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Identification and Properties of *Trichomonas vaginalis* Proteins Involved in Cytadherence

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Trichomonas vaginalis **NYH286 surface proteins which are candidates for mediating parasite cytadherence (adhesins) were identified. At least four trichomonad protein ligands ranging in relative molecular mass from 65 to ≤21 kilodaltons were found to selectively bind to chemically stabilized HeLa cells. The proteins were present on the surfaces of 10 different isolates of *T. vaginalis* examined; however, the nonpathogenic trichomonad *T. tenax* did not possess similar HeLa cell-binding proteins under identical experimental conditions, suggesting that these proteins are unique to the pathogenic human trichomonads. The surface nature of the candidate adhesins was confirmed by the ability of the proteins on intact, live organisms to be radioiodinated and to be removed with trypsin treatment. Rabbit antiserum (immunoglobulin G fraction) generated against adhesin proteins electroeluted from acrylamide preparations inhibited cytadherence compared with control immunoglobulin G. An adherence-negative subpopulation of *T. vaginalis* NYH286 organisms was also isolated. These nonadherent trichomonads did not synthesize the adhesin proteins. Interestingly, absence of adhesins from these parasites paralleled expression of a major immunogen known to undergo phenotypic variation. Revertant organisms derived from the adherence-minus subpopulation synthesized the adhesins and attached to HeLa cells. The emergence of revertant adherent *T. vaginalis* organisms also corresponded with the appearance of parasites which were without the major immunogen on their surface. Finally, it was determined that only those parasites lacking the major surface immunogen were capable of adherence and toxicity to HeLa cells.**

Trichomonas vaginalis is a mucosal parasite of the urogenital-vaginal tract. Recent studies have shown that receptor-ligand type interactions are important to *T. vaginalis* parasitism of host cells (3, 6, 17). The ability of the pathogenic human trichomonads to cytadhere is essential and prerequisite to host cell killing (3, 6, 17). Proteins on the surface of live trichomonads have been implicated as mediators of host cytadherence (3). Clearly, identification of these biofunctional parasite molecules would be important not only for allowing us to understand the biology of the initial key step in *T. vaginalis* recognition of host cells and tissues, but also in determining the trichomonad ligands that might represent reagents for vaccine development for interference with an initial key step during infection.

In this report evidence is presented to implicate at least four *T. vaginalis* surface proteins in cytadherence. No similar biofunctional adhesin candidates were detected in nonpathogenic *Trichomonas tenax* parasites, reinforcing the idea that the adhesins along with prominent immunogens (1, 4, 5, 7) are bona fide virulence factors of the pathogenic human trichomonads. Furthermore, data obtained following the isolation of adherence-minus trichomonads from the parent parasite population support the view that alternating phenotypic expression of adhesins and possibly a repertoire of major immunogens (1, 3–5; J. F. Alderete, J. Infect. Dis., in press) occur on *T. vaginalis* surfaces.

MATERIALS AND METHODS

Parasites. *T. vaginalis* NYH286 has been extensively used and recently described by us (1–8). Other isolates representing both long-term-grown and fresh isolates from patients with trichomoniasis have also been described (2–8).

Parasites were passaged daily as required (19) in Diamond Trypticase-yeast extract-maltose (TYM) medium (13) without agar and supplemented with 10% heat-inactivated donor horse serum (Hazelton Research Products, Inc., Lenexa, Kan.). For all experiments, only mid- to late-logarithmic-phase trichomonads (19) were used. Nonpathogenic *T. tenax* was obtained from the American Type Culture Collection, Rockville, Md., and was passaged in Trypticase-Panmede (Harcos, Inc., Bronxville, N.Y.) medium with 10% serum (3, 5). Procedures for intrinisic and extrinsic radiolabeling of trichomonads for the various assays have been described recently (2, 5, 8, 15). Also, trypsinization of intact, live parasites was performed by established conditions known to abolish cytadherence (3).

