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Trichomonas vaginalis Surface Proteinase Activity Is Necessary for Parasite Adherence to Epithelial Cells

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The role of cysteine proteinases in adherence of *Trichomonas vaginalis* NYH 286 to HeLa and human vaginal epithelial cells was evaluated. Only pretreatment of trichomonads, but not epithelial cells, with *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), an inhibitor of trichomonad cysteine proteinases, greatly diminished the ability of *T. vaginalis* to recognize and bind to epithelial cells. Leupeptin and L-1-tosylamide-2-phenylethyl chloromethyl ketone, other cysteine proteinase inhibitors, also decreased *T. vaginalis* cytadherence. Parasites incubated with TLCK and washed extensively still did not adhere to cells at levels equal to those seen for control trichomonads treated with phosphate-buffered saline or culture medium alone. Exposure of TLCK-treated organisms with other cysteine proteinases restored cytadherence levels, indicating that proteinase action on the parasite surface is prerequisite for host cell attachment. Concentrations of TLCK which inhibited cytadherence did not alter the metabolism of *T. vaginalis*, as determined by metabolic labeling of trichomonad proteins; the protein patterns of *T. vaginalis* in the presence and absence of TLCK were identical. Kinetics of TLCK-mediated inhibition of cytadherence of other *T. vaginalis* isolates with different levels of epithelial-cell parasitism were similar to the concentration-dependent inhibition seen for isolate NYH 286. Incubation of TLCK-treated, washed organisms in growth medium resulted in regeneration of adherence. Finally, treatment of *T. vaginalis* organisms with proteinase inhibitors for abrogation of cytadherence effectively rendered the trichomonads unable to kill host cells, which is consistent with the contact-dependent nature of host cytotoxicity. These data show for the first time the involvement of *T. vaginalis* cysteine proteinases in parasite attachment to human epithelial cells. These results have implications for future pharmacologic intervention at a key step in infection.

Trichomonas vaginalis is a mucosal protozoan parasite of the urogenital-vaginal tract. This pathogen is responsible for one of the most common sexually transmitted diseases in humans (1, 19, 23). Trichomonal cytadherence to epithelial cells is a highly specific, key step during initiation of infection and disease pathogenesis (4, 5). Cytopathogenicity of host cells in monolayer cultures has been shown to be contact dependent (9, 20).

T. vaginalis and other flagellates (13, 24) possess very high levels of proteolytic activity. At least 11 cysteine proteinases for the pathogenic human trichomonads have been identified. These proteinases differ on the basis of their stimulation with dithiothreitol, molecular weights, pH optima, and relative sensitivities to inhibitors. Trichomonad proteinases, for example, do not appear to be affected by pepstatin, an aspartic proteinase inhibitor, or by serine proteinase inhibitors such as phenylmethylsulfonyl fluoride. However, proteinase activities were inhibited by *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), iodoacetic acid, leupeptin, chymostatin, and antipain, each of which has been shown to inactivate several but not all trichomonad cysteine proteinases (15, 22).

Trichomonad cysteine proteinases degrade a prominent surface immunogen of *T. vaginalis* which undergoes phenotypic variation in some isolates (10). Degraded immunogen was found in culture supernatants (8), indicating that proteolytic activity for some trichomonad molecules may be common during normal growth of this parasite and that one or more proteinases may reside on the *T. vaginalis* surface. The particular cysteine proteinase responsible for degradation of this immunogen is unknown, however.

The role and involvement of any *T. vaginalis* cysteine proteinase during infection and disease pathogenesis have remained undefined. During experiments identifying putative adhesin proteins of *T. vaginalis* that participate in cytadherence, we obtained poor resolution of the candidate adhesins after electrophoresis and autoradiography (6). We reasoned that trichomonad proteinases may in part be responsible for the absence of well-defined protein bands, and we attempted to solve the problems by using an effective inhibitor of most cysteine proteinases of *T. vaginalis*, TLCK, during detergent extraction of total parasite proteins. Because of the possibility that trichomonad surface proteinases might digest the adhesins, as was observed for the parasite immunogen (8), we also monitored the effect of exogenous cysteine proteinase inhibitors on overall *T. vaginalis* cytadherence levels. To our surprise, pretreatment of parasites with cysteine proteinase inhibitors decreased trichomonal recognition and attachment to epithelial cells. Data presented in this report show that one or several trichomonad cysteine proteinases are involved during the initial event important to host parasitism and establishment of infection.

