model system.

The four adhesins may be distinct proteins

That the adhesins might be distinct gene products was suggested by the previous results of pulse-chase experiments in which no common precursor peptide was found among the adhesins (Alderete and Garza, 1988). The immunoblot data (Fig. 3A) also reinforced the idea that the adhesins were distinct proteins. Peptide digestion analysis, therefore, was performed on the four adhesins (Fig. 6). Silver-stained profiles of gels after proteolytic digestion with chymotrypsin, papain, and *Streptomyces griseus* protease (Fig. 6) revealed that while most of the patterns were different, some common peptides were detected among the adhesins. Immunoblots of duplicate gels of the adhesin digests using the affinity-purified, anti-adhesin antibodies were then performed to show the absence of any relationship, especially among the comigrating fragments after enzyme treatment. No evidence was obtained of any immuno-cross-reactivity between the adhesins or adhesin fragments (data not shown). Preimmune sera did not react with any of the adhesins or adhesin fragments, as expected.

HeLa cell molecules may function as receptors for trichomonad adhesins

A HeLa cell extract was incubated with blots of the adhesins (*Experimental procedures*) to test whether epithelial cells possessed molecules capable of recognizing the immobilized ligands. As seen in Fig. 7, molecules present on the deoxycholate extract of HeLa cells, as

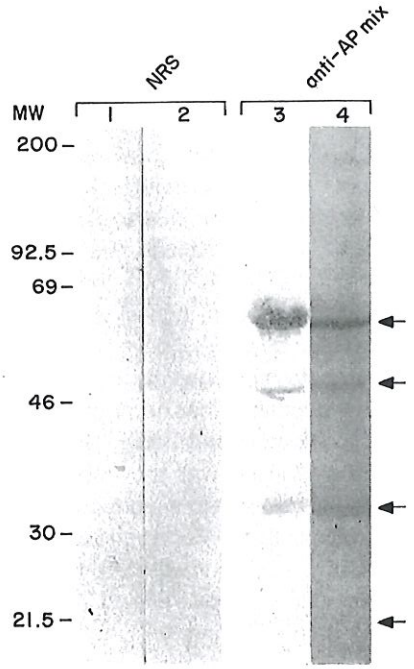

Fig. 5. Comparative analysis of trichomonad adhesins which recognize and bind to HeLa cells and vaginal epithelial cells (VECs). Immobilon-P (Millipore) membrane blots of HeLa cell- (lanes 1 and 3) and VEC-binding (lanes 2 and 4) parasite proteins were probed with a pooled preparation of IgG fraction from prebleed, control antiserum of all rabbits (lanes 1 and 2) and were compared with pooled IgG fraction from antiserum to the trichomonad adhesins (lanes 3 and 4). As expected, only AP65, AP51, and AP33 were detected based on Fig. 3. The ligand assay was performed under identical conditions for both cell types. Host cell-binding proteins from the ligand assay were electrophoresed in 10% acrylamide gels prior to blotting and probing with antibodies.

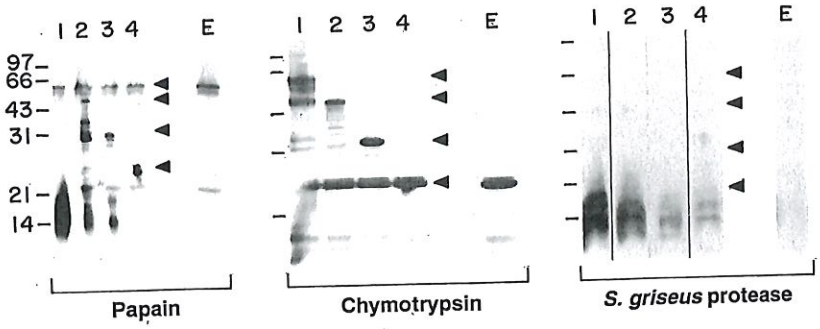

Fig. 6. Peptide mapping of the four *T. vaginalis* adhesins digested with papain, chymotrypsin and pronase E from *Streptomyces griseus*. Peptides of the adhesins digested *in situ* using 12% SDS–PAGE and under the conditions described in the *Experimental procedures* were visualized by silver stain. Lane numbers indicate the corresponding adhesins, i.e. 1, AP65; 2, AP51; 3, AP33; and 4, AP23. 'E' refers to the particular enzyme used. Arrows on the right-hand side of the gels indicate the positions of undigested adhesins. Lines on left-hand side represent the positions of the molecular mass markers.