Ligand assay. NET-PMSF buffer (7, 8) (500 μl) and 50 μl of 10% sodium deoxycholate (Sigma Chemical Co., St. Louis, Mo.) prepared in H_2O were added to pellets containing 2×10^7 radiolabeled or unlabeled, washed *T. vaginalis* organisms (~0.5 mg of protein). After gentle homogenization (8), the detergent extract was layered on a sucrose cushion and centrifuged at 100,000 × *g* for 30 min at 4°C in a Beckman SW55.1 swinging-bucket rotor. The supernatant was recovered and represented ~70 to 90% of total radioactivity. NET-PMSF buffer (8) was then added to the extract to give a 1-ml final volume, and the 1-ml extract was used to suspend 10^6 fixed HeLa cells prepared as described below. After a 1-h incubation at 37°C with occasional gentle stirring, cells were pelleted by centrifugation at 1,000 rpm for 5 min and washed well three times with NET-PMSF buffer containing 0.05% sodium deoxycholate. Finally, pelleted HeLa cells were suspended in electrophoresis dissolving buffer (18) and boiled, and radioactivity eluted from fixed cell surfaces was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) and autoradiography or fluorography (10). Specific activities for iodinated

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and ^{35}S-labeled parasites ranged from 1×10^6 to 3×10^6 cpm/2×10^7 trichomonads and 3×10^5 to 7×10^5 cpm/2×10^7 trichomonads, respectively. Radioactivity recovered from HeLa cells after incubation with extract represented approximately 0.2 to 0.5% of the amount of radioactivity originally present in the extract.

HeLa and HEp-2 epithelial cells which were used in an earlier study (3) were washed well with phosphate-buffered saline (PBS) and suspended to a density of 5×10^6/ml in PBS. Cells were gently stirred at 4°C, and glutaraldehyde was added to a 2.5% final concentration. Cells were triturated, if necessary, to avoid clumping, and after a 1-h fixation, the cells were washed extensively five times with ice-cold phosphate-buffered saline. Cells were then resuspended to the original density and treated overnight at 4°C with a 2-mg/ml solution of ovalbumin (Sigma). Cells were then washed three additional times with NET-PMSF buffer, and 10^6 cells were added to individual Microfuge tubes. Only siliconized tubes were used for handling host cells during and after treatment with glutaraldehyde.

Antiserum to trichomonad HeLa cell-binding proteins. In duplicate ligand assays as just described except with unlabeled organisms, regions of acrylamide gels corresponding to HeLa cell-binding proteins (Fig. 1, lane 2) were electroeluted (21), and ligands were individually mixed with Freund complete adjuvant. Rabbits were immunized subcutaneously and intramuscularly, followed by booster immunizations in Freund incomplete adjuvant 2 weeks later and at monthly intervals until evidence of specific antibody production was obtained. Antiserum or the immunoglobulin G (IgG) fraction (14) of antiserum was tested for inhibition of parasite cytadherence (3). Prebleed serum or antiserum generated from a rabbit immunized to a protein other than those implicated as adhesins (Fig. 1, lane 2) served as controls.

Flow cytofluorometry and fluorescence-activated cell sorting. Trichomonads were prepared as for indirect immunofluorescence as detailed in an earlier report (5). Monoclonal antibody C20A3 recognizes a prominent immunogen (7) which undergoes phenotypic variation among parasites of some isolates (5). Hybridoma supernatant with antibody of the same isotype but nonreactive with *T. vaginalis* was used as a control (5). Flow cytofluorometric analyses were always performed simultaneously on duplicate samples of parasites used for other assays. Fractionated C20A3-reactive and nonreactive subpopulations of *T. vaginalis* were isolated as described recently (5).

HeLa cell adherence and killing. The ability of *T. vaginalis* to parasitize HeLa cells and cause contact-dependent host cell killing was measured by established procedures (3, 6). For antibody inhibition experiments, [^{3}H]thymidine-labeled parasites (3) were first pretreated with IgG antibody, followed by addition of the trichomonad-antibody mixture to HeLa cell monolayers (3). After 30 min of incubation, the extent of trichomonal attachment to HeLa cells was determined by measuring HeLa cell-associated counts.