MATERIALS AND METHODS

Organisms. *T. vaginalis* isolates NYH 286, IR 78, and RU 375 have been used in recent studies (2, 3, 5–9, 26–28). Parasites were passaged daily (2, 3) in Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract-maltose (TYM) medium supplemented with heat-inactivated horse serum (Hazleton Biologics, Inc., Lenexa, Kans.) (3, 16). For all experiments, only parasites in mid- to late

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logarithmic phases of growth (5) were used. Radiolabeling of *T. vaginalis* with [^{35}S]methionine (specific activity, 1,134 Ci/mmol; Dupont, NEN Research Products, Wilmington, Del.) was accomplished as described before (5), except that labeling was for only 2 h at 37°C. Trichomonads were then washed with cold phosphate-buffered saline (PBS), and total proteins were precipitated as described previously with 10% trichloroacetic acid (25). Radiolabeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 3% stacking and 7.5% separating acrylamide gels (21). After Coomassie brilliant blue staining, gels were processed for fluorography as previously described (25).

Cytadherence assay. HeLa epithelial cells were obtained from the American Type Culture Collection, Rockville, Md., and maintained in Dulbecco modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Hazleton), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cultures were kept in a 7.0% CO_2 atmosphere at 37°C, and cells were passaged and used for cytadherence assays as previously described (5). *T. vaginalis* surface parasitism of HeLa epithelial cells in monolayer culture was monitored either by an earlier published procedure with HeLa cells on 12-mm cover slips (5) or by a recent modification as described below. Briefly, 4×10^4 HeLa cells were seeded in each well of a 96-well microdilution plate (Dynatech Laboratories, Inc., Chantilly, Va.) and incubated overnight to achieve confluency. A sample (100 μl) containing 4×10^5 [^{3}H]thymidine-labeled parasites (5) suspended in a 2:1 (vol/vol) medium mixture (DMEM-TYM) without serum previously found suitable for both trichomonads and host cells (5) was then added to HeLa cell monolayers washed with warm DMEM. After incubation for 30 min at 37°C in a 7% CO_2 atmosphere, unbound parasites were removed, and wells with adherent trichomonads were washed three times with warm DMEM. After the plates dried, the extent of cytadherence was determined by counting individual wells for radioactivity (5). Purification of human vaginal epithelial cells obtained from vaginal swabs of healthy women has been described elsewhere (4), and trichomonal cytadherence of vaginal epithelial cells was determined as recently detailed (4).

Trichomonads washed with PBS (137 mM NaCl–2.7 mM KCl–4 mM Na_2HPO_4–1.5 mM KH_2PO_4 at pH 7.0) were suspended in PBS or TYM medium and treated with proteinase inhibitors for 20 min at 37°C. Parasites were then either added to host cells or washed again to remove inhibitors prior to addition to host cells. Proteinase inhibitors, such as TLCK, TPCK, leupeptin, and soybean trypsin inhibitor (all from Sigma Chemical Co., St. Louis, Mo.), were prepared as stock solutions in distilled water. Control trichomonads were similarly handled, but inhibitors were omitted. Calculations of numbers of trichomonads adherent to host cells indicated that values ranging from ~1.0 to 3.0 parasites per cell were obtained for controls. These values were very consistent with those obtained in earlier studies (4, 5) and affirmed the usefulness of this assay for these experiments.

For each experiment, parasite motility in the presence of inhibitors was evaluated by microscopic observation and compared with motility of controls without inhibitors (5, 6). All experiments presented in this study were performed at least five times with triplicate samples.

Also, Student *t*-test analysis was performed to determine the significance of the effect of inhibitors on cytadherence and cytotoxicity (discussed below). For all experiments presented in this report, values of $P < 0.005$ were obtained.