detected with anti-HeLa cell serum, indeed bound to adhesins (lane 3). Preimmune serum (lane 1) and anti-epithelial cell serum (lane 5) were non-reactive with duplicate blots of adhesins alone. As expected, anti-adhesin IgG antibodies recognized the adhesins, except for AP23 (lane 2). It is especially noteworthy that the previously undetected AP23 adhesin (lane 2 and Figs 3A and 7) was now identifiable by this assay (lane 3). These results reinforce the idea that host cells have adhesin-binding molecules (receptors).

Discussion

This report presents evidence showing that the four epithelial cell-binding proteins of *T. vaginalis* anchor motile organisms to vaginal epithelial cells. Our work provides a molecular basis for understanding a critical first step of infection for this sexually transmitted human parasite, and criteria which prove the existence and identity of specific microbial adhesins involved in host cytoadherence (Beachey, 1989) have been satisfied.

Earlier experiments implicated the four parasite-synthesized proteins as putative adhesins mediating attachment to epithelial cells (Alderete and Garza, 1988). The surface localization of the adhesins was shown by the removal of the proteins from the parasite surface by proteinase treatment of live parasites (Alderete and Garza, 1988) and has now been supported by visualization of the adhesins by indirect immunofluorescence (Fig. 2). Antibody to each adhesin inhibited the binding of live trichomonads to epithelial cells (Fig. 3) and, predictably (Alderete and Pearlman, 1984; Krieger *et al.*, 1985), the same antibodies protected HeLa cells from contact-dependent cytotoxicity. The lack of immuno-cross-reactivity among the adhesins (Fig. 3A) was consistent with the previous suggestion (Alderete and Garza, 1988) that the adhesins were distinct gene products (Fig. 6). Purified adhesins also inhibited the interaction of *T. vaginalis* with epithelial cells (Fig. 4), and this was important proof that these molecules are involved in cytoadherence. It was equally noteworthy that anti-adhesin antibodies recognized the *T. vaginalis* adhesins obtained when VECs were used in the ligand assay (Fig. 5). These data demonstrate that the characterization of the parasite adhesins with an *in vitro* system also reflected the *in vivo* situation. Finally, HeLa cell molecules recognized the adhesins (Fig. 7), showing the existence of possible host receptors, as has been done for other protozoan parasites (Davis and Kuhn, 1990). Collectively,

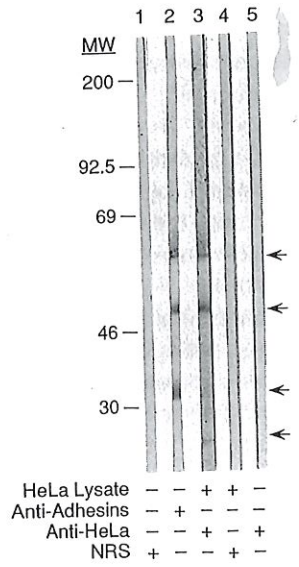

Fig. 7. Detection of adhesin-binding, HeLa cell molecules. Nitrocellulose blots of the adhesins were incubated with a detergent extract of HeLa cells (HeLa Lysate) followed by probing with rabbit anti-HeLa cell serum (lane 3) and prebleed, normal rabbit serum (NRS) as a control (lane 4). Duplicate blots were probed with the pooled antiserum to the four adhesins (lane 2) and compared with the respective pooled NRS (lane 1) to show the presence and localization of adhesin proteins on blots. Lane 5 shows the absence of adhesin detection with the anti-HeLa cell serum, illustrating the specificity of the antiserum to HeLa cells. Note the recognition of a band comigrating with AP23 which was detected with HeLa cell molecules and anti-HeLa cell serum but not with the anti-AP23 serum, as before. Details regarding the incubation of the epithelial cell extract with the blotted adhesins and incubation with antiserum are provided in the *Experimental procedures* section. Arrows on the right of the gel point to the adhesins. The + and – symbols refer to the presence or absence of the reagents, respectively.

the data reinforce the receptor-ligand nature of the *T. vaginalis*/target cell interaction.