Isolation of nonadherent NYH286 trichomonads was accomplished by sequential passage of 2×10^7 organisms on 75-cm^2 flasks containing confluent HeLa cell monolayers. Remaining unbound organisms were washed and grown overnight, followed by readsorption on HeLa cells as above. This procedure was repeated at least three times to ensure removal of all trichomonads capable of parasitizing HeLa cells. Incubation conditions were identical to those used to monitor cytadherence (3, 6). Nonadherent parasites were then passaged daily and screened at least weekly for reversion to the adherence phenotype, as well as evaluated by flow cytofluorometry with monoclonal antibody C20A3 as before (5).

RESULTS

Selective recognition of HeLa cells by *T. vaginalis* proteins. Experiments were first performed which showed that live *T. vaginalis* NYH286 organisms were capable of attaching to HeLa cells treated with glutaraldehyde. This suggested that it might be possible to use the chemically stabilized HeLa cells in a ligand assay for selective enrichment of trichomonad proteins possibly involved in cytadherence, as has been done by others (9, 10). Our first attempts involved incubation of fixed HeLa cells with dipolar-ionic (Zwitterionic 3-12; Calbiochem-Behring, La Jolla, Calif.) detergent extracts of radioiodinated *T. vaginalis* organisms, since this detergent is efficient in solubilizing immunogens for a variety of assays (7, 8, 16, 20). Little, if any, radioactivity was associated with the host cells, and no protein bands were visualized after SDS-PAGE and autoradiography (Fig. 1A, lane 1).

An alternative detergent system with sodium deoxycholate (9) was then used for solubilization of radiolabeled *T. vaginalis*. Figure 1A (lane 2) shows a representative SDS-PAGE gel pattern of iodinated trichomonad proteins in the deoxycholate extract which bound to fixed HeLa cells.

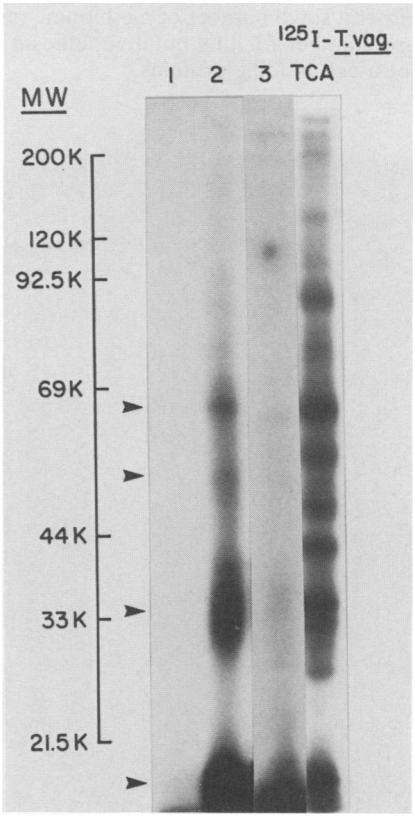

FIG. 1. Representative autoradiograms showing HeLa cell binding, iodinated proteins from extracts of Zwittergent 3-12 (lane 1)- versus sodium deoxycholate (lane 2)-solubilized *T. vaginalis* NYH286 (T. vag.). Lane 3 shows the diminished intensity of the four putative adhesins when trypsinization of iodinated parasites solubilized with deoxycholate was used in the ligand assay. ^{125}I-*T. vag.* TCA represents total trichloroacetic acid-precipitated proteins of iodinated organisms. Molecular weight standards (in thousands) are shown to the left. Arrows indicate positions of the four putative adhesins.

Similar results were obtained with an SDS-Triton X-100 detergent combination (10) (data not shown). In no less than 20 different experiments, all four proteins were identified, and areas on 7.5% acrylamide gels corresponding to proteins with relative molecular masses of ~35 and ≤21 kilodaltons (kDa) always produced pronounced intensities in autoradiograms. Also, similar results were obtained in the ligand assay with HEp-2 epithelial cells, as would be expected due to the ability of HEp-2 cells to accommodate *T. vaginalis* in surface parasitism (3).