TABLE 1. Effects of cysteine proteinase inhibitors on *T. vaginalis* isolate NYH 286 cytadherence to epithelial cells[a]

Expt no.	Host cell type	Inhibitor[b]	% Parasite cytadherence[c]
1	HeLa		100.0 ± 8.2
	HeLa	TLCK	6.1 ± 0.4
	VEC		100.0 ± 0.7
	VEC	TLCK	7.8 ± 1.2
2	HeLa		100.0 ± 8.1
	HeLa	TLCK	53.2 ± 5.8
	HeLa	TPCK	57.0 ± 2.3
	HeLa	Leupeptin	45.1 ± 2.4
3	HeLa		100.0 ± 5.1
	HeLa	TLCK	24.0 ± 1.2
	HeLa	TPCK	56.6 ± 6.0
	HeLa	Leupeptin	33.1 ± 3.6
	HeLa	Soybean trypsin inhibitor	86.2 ± 7.0

[a] Epithelial cells used in these experiments were HeLa cells cultured on cover slips (experiments 1 and 2) or in 96-well plates (experiment 3), as described in Materials and Methods. Vaginal epithelial cells (VEC) were from healthy women, and conditions for monitoring vaginal-epithelial-cell parasitism were recently detailed (4).

[b] Approximately 2×10^6 washed organisms suspended in PBS (experiment 1) or TYM medium (experiments 2 and 3) were treated and coincubated with host cells (experiment 1) or pretreated for 20 min and washed before addition to cells (experiments 2 and 3). A concentration of 1 mM TLCK, 1 mM TPCK, 0.2 mM leupeptin, or 1 mg of soybean trypsin inhibitor per ml was added to parasites, which were then incubated at 37°C. Control parasites were handled similarly but without inhibitors.

[c] Obtained by normalization of the bound radioactivity to that of control samples without inhibitors, which represented 100% adherence levels. Numbers represent the means ± standard deviations of triplicate samples.

Contact-dependent HeLa cell killing. The quantitative colorimetric assay to measure cytadherence-mediated cytotoxicity of HeLa cells in monolayer culture was performed as described recently (9). In these experiments, *T. vaginalis* organisms were pretreated with either 1 mM TLCK or 0.2 mM leupeptin as described above and compared with untreated control parasites.

RESULTS

Inhibitors of trichomonad cysteine proteinases greatly reduce *T. vaginalis* cytadherence. *T. vaginalis* organisms were coincubated with HeLa cells in monolayer cultures in the presence of a trichomonad cysteine proteinase inhibitor. TLCK at a 1 mM concentration has been found to inhibit most or all of the trichomonad proteinases (15, 22), so this concentration of inhibitor was used initially. Table 1 presents the results of three representative experiments which show that *T. vaginalis* cytadherence to HeLa cells in the presence of TLCK was dramatically reduced compared with cytadherence of control parasites handled similarly but without TLCK. In some cases, a greater-than-90% inhibition of cytadherence was observed (experiment 1). Similar levels of diminished attachment to vaginal epithelial cells by trichomonads in the presence of TLCK were also observed (experiment 1). Other trichomonad cysteine proteinase inhibitors such as TPCK and leupeptin were also effective inhibitors of cytadherence. Experiments such as these have been performed a minimum of 20 times, and in all cases, cysteine proteinase inhibitors reduced the level of trichomonal cytadherence by 50 to 90%. Although the cytadherence assay was done at pH 6.0 as reported earlier by us (5), we also examined levels of parasitism in the presence or

TABLE 2. Effects of pretreatments of *T. vaginalis* isolate NYH 286 or HeLa epithelial cells on cytadherence[a]

Expt no.	Sample no.	Pretreatment of:		% Cytadherence[b]
		Parasites[c]	Host cells[d]	
1	1			100.0 ± 13.1
	2	TLCK		4.4 ± 0.1
	3		TLCK	105.7 ± 2.3
	4	TLCK, washed		3.8 ± 0.3
2	5			100.0 ± 11.2
	6		Papain	99.3 ± 6.1
	7		Extract[e]	91.9 ± 9.9

[a] Cytadherence levels were determined by using HeLa cell monolayers on cover slips (experiment 1), as described in Materials and Methods.

[b] The level of cytadherence achieved with untreated parasites handled similarly to treated organisms was taken to be 100%. Numbers represent the means ± standard deviations of triplicate samples.

[c] Approximately 2×10^6 washed organisms suspended in TYM medium were pretreated with 1 mM TLCK for 20 min at 37°C. Parasites were then centrifuged and suspended in DMEM-TYM (2:1) medium mixture for addition to host cells.

[d] Cover slips with HeLa cell monolayers were pretreated with 1 mM TLCK, or HeLa cell monolayers in 96-well microdilution plates (experiment 2) were treated with 0.25 μg of papain or 0.3 μg of parasite extract which was first dialyzed for 24 h. Treatment for 10 min at 37°C was followed by extensive rinsing of host cells with DMEM before parasite interaction. These conditions did not disrupt the host cell monolayer.