That adhesin proteins were present in greater amounts on fresh isolates (Fig. 1) was critical to the success of this study. This allowed for the actual detection of adhesins by various assays through the generation of hyperimmune anti-adhesin serum. That the four adhesins appear to be encoded by distinct genes cannot be stated categorically on the basis of the comigration of peptides after proteolytic digestion experiments (Fig. 6). However, if this is the case, the increase in adhesins among fresh isolates perhaps suggests the possibility of co-ordinated regulation of gene expression. The higher amounts of adhesins on fresh isolates, and their loss during *in vitro* cultivation, may also suggest that the adhesins may be environmentally regulated. Factors in the vagina may allow for the enhanced expression of adhesin genes. *In vitro* cultivation, then, appears to result in loss of a signal which promotes or influences the expression of adhesins or, alternatively, may select for trichomonads synthesizing lower amounts. Indeed, a recent report documented that growth of trichomonads under *in vivo*-like conditions in a continuous flow chemostat fermentor influenced *T. vaginalis* properties (Lehker and Alderete, 1990), supporting the idea that the host environment or factors may affect expression of virulence factors, such as the adhesins.

That *T. vaginalis* recognition and binding to host cells is more complex than just adhesin–receptor associations, however, is further exemplified by the requirement for a trichomonad cysteine proteinase during the cytoadherence event (Arroyo and Alderete, 1989). The proteinase action was directed towards the parasite and not the host-cell surface (Arroyo and Alderete, 1989). Since a precursor form of the adhesins was not previously detected (Alderete and Garza, 1988) and was not observed in this study, the exact relationship between the proteinase(s) and adhesins remains unknown. Because the adhesins have been shown to be labile to proteinases of the parasite (data not shown), it is possible that the adhesins on live organisms may be protected from non-specific proteinase activity by a 'masking' protein, which requires degradation before expression of adhesin functionality. At any rate, it is clear that the property of cytoadherence, apart from the receptor-ligand features, is multifactorial and complex.

Because of this complexity, it may never be possible to totally abolish cytoadherence (Fig. 3B). The combination of release of proteinases (Lockwood *et al.*, 1988) and masking of the adhesins (Arroyo and Alderete, 1989) may not allow the antibody to neutralize the entire potential source of adhesin molecules. Moreover, a continuous protein synthesis of adhesins is required for attachment of trichomonads to vaginal epithelial cells (Alderete and Garza, 1985; 1988; Alderete *et al.*, 1988). It is possible that parasites lose the adhesin–antibody complexes through capping or other membrane perturbations. It is equally possible that other adhesins, yet to be characterized, may be responsible for the remaining levels of adherence not inhibitable by the anti-adhesin antibodies described here. Nonetheless, these data confirm the involvement of these four epithelial cell-binding proteins as bona fide adhesins.

The precise inter-relationships between the four adhesins await further characterization of the structure–function properties of each of the adhesins. It has been established, however, that the adhesins are surface-exposed and participate in the cytoadherence event. It is possible that the four adhesins form a multimeric complex or, alternatively, some of adhesins may be accessory or support proteins for those directly recognizing and binding to one or several distinct host cell receptors. A combination of approaches, including identification of the epithelial cell receptor(s) and the synthesis of sufficient amounts of adhesins through recombinant DNA techniques, will be required for future delineation of the exact role of each adhesin in cytoadherence.

Experimental procedures

Parasites and surface labelling

Isolates of *T. vaginalis* have been described before (Alderete, 1983; Alderete and Garza, 1985; Neale and Alderete, 1990). Parasites were passaged daily in a serum-based complex medium (Diamond, 1957), and only trichomonads from the late-logarithmic phase of growth were used. Fresh isolates were passaged *in vitro* for ≤1 week, although prolonged cultivation was also done on randomly selected isolates over a 6–8-week period. Extrinsic radiolabelling of trichomonad surface proteins was performed by the Chloramine T procedure as previously described (Garvey *et al.*, 1977; Alderete, 1983).