The surface location of the four HeLa cell-binding, iodinated proteins was further confirmed by the diminished or absent protein bands in autoradiograms obtained by trypsinization of intact, live parasites prior to deoxycholate solubilization (Fig. 1, lane 3). In duplicate experimental samples, protease-treated *T. vaginalis* were incapable of adherence to HeLa cell monolayers, as reported earlier (3).

Attempts to confirm the parasite origin of the four ligands which recognize HeLa cells were then performed with deoxycholate extracts of [^{35}S]methionine-labeled *T. vaginalis*. Figure 2B (lane 1) shows a representative fluorogram with three of four of the proteins present compared with the use of iodinated trichomonad proteins in the ligand assay (Fig. 2A, lane 1). Prolonged exposure of the X-ray film during autoradiography and experiments with ^{3}H-amino acids for labeling *T. vaginalis* gave a protein of 65 kDa, suggesting either a small number of methionine residues or a very low copy number for this putative adhesin relative to the other HeLa cell-binding proteins.

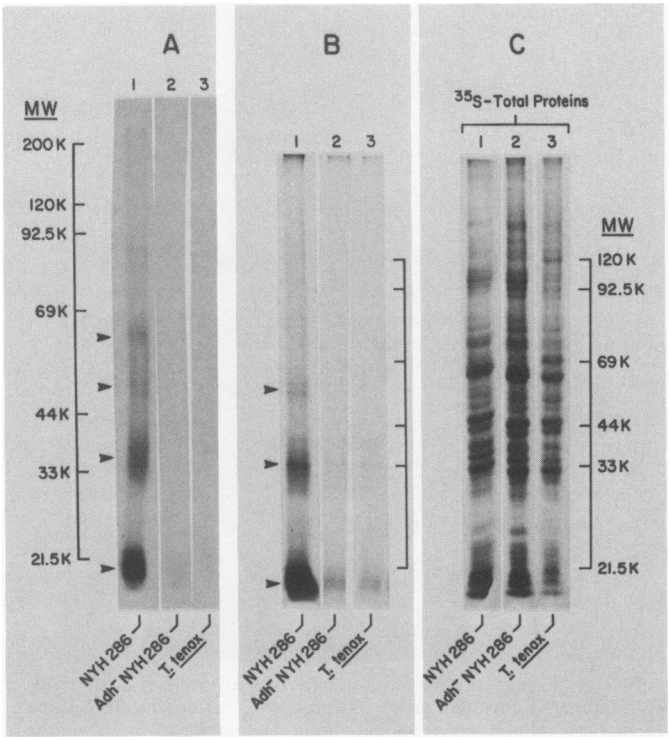

FIG. 2. SDS-PAGE patterns of HeLa cell-binding proteins from ^{125}I- (A) and ^{35}S (B)-labeled proteins of *T. vaginalis* NYH286, a nonadherent subpopulation of *T. vaginalis* NYH286 (Adh⁻ NYH286), and *T. tenax*. (C) Fluorographic patterns of total acid-precipitated proteins. Molecular weight standards (in thousands) are shown. Arrows in panels A and B indicate adhesin candidates identified by the ligand assay.

Under identical experimental conditions, it was also of interest to evaluate whether the nonpathogenic trichomonad *T. tenax* possessed proteins with affinity for HeLa cell surfaces. Figure 2A and B (lanes 3) show no iodinated or ^{35}S-labeled proteins when deoxycholate extracts of *T. tenax* organisms were used.

Competitive binding experiments. Increasing amounts of unlabeled deoxycholate-solubilized trichomonad proteins were mixed with the same amount of detergent extract from iodinated *T. vaginalis*, and the mixture added to fixed HeLa cells in the ligand assay. Stoichiometric decreased binding of all proteins was observed in the presence of increasing levels of unlabeled extract (Fig. 3). The extent of inhibition was similar for all four gel areas corresponding to HeLa cell-binding proteins (Fig. 1, lane 2), further reinforcing the idea of receptor-ligand interactions between these trichomonad proteins and possible HeLa cell surface receptors.