[e] Protein solubilization with 1% deoxycholate of 2×10^7 organisms in PBS was performed at 4°C for 30 min (6). Insoluble material was eliminated by centrifugation in a 10% sucrose cushion at 17,000 × *g* for 30 min at 4°C. Excess detergent was removed by dialysis against PBS for 24 h at 4°C. The proteinase activity in the extract was monitored with the Bio-Rad kit with casein as substrate.

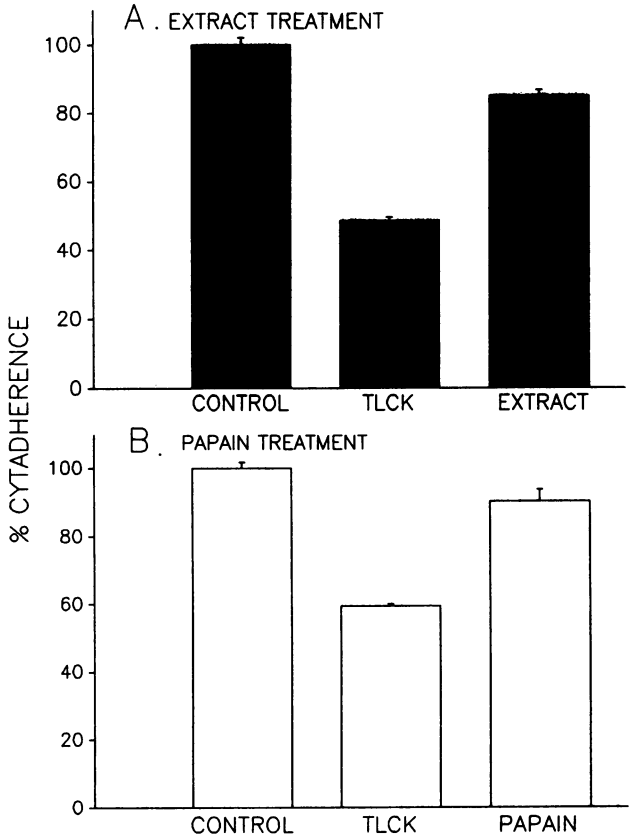

FIG. 1. Restoration of cytadherence to HeLa cell monolayers of TLCK-treated *T. vaginalis* NYH 286 following incubation with trichomonad detergent extract containing proteinases (A) or papain (B). For these experiments, parasites were first treated with 1 mM TLCK in TYM, as described in Materials and Methods. Trichomonads were then washed with PBS and suspended in 1.0 μg of extract dialyzed to remove detergent or 0.25 μg of papain. After 5 min at 37°C, the parasites were washed with PBS and suspended in medium mixture for addition to cell monolayers in a 96-well microdilution plate assay. The preparation of a trichomonad extract for these experiments was as described in Table 2.

absence of inhibitors at pH values of 5.0 to 7.0. The inhibitors were effective at all pH values in this range, ensuring that the observations were relevant to in vivo conditions, since the vaginal pH during trichomoniasis is approximately 5.5. These initial data suggested that one or more proteinases of either parasite or host cell origin were involved in recognition and binding events between parasite and epithelial-cell surfaces.

The next series of experiments was performed in order to determine the origin of the proteinase(s) involved in trichomonal cytadherence. Inhibition of attachment was obtained only by pretreatment of *T. vaginalis* parasites (sample 2) and not HeLa cells (sample 3) (Table 2). Extensive washing of TLCK-pretreated organisms did not alleviate the inhibitory effect of TLCK (sample 4). These data suggest that proteinases may reside on the parasite surface.

To test whether the proteinase, possibly of parasite origin, was affecting the host cell surface, we treated HeLa cells with papain (sample 6), a cysteine proteinase, or a trichomonad detergent extract (sample 7) which possessed proteinase activity (proteinase detection kit; Bio-Rad Laboratories, Richmond, Calif.). The level of *T. vaginalis* cytadherence was similar to that of untreated control HeLa cells (sample 5), indicating that proteinase action on epithelial-cell surfaces was not necessary for cytadherence. No competition of cytadherence was seen when HeLa cells were pretreated with extract, presumably because of either the low copy number of adhesins (6) or the susceptibility of these molecules to proteinases found in *T. vaginalis* extracts. These data indicate that one or several proteinases interacting with the parasite and not the host cell surface are involved in cytadherence.