Ligand assay

To analyse the susceptibility of the adhesins to parasite proteinases, the trichomonal deoxycholate extract (Alderete and Garza, 1988) was prepared in NET (50 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA) buffer, pH 7.4, without proteinase inhibitors. The parasite detergent extract was incubated for various time points up to 60 min at 0, 25 and 37°C, prior to addition to fixed HeLa cells (Alderete and Garza, 1988). Results from this experiment, performed numerous times, resulted in a modification of the original ligand assay (Alderete and Garza, 1988). Briefly, fixed HeLa cells or purified vaginal epithelial cells (VECs) (Alderete *et al.*, 1988) were incubated with a deoxycholate extract of total parasite proteins prepared using 1 mM TLCK (Sigma) for 18 h at 4°C (Alderete and Garza, 1988). Host cells were then washed with NET-0.05% deoxycholate buffer (Alderete and Garza, 1988), and the epithelial cell-adsorbed parasite proteins were eluted by boiling the fixed cells in electrophoresis dissolving buffer (Laemmli, 1970) for 3 min. Epithelial cell-binding proteins were then

subjected to sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS–PAGE) using 7.5% acrylamide for the separating gel, as previously reported for trichomonad proteins (Alderete and Garza, 1988; Laemmli, 1970). For this and other assays performed in this study, experiments were done at least three times with triplicate or quadruplicate samples.

Antibodies

Adhesin protein bands from the ligand assay were separated by SDS–PAGE, and bands were excised, rinsed in water, and frozen in liquid nitrogen before being individually mascerated and suspended in sterile phosphate-buffered saline (PBS). Polyclonal, monospecific antiserum to each of the individual adhesins was generated in rabbits immunized as before (Alderete and Garza, 1988) by intramuscular injections followed by booster immunizations two weeks later and at monthly intervals until evidence of high-titred antibody to the specific adhesin was evident.

Antibodies in sera to adhesins were detected by immunoblotting (Towbin *et al.*, 1979; Alderete *et al.*, 1986b). For some experiments the IgG fraction of each anti-adhesin serum was purified by protein A–Sepharose affinity chromatography (Pharmacia). Alternatively, preparative adhesins or total parasite extract immunoblots were performed with each antiserum, and antibody specifically bound to adhesins or other surface immunogens, respectively, were eluted (Olmsted, 1981). This purified antibody was then retested to insure specific recognition of the adhesins or other surface immunogens. Purified antibody was also used for inhibiting trichomonal cytoadherence and contact-dependent cytotoxicity, as before (Alderete and Garza, 1985; Arroyo and Alderete, 1989; Alderete and Pearlman, 1984). Finally, anti-adhesin IgG antibodies were used for indirect immunofluorescence to visualize the surface expression of the adhesins on live parasites (Alderete *et al.*, 1986a).

Prebleed serum, purified IgG from prebleed serum of all rabbits, or blot-purified antibodies directed toward other surface immunogens, such as P270, a prominent immunogen that undergoes phenotypic variation (Alderete *et al.*, 1986a; Alderete and Neale, 1989), P230, the protein recognized by antibodies present in vaginal secretion of patients with trichomoniasis (Alderete *et al.*, 1991), and which also undergoes epitope variation (Alderete *et al.*, 1987), or P65, a protein distinct from the AP65 adhesin (Alderete *et al.*, 1987; Alderete and Neale, 1989) were used as controls in duplicate experiments on parasite samples handled identically. Under our experimental conditions, all of the antibody reagents, including the anti-adhesin serum IgG antibodies, blot-purified anti-adhesin antibodies, and antibodies to surface immunogens handled similarly did not affect parasite motility or cause agglutination. Mock controls were also included where necessary in experiments, and these consisted of buffer alone used for elution of antibodies from blots or detergents used for purification of adhesins from acrylamide gels.

Detection of HeLa cell molecules recognizing adhesin proteins

To detect any HeLa cell molecules recognizing the adhesins, the ligand assay was performed and adhesins electrophoresed as above. After blotting, nitrocellulose blots were blocked by treatment with PBS containing 0.05% Tween 20 and 5% non-fat powdered milk (Blotto) for 1 h at 22°C (Towbin *et al.*, 1979; Alderete *et al.*, 1986b). This blot was then incubated with a detergent extract of HeLa cells (5 μg ml^{-1} protein) prepared as above for the parasites. The protein concentration in the extract was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce). After incubation for 18 h at 4°C, the blots were washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with a 1:100 dilution of rabbit antiserum to HeLa cells prepared in Blotto for 3 h at 22°C (Hayman and Ruoslahty, 1988; Davis and Kuhn, 1990). The blots were again washed and this was followed by incubation for 3 h at 22°C with a 1:2000 dilution of horseradish peroxidase-conjugated, goat anti-rabbit IgG (Bio-Rad), also prepared in Blotto. After additional washes in PBS-T, the blots were developed with 4-chloro-1-naphthol (3 mg ml^{-1}) (BioRad) in methanol as a substrate.