Additional evidence that trichomonad proteins are adhesins was obtained from competition experiments with an aqueous extract of trichomonad proteins. French pressure cell treatment of *T. vaginalis* (6) followed by size fractionation of solubilized proteins via gel permeation chromatography was performed. An aqueous extract containing proteins <70 kDa in size produced a dose-dependent inhibition of trichomonal cytadherence (data not shown).

Antibody inhibition of cytadherence by *T. vaginalis*. Attempts were then made to produce antiserum to the quantitatively prominent ≤21-kDa protein ligand to demonstrate specific inhibition of cytadherence. Figure 4 shows a dose-dependent decreased attachment to HeLa cells by parasites when IgG to the ≤21-kDa adhesin was used. IgG (100 μg) resulted in approximately 80% reduction of parasites bound to HeLa cells. Similar extents of inhibition (~80%) were also achieved when *T. vaginalis* were pretreated with IgG of individual antiserum to the three other putative adhesins (Fig. 1). Also, the use of a mixture of IgG of the different antisera resulted in almost total abolition of trichomonal cytadherence, with numbers of adherent organisms representing only ~5% of control levels. No similar effect was seen when using IgG of control prebleed serum or IgG from antiserum generated to a trichomonad protein other than the four HeLa cell-binding proteins. All of these data suggest that the HeLa cell-binding proteins may be unique biofunctional molecules of the pathogenic human trichomonads.

Isolation of nonadherent trichomonads. Purification of adherent (Adh^{+}) and nonadherent (Adh^{-}) subpopulations of trichomonads from the parent NYH286 population was then attempted. This was done because repeated incubations of parasites with HeLa cell monolayers failed to achieve 100% adsorption of all trichomonads. Also, any Adh^{-} organisms would be useful in the identification of putative adhesins.

Repeated incubations of the parent population of isolate NYH286 with HeLa cell monolayers gave Adh^{-} *T. vaginalis* (Fig. 5). Figure 5 also shows that the Adh^{-} subpopulation of trichomonads failed to kill HeLa cells, consistent with earlier reports on the contact-dependent nature of host cell killing (3, 6).

Flow cytofluorometric patterns obtained with monoclonal antibody C20A3, which recognizes a prominent immunogen known to undergo phenotypic variation (5), showed both fluorescent and nonfluorescent parasites in the parent NYH286 population; however, purified Adh^{-} NYH286 organisms were a homogeneous fluorescent subpopulation.

Next, Adh^{-} parasites were examined for expression of any of the four trichomonad adhesin candidates. Figure 2A and B (lanes 2) illustrate the absence of detectable ligands in the gel