Finally, TLCK-treated organisms were incubated with extract containing proteinase activity or papain. Parasites treated with 1.0 μg of protein in the extract (Fig. 1A) or 0.25 μg of papain (Fig. 1B) recovered their levels of cytadherence. However, higher amounts of extract or papain reduced attachment, probably because of removal of adhesin proteins from trichomonal surfaces, as has been demonstrated previously (4, 5). This reversal of TLCK-mediated inhibition of a *T. vaginalis* proteinase involved in cytadherence strongly supports the idea that proteinase action on the parasite surface is prerequisite for host cytadherence. As expected, the addition of TLCK to papain and extract prior to incubation of these enzyme preparations with parasites did not restore cytadherence to levels above those seen only for TLCK-treated organisms (data not shown).

It was important to show that exposure of parasites to TLCK did not impair the motility and protein synthesis of *T. vaginalis*, since metabolic integrity is necessary for efficient trichomonal cytadherence (5). Incubation of trichomonads with up to 2 mM TLCK for 20 min, the time needed to inhibit cytadherence to levels shown in Tables 1 and 2, did not affect growth and multiplication of *T. vaginalis* (Fig. 2A). Indeed, in several different experiments, TLCK-pretreated organisms showed slightly enhanced overall parasite densi-

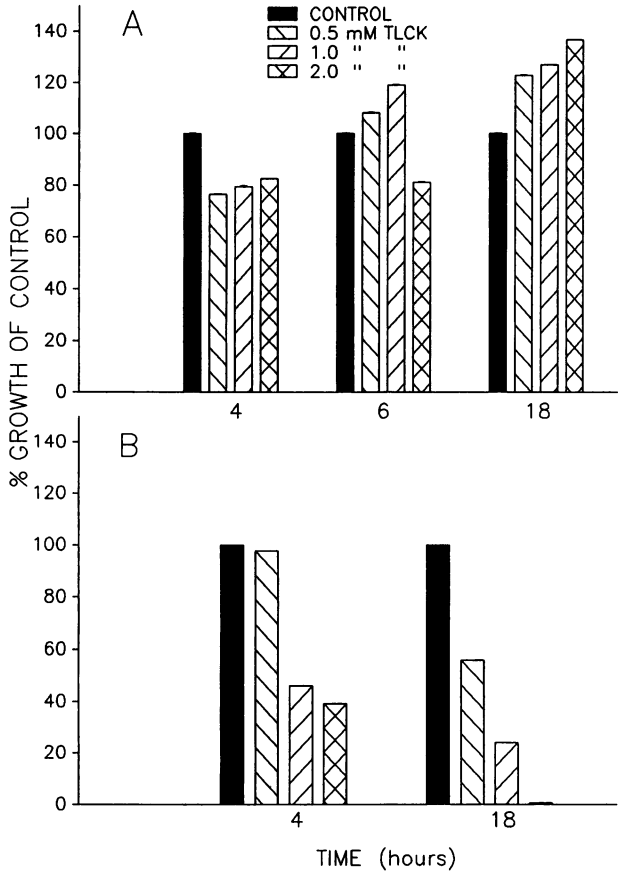

FIG. 2. Growth of *T. vaginalis* NYH 286 organisms in the presence of TLCK concentrations which inhibit cytadherence. The trichomonads were either pretreated for 20 min (A) or incubated for 4 h (B) at 37°C with different concentrations of TLCK in TYM. The parasites were counted at various times following TLCK treatment. The total number of organisms of the control group without TLCK treatment was assumed to represent 100% growth.

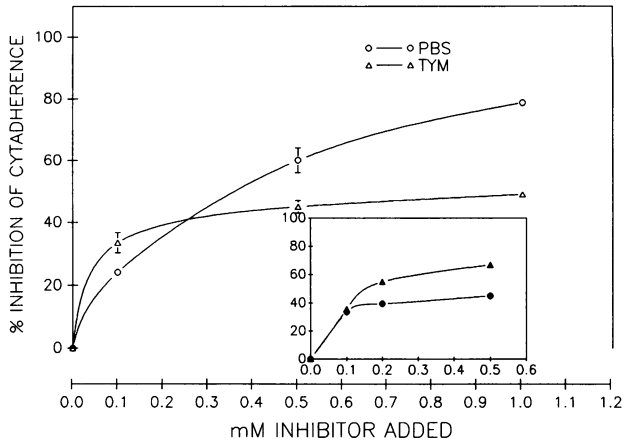

FIG. 3. Inhibition of cytadherence after pretreatment of *T. vaginalis* NYH 286 suspended in PBS (○) or TYM (△) with TLCK. In this case, confluent monolayers of HeLa cells in 96-well microdilution plates were used for the adherence assay, as described in Materials and Methods. The insert shows a comparison of inhibition kinetics with TLCK (●) and leupeptin (▲) under the same experimental conditions.