Cytoadherence and cytotoxicity assays

HeLa epithelial cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained as before in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Alderete and Garza, 1985; Arroyo and Alderete, 1989). Human VECs were obtained from vaginal swabs of healthy, uninfected women and were purified using a recently described procedure (Alderete *et al.*, 1988).

Epithelial cells were fixed with 2.5% glutaraldehyde in PBS for 1 h at 4°C, using conditions previously described (Alderete and Garza, 1988; Alderete *et al.*, 1988), prior to use in the ligand assay. HeLa cells were also cultured as monolayers in 96-well microtitre plates for parasite cytoadherence and cytotoxicity assays (Alderete and Pearlman, 1984; Alderete and Garza, 1985; Arroyo and Alderete, 1989).

Attachment of *T. vaginalis* to HeLa cells and the contact-dependent destruction of HeLa cell monolayers were measured by established procedures (Alderete and Garza, 1985; Arroyo and Alderete, 1989; Alderete and Pearlman, 1984). For these experiments, [^{3}H]-thymidine-labelled parasites and unlabelled parasites were used for cytoadherence and cytotoxicity, respectively. Organisms were suspended to a density of 4×10^6 parasites per 800 μl in a mixture of 2:1 parts cell culture medium to trichomonal medium without serum (DMEM:TYM) (Alderete and Garza, 1985; Arroyo and Alderete, 1989), then mixed with different amounts of up to 100 μg ml^{-1} of blot-purified anti-adhesin antibodies. Antibodies to other surface protein immunogens, processed similarly, were also used as controls. Buffer used during elution of antibody was handled identically and also used as a mock control. The volume was then adjusted with DMEM:TYM to 1 ml. Trichomonads and antibody were then added immediately to HeLa cell monolayers (4×10^5 organisms in 100 μl per well) to give a final host cell: parasite ratio of 1:5, as described before for these assays (Alderete and Garza, 1985; Alderete and Pearlman, 1984). After 30 min at 37°C, the radioactivity associated with HeLa cells was determined for quantification of the extent of cytoadherence (Alderete and Garza, 1985).

The ability of purified adhesins to inhibit cytoadherence was also tested. In this case the adhesins from a preparative ligand assay (500 μl) or just electrophoresis dissolving buffer without adhesins (mock control) was dialysed against two changes of 1 l of 2.5% Triton X-100 for 18 h at 22°C followed by dialysis against

eight changes of 1 l of PBS for another 48 h at 4°C. The presence and stability of all adhesins were monitored by SDS–PAGE and immunoblotting. Fixed HeLa cells in monolayers were pretreated with increasing amounts of purified adhesins (180 μg ml^{-1}) for 18 h at 4°C. These pretreated cell monolayers were then washed three times with PBS before incubation with live organisms as above (Alderete and Garza, 1985). Bovine serum albumin and horse serum were also used as negative controls in these experiments.

For cytotoxicity experiments, the time of incubation between parasites and HeLa cells was 1–2 h. The time needed for maximal killing to occur in samples with parasites without antibody was always determined for each experiment. Microtitre wells were washed and the remaining HeLa cells fixed and stained as previously described for quantitative measurement of cellular destruction by colorimetric methods. The optical density measurement of cells remaining in the wells has been determined to correlate with cell death (Alderete and Pearlman, 1984). Trypan blue staining of released and adherent host cells was always performed to assure the validity of the assay, as previously established.

Peptide mapping

The adhesins were analysed in peptide mapping experiments using standard procedures (Cleveland *et al.*, 1977). Proteolysis was allowed to occur *in situ* using papain, chymotrypsin, and *Streptomyces griseus* protease (Sigma). A preparative gel containing adhesins was stained with Coomassie brilliant blue for 30 min followed by destaining for 30 min. Individual bands were excised from the gel and soaked for 30 min in 125 mM Tris-HCl, pH 6.8, containing 0.1% SDS and 1 mM EDTA. Individual adhesin gel bands were then re-electrophoresed in a 12% SDS–PAGE and digested through a stacking gel containing either papain (1 μg), chymotrypsin (3.4 μg), or pronase E (2.5 μg). These concentrations were found to be optimal for proteolysis of the adhesins under these conditions. During migration of proteins through the stacking gel, the current was turned off for 30 min in order to allow for proteolytic digestion. After electrophoresis, the gel was stained with silver nitrate (Oakley *et al.*, 1980). Duplicate experiments were also performed in which the proteinase-degraded adhesins were transferred to nitrocellulose for immunoblotting with specific antibody.