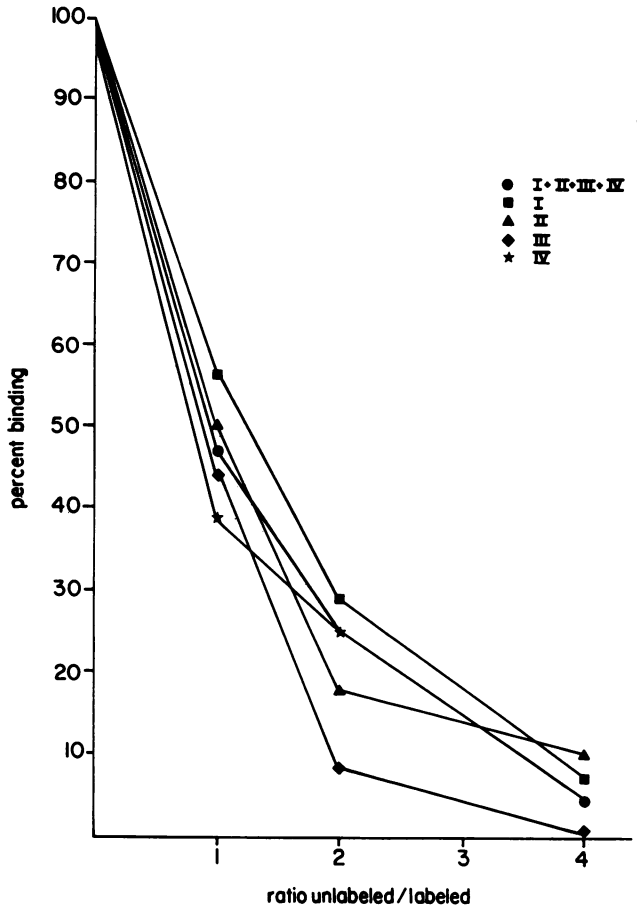

FIG. 3. Representative competition experiment with fixed HeLa cells incubated with a mixture of increasing amounts of unlabeled *T. vaginalis* extract with a constant amount of iodinated trichomonad proteins. Parasites were solubilized with sodium deoxycholate as described in Materials and Methods and mixed to give a final volume of 1 ml prior to addition to fixed HeLa cells. Bound material was subjected to SDS-PAGE, followed by autoradiography and densitometric scanning of X-ray film. The area under each gel region (Fig. 1, lane 2) was quantitated, and absence of unlabeled extract yielded maximum binding (100%). Adhesin symbol designations: I, ≤21.5 kDa; II, ~33 kDa; III, ~50 kDa; IV, ~65 kDa.

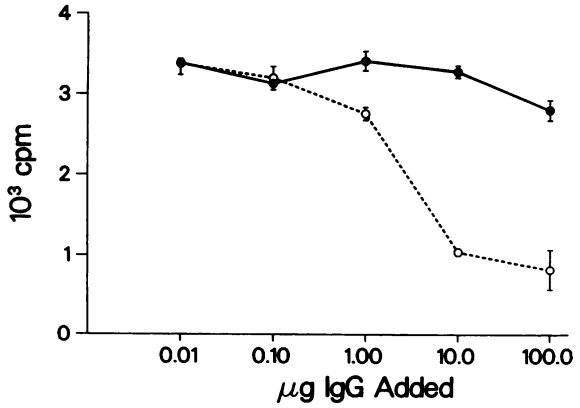

FIG. 4. Inhibition of *T. vaginalis* NYH286 adherence to HeLa cell monolayers with IgG from rabbit antiserum generated to the adhesin with a relative molecular mass of ≤21 kDa (○) compared with control IgG (●).

fluorograms when deoxycholate extracts of ^{125}I- or ^{35}S-labeled Adh$^-$ *T. vaginalis* proteins were incubated with fixed HeLa cells.

Properties of revertant Adh$^+$ trichomonads. Revertant Adh$^+$ organisms also capable of killing HeLa cells were derived from the Adh$^-$ subpopulation after 3 months of in vitro cultivation (Fig. 5). Interestingly, flow cytofluorometry with C20A3 of the revertants indicated that the population was heterogeneous, comprising both fluorescent and nonfluorescent organisms (Fig. 5).

Fluorescence-activated cell sorting was then performed on the revertant C20A3-fluorescent and nonfluorescent parasites. Only nonfluorescent trichomonads expressed the Adh$^+$ phenotype and produced levels of cytadherence equal to or better than that seen with the original parent NYH286 parasites (Fig. 6). On the other hand, fluorescent organisms were unable to bind to HeLa cells, and this result was similar to that seen originally for Adh$^-$ parasites (Fig. 5). As expected, nonfluorescent Adh$^+$ organisms possessed the four adhesins compared with C20A3-reactive, Adh$^-$ trichomonads (Fig. 2). Finally, purification of Adh$^-$ and Adh$^+$ subpopulations was also achieved with other isolates known to be heterogeneous and capable of phenotypic variation for the C20A3 surface marker (3).