ties compared with control densities after 6 and 18 h of incubation in TYM-serum medium. Only exposure to TLCK for more than 2 h decreased parasite densities compared with those of untreated control organisms (Fig. 2B). Absence of motility was observed only by treatment of parasites with TLCK at 18 h.

Protein synthesis of *T. vaginalis* was also monitored at the times indicated for the growth kinetics shown in Fig. 2. The levels of [^{35}S]methionine incorporation into proteins, as determined by band intensities of fluorograms of total trichloroacetic acid-precipitated proteins of untreated parasites (25), were identical for parasites exposed to 2 mM TLCK for 20 min. Not unexpectedly, only a pretreatment of trichomonads for more than 2 h with 2 mM TLCK (Fig. 2B) resulted in reduction of band intensities of all proteins (data not shown).

Inhibition of *T. vaginalis* cytadherence is dependent on TLCK concentration. Maximal inhibition was achieved by using 1 mM TLCK regardless of whether parasites were suspended in TYM medium or PBS (Fig. 3). The TLCK inhibitor at >1 mM did not give inhibition values other than those seen at 1 mM (Fig. 3). Organisms in TYM medium were less inhibited than trichomonads in PBS, possibly because of the presence of cysteine in TYM medium (24) or to the binding of TLCK by macromolecules in TYM complex medium. TYM medium was selected instead of PBS for all remaining experimental manipulations, however, because trichomonads appeared more motile in TYM medium.

It was also of interest to determine the concentration-dependent inhibition activities of TLCK and leupeptin, since a differential inhibitory effect among trichomonad cysteine proteinases by these inhibitors has been reported elsewhere (15, 22). Leupeptin was more effective at reducing cytadherence than TLCK at the same molar concentrations (Fig. 3, insert).

Regeneration of cytadherence among TLCK-treated parasites. The irreversible inhibition of proteinases involved in cytadherence achieved by TLCK treatment of parasites prompted us to examine whether regeneration of cytadherence could result from placing trichomonads in growth medium. *T. vaginalis* pretreated with 1 mM TLCK for 20 min and then incubated in growth medium for different lengths of time did indeed recover its former level of cytadherence (Fig. 4). After 3 h, the extent of cytadherence of TLCK-pretreated trichomonad equaled those of untreated control parasites.

Similar TLCK-mediated inhibition of cytadherence among other isolates. It was important to test representative isolates of *T. vaginalis* with known differences in cytadherence levels (Fig. 5, insert). The kinetics of TLCK inhibition of host cell attachment for the three isolates was also dependent on TLCK concentration (Fig. 5). The amount of TLCK needed to achieve a 50% reduction of parasite attachment to target cells differed for each isolate, however, as NYH 286, IR 78, and RU 375 required 0.5, 1.0, and 2.0 mM TLCK, respectively.

Cysteine proteinase inhibitors greatly reduce *T. vaginalis* contact-dependent cytotoxicity. It has been shown by us (5, 9) and others (20) that *T. vaginalis* killing of host cells is a contact-dependent event. For these reasons, we decided to test the effect of TLCK and leupeptin preincubation of *T. vaginalis* in a parasite cytotoxicity assay. Both inhibitors were able to effectively protect the monolayers from parasite destruction (Table 3), which is consistent with the view that cytadherence is an important prerequisite for cytotoxicity.