Acknowledgements

This study was supported by Public Health Service grant AI18768 from the National Institute of Allergy and Infectious Disease, National Institutes of Health. The excellent secretarial assistance of Diana Hinojosa is gratefully acknowledged.

References

Alderete, J.F. (1983) Identification of immunogenic and antibody-binding membrane proteins of pathogenic *Trichomonas vaginalis*. *Infect Immun* **40:** 284–291.

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 cytoadherence. *Infect Immun* **56:** 28–33.

Alderete, J.F., and Neale, K.A. (1989) Relatedness of major immunogen among all *Trichomonas vaginalis* isolates. *Infect Immun* **56:** 1849–1853.

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., Kasmala, L., Metcalfe, E., and Garza, G.E. (1986a) Phenotypic variation and diversity among *Trichomonas vaginalis* and correlation of phenotype with trichomonal virulence determinants. *Infect Immun* **53:** 285–293.

Alderete, J.F., Suprun-Brown, L., and Kasmala, L. (1986b) 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., Gombošova, A., Valent,M., Janoška, A., Fabušova, H., Kasmala, L., Garza, G.E., and Metcalfe, E.C. (1987) Phenotypes and protein/epitope phenotypic variation among fresh isolates of *Trichomonas vaginalis*. *Infect Immun* **55:** 1037–1041.

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

Alderete, J.F., Newton, E., Dennis, C., Engbring, J., and Neale, K.A. (1991) Vaginal antibody of patients with trichomoniasis is to a prominent surface immunogen of *Trichomonas vaginalis*. *Genitourin Med* **67:** 220–225.

Arroyo, R., and Alderete, J.F. (1989) *Trichomonas vaginalis* proteinase activity is necessary for parasite cytoadherence. *Infect Immun* **57:** 2991–2997.

Beachey, E.H. (1989) Bacterial adherence. In *Molecular Mechanisms of Microbial Adhesion*. Switalski, L., Hook, M., and Beachey, E.H. (eds). New York: Springer-Verlag, pp. 1–4.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W., and Laemmli, U.K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J Biol Chem* **252:** 1102–1106.

Davis, C.D., and Kuhn, R.E. (1990) Selective binding of *Trypanosoma cruzi* to host cell membrane polypeptides. *Infect Immun* **58:** 1–6.

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

Garvey, J.S., Cremer, N.E., and Sussdorf, D.H. (1977) ^{125}I or ^{131}I-labeled proteins. In *Methods in Immunology*. Campbell, D.H. (ed.). Reading, MA: Benjamin, W.A., Inc., pp. 171–182.

Hayman, E.G., and Ruoslahty, E. (1988) Cell attachment to blotted proteins. In *Handbook of Immunoblotting of Proteins*. Bjerrum, O.J., and Heegaard, N.H.H. (eds). Florida: CRC Press, Inc., pp.81–86.

Heath, J.F. (1981) Behavior and pathogenicity of *Trichomonas vaginalis* in epithelial cells. *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.

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.

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 growth conditions. *Genitourin Med* **66:** 193–199.

Lockwood, B.C., North, M.J., Scott, K.I., Bremner, A.F., and Coombs, G.H. (1987) The use of a highly sensitive electrophoretic method to compare the proteinases of trichomonads. *Mol Biochem Parasitol* **24:** 89–95.

Lockwood, B.C., North, M.J., and Coombs, G.H. (1988) The release of hydrolases from *Trichomonas vaginalis* and *Trichomonas foetus*. *Mol Biochem Parasitol* **30:** 135–142.

Neale, K.A., and Alderete, J.F. (1990) Analysis of the proteinases of representative *Trichomonas vaginalis* isolates. *Infect Immun* **58:** 157–162.

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

Oakley, B.R., Kirsch, D.R., and Morris, N.R. (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal Biochem* **105:** 361–363.

Olmsted, J.B. (1981) Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J Biol Chem* **256:** 11955–11957.

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.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:** 4350–4354.