DISCUSSION

Evidence is presented which supports the idea that trichomonad proteins are involved in host cytadherence. Assay systems with chemically stabilized epithelial cells have been described by others for the study of microbial receptor-binding proteins (9, 10). Four *T. vaginalis* adhesin candidates were identified (Fig. 1, lane 2). Identical comigrating proteins have been observed for 10 other isolates of *T. vaginalis*. The ability to achieve stoichiometric competition of the adhesins when different ratios of unlabeled to labeled *T. vaginalis* extracts were used (Fig. 3) strengthens the view that all four proteins are indeed involved in HeLa cell surface recognition. No similar inhibition was seen with

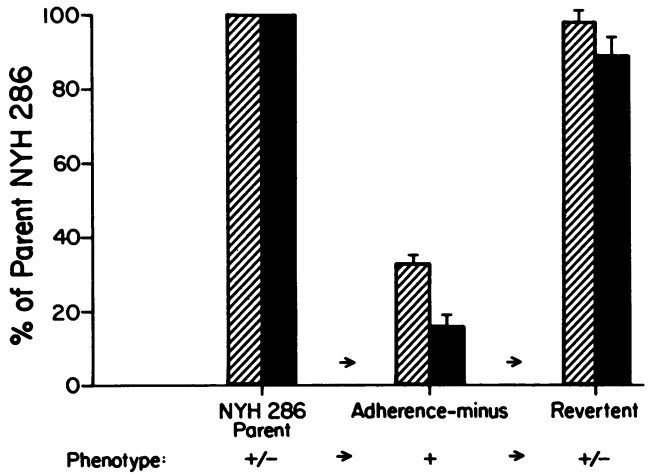

FIG. 5. Comparative adherence (hatched bars) and toxicity (solid bars) to HeLa cells of *T. vaginalis* NYH286 parent, of the nonadherent subpopulation of trichomonads derived from NYH286, and the revertant adherent parasites obtained after 3 months of in vitro cultivation of Adh$^-$ organisms. Phenotype refers to the expression of a prominent immunogen on *T. vaginalis* surfaces as defined by flow cytofluorometry with monoclonal antibody C20A3 (2, 5, 7).

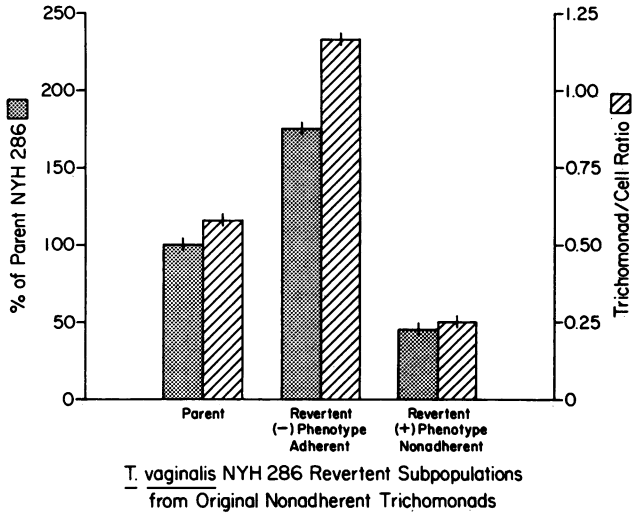

FIG. 6. Comparative cytadherence levels of sorted subpopulations of revertant adherent *T. vaginalis* NYH286 derived from the Adh^- subpopulation (Fig. 4). Revertant parasites were sorted on the basis of fluorescence with monoclonal antibody C20A3 (5). Parent refers to the original heterogeneous NYH286 population (Fig. 4) from which all subsequent parasite fractions were derived.

detergent extracts of *T. tenax*, as might be predicted by the lack of similar HeLa cell-binding proteins in this nonpathogenic trichomonad (Fig. 2). The absence of similar proteins which recognize epithelial cell surfaces in the nonpathogenic trichomonad *T. tenax* suggests that the HeLa cell-binding ligands are virulence factors of the pathogenic human trichomonads.