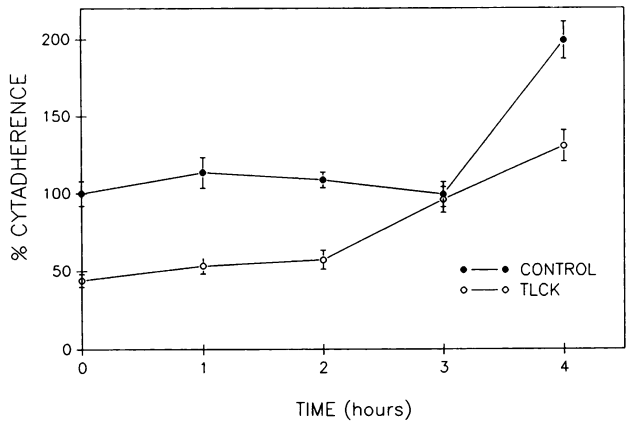

FIG. 4. Regeneration of cytadherence of TLCK-pretreated *T. vaginalis* isolate NYH 286. After incubation with 1 mM TLCK for 20 min at 37°C, trichomonads were washed and suspended in growth medium. *T. vaginalis* cytadherence was then measured with HeLa cell monolayers in 96-well microdilution plates. One hundred percent of attachment represents the level of cytadherence of control trichomonads (●) handled similarly but without pretreatment with TLCK. All other values at different times were compared with values for the control at time zero. The level of cytadherence of *T. vaginalis* pretreated with TLCK (○) shows the extent of inhibition resulting from proteinase inhibition.

TABLE 3. Cysteine proteinase inhibitor reduction of *T. vaginalis* NYH 286 contact-dependent cytotoxicity[a]

Assay time (h)[b]	Pretreatment reagent[c]	% Cytotoxicity[d] (% inhibition)
1		100.0 (0)
	TLCK	8.7 (91.3)
	Leupeptin	69.6 (30.4)
2		100.0 (0)
	TLCK	21.5 (78.5)
	Leupeptin	98.4 (1.6)

[a] The colorimetric assay to measure cell killing was performed by using a HeLa cell monolayer in 96-well microdilution plates (9). After incubation of cells with trichomonads, wells were washed, and the remaining cells were fixed with formaldehyde and then stained with crystal violet. Stained material was solubilized in 1% sodium dodecyl sulfate and monitored at a wavelength of 570 nm.

[b] Because we wanted to observe monolayer destruction in a period of 1 to 2 h in order to avoid interference due to proteinase regeneration (Fig. 4), the host cell/parasite ratio was 1:50, which was higher than that for the standard published procedure (9).

[c] Parasites were treated with 1.0 mM TLCK or 0.2 mM leupeptin in TYM medium for 20 min at 37°C, washed with PBS, and suspended in interaction medium before being added to host cells.

[d] The values for cytotoxicity as previously described (9) [1 − (E/C)] were 0.867 ± 0.02 and 0.983 ± 0.03 for the 1- and 2-h controls, respectively. These values were assumed to be 100% when the levels of protection provided by proteinase inhibitors were calculated.

DISCUSSION

To our knowledge, the cysteine proteinases of *T. vaginalis* have received little attention as potential virulence factors (15, 22). In this report, we present evidence for the involvement of *T. vaginalis* proteinases, which may reside on the parasite surface, in events leading to recognition of and binding to host cells, key steps of host parasitism. Our results suggest that these proteinases represent important determinants of virulence and disease pathogenesis.

Data described here show that some members of this repertoire of molecules (proteolytic enzymes) may have biofunctional roles other than the digestion of proteins for nutritional purposes (24). Proteinases are already appreciated for a variety of functions related to those proposed by us for trichomonal cytadherence. For example, proteinases have been implicated in *Candida* spp. attachment to tissues (11, 30) and in the invasion of malaria parasites into erythrocytes (17, 31). *Trypanosoma cruzi* requires the action of a proteinase during maturation in order to successfully parasitize host cells (12, 29).

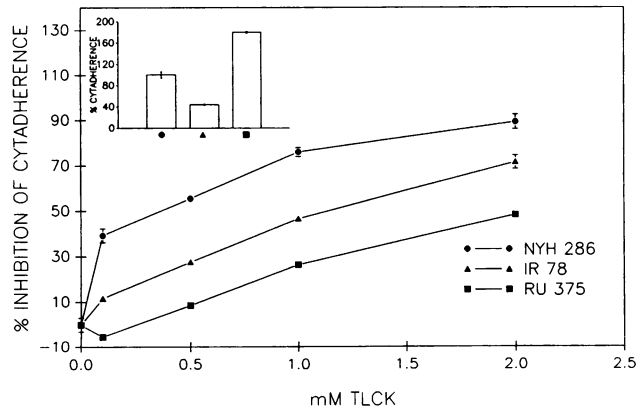

FIG. 5. TLCK cytadherence inhibition of *T. vaginalis* isolates NYH 286 (●), IR 78 (▲), and RU 375 (■). Different isolates of trichomonads were pretreated with TLCK in TYM medium, and parasite cytadherence levels were measured using a 96-well assay. Individual levels of attachment for each isolate in the absence of inhibitor were normalized to represent 100% cytadherence. Insert shows relative attachment levels for the respective isolates.