The lack of well-defined protein bands in the four acrylamide gel areas corresponding to the adhesins (Fig. 1, lane 2) was a concern. The use of extracts from iodinated *T. vaginalis* organisms generally gave more diffuse gel regions than detergent preparations of intrinsically labeled proteins (Fig. 2). A possible explanation for the absence of resolution of proteins by our ligand assay might be the variety of proteases known to be produced by this parasite (12). Clearly, gentle solubilization of the organisms may cause the release of proteases active against the adhesins. This view is supported by the protease lability of the ligands, since protease treatment of trichomonads diminishes cytadherence (3) and removes iodinated proteins with molecular masses similar to those of the adhesins from the parasite surface (4) (Fig. 1, lane 3). Furthermore, parasite-derived protease activity on surface proteins of *T. vaginalis* was recently observed (J. F. Alderete, unpublished observations); thus, multiple bands might be due to degradation of the individual adhesins. Additional experimentation is required to understand the absence of precise protein bands in autoradiograms.

The ability to inhibit cytadherence with IgG from antiserum to the protein of ≤21 kDa (Fig. 4) and to the other adhesin candidates (data not shown) provided additional evidence for the biofunctionality of these proteins. That only IgG at protein concentrations of 100 μg/ml was effective at inhibiting trichomonal cytadherence may be indicative of a low titer of specific antibody. In fact, long-term boosting (1 year) of the individual rabbits with the respective purified proteins was required before antibody which inhibited cytadherence was detected. These data perhaps suggest an immunorecessive nature for these adhesins.

Serum from patients with trichomoniasis does not appear to have significant levels of antibody to proteins with molecular weights in the range of the putative adhesins (Fig. 1) (8). Also, antiserum from mice subcutaneously infected with *T. vaginalis* NYH286, which contains antibody to proteins of <70 kDa, failed to recognize these immunogens on intact, live organisms (1, 8). Thus, an immunorecessive nature coupled with the possibility of inaccessible epitopes on these biofunctional molecules while residing on *T. vaginalis* surfaces might be beneficial to the parasite, and in this way little or no interference of host parasitism even in the possible presence of antibody will occur during infection. It is important to note that no data are available on the precise role, if any, of acquired immunity by humans to trichomoniasis. Furthermore, the exact reactivity of existing vaginal mucosal anti-*T. vaginalis* antibody to specific trichomonad proteins is not known. The idea that this pathogen may evade any host immune surveillance of biofunctional molecules, however, is supported by the recent finding that all patients are infected with Adh^+ parasites (2).

Adh^- organisms (Fig. 5) were isolated by consecutive, sequential adsorption of NYH286 trichomonads on HeLa cell monolayers. This Adh^- phenotype and revertant Adh^+ parasites showed the absence and presence, respectively, of the HeLa cell-binding proteins, confirming the role of these proteins in cytadherence. Interestingly, Adh^- trichomonads were positive for expression of a prominent immunogen on their surface (Fig. 5), consistent with recent findings on the relationship of immunogens with host cell parasitism and killing (1, 3, 6). The emergence of *T. vaginalis* without the C20A3-reactive molecule and which were responsible for cytadherence during the reversion of Adh^- to Adh^+ provides support for the hypothesis that alternating expression of groups of surface proteins occurs in *T. vaginalis* (Alderete, in press). For example, the presence on *T. vaginalis* surfaces of only one of two repertoires of proteins corresponds to the formula $A^+B^- \rightleftarrows A^-B^+$, where the major immunogens, such as those seen with the C20A3 (Fig. 2 and 5) (1), are designated A and the adhesins are represented by B (Fig. 5). Phenotypic variation also occurring for the adhesin proteins is demonstrated in this study, since purified Adh^- (A^+B^-) and Adh^+ (A^-B^+) parasites give rise to subpopulations of opposite phenotypes during in vitro growth and multiplication (Fig. 2, 5, and 6).

This paper describes the identification and properties of biofunctional molecules of *T. vaginalis*. These proteins, along with key immunogens which undergo phenotypic variation (1, 6), appear to be unique to the pathogenic human trichomonads. Indeed, these repertoires of *T. vaginalis* proteins and their biologic properties represent important areas for further investigation.

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