Attention by us and others has already focused on the specific manner by which the pathogenic human trichomonads parasitize epithelial cells (5, 9, 18, 20). We took advantage of the reported phenotype distinctions that exist among some *T. vaginalis* isolates (7), and trichomonad surface proteins which recognize and bind to HeLa (6) and vaginal epithelial (4) cells in a receptor-ligand fashion were identified. A ligand assay employing detergent extracts of radiolabeled trichomonads and glutaraldehyde-fixed cells previously identified putative trichomonad adhesins (4, 6), and these results suggested that the putative adhesin proteins were indeed sensitive to proteinases present in the detergent extract (15, 22). During the course of this study, we also observed the degradation of a prominent *T. vaginalis* surface immunogen (10) by trichomonad proteinases (8), which was occurring during growth and multiplication of the organisms (8). Indeed, degraded immunogen fragments were readily detected in culture supernatants, suggesting that the proteolysis was occurring on the parasite surface (8). The possibility of *T. vaginalis* proteinase activity on the trichomonad surface also degrading adhesin proteins, like that demonstrated for the immunogen (8), prompted us to examine whether cytadherence of epithelial cells by *T. vaginalis* was affected by known inhibitors of trichomonad cysteine proteinases (15, 22). In experiments in which inhibitors such as TLCK were added to the parasite-host cell mixture, we were surprised to see almost total abolishment of the ability of *T. vaginalis* to attach to HeLa or vaginal epithelial cells (Table 1).

Only pretreatment of live parasites, but not epithelial cells, with cysteine proteinase inhibitors caused reduction in trichomonal cytadherence (Table 1 and Fig. 3). Under no circumstances was the concentration of inhibitor detrimental

to the biosynthetic capabilities and energy metabolism of the organisms, as both motility and protein synthesis were unaffected. This was true for all isolates examined in this study. Interestingly, trichomonads pretreated with TLCK were able to regenerate the ability to cytadhere (Fig. 4), which is consistent with earlier data from our laboratory (5, 6). It was previously shown that organisms which were trypsinized to release surface adhesins were capable of synthesis and reexpression of these proteins after a certain time (5, 6).

Our results do not allow us to determine the number of cysteine proteinases that may be involved in the observations reported here. Approximately 11 proteinases of *T. vaginalis* have been identified by substrate digestion gel electrophoresis (22), and characterization was performed on the basis of proteinase activity in the presence of known inhibitors (15, 22). That leupeptin is a more effective inhibitor than TLCK of trichomonal targeting of host cell surfaces is noteworthy, however, as all cysteine proteinases of *T. vaginalis* are not equally inhibited by leupeptin and TLCK (15, 22). Therefore, the screening of multiple inhibitors may be important for future identification of the proteinases involved in cytadherence.

The exact function or the precise step of trichomonad proteinase involvement during parasite recognition of and binding to epithelial-cell surfaces is not known. It is conceivable that unmasking of adhesins by proteinases residing on the parasite surfaces is required for host cell recognition and binding. It is equally possible that adhesins on trichomonad membranes exist as precursor forms which must be activated by specific proteinase digestion. Modification of target cell receptors represents yet another mechanism by which the trichomonad proteinases may be involved. However, our results show that treatment of host cells with papain or parasite detergent extracts containing proteinases do not abrogate *T. vaginalis* cytadherence (Table 2) or reverse the inhibition of cell attachment of TLCK-treated organisms (data not shown). On the other hand, papain or trichomonad extract incubated with TLCK-treated organisms resulted in the recovery of previous host cell parasitism levels (Fig. 1). These data reinforce the idea that modifications which are mediated by trichomonad proteinases occur on the *T. vaginalis* surface. These surface alterations appear necessary for successful and efficient host cell attachment. Clearly, dissecting the steps of proteinase action in this host-parasite interaction represents an important area of investigation.

Cytopathogenicity of host cells in monolayer cultures by *T. vaginalis* has been shown to be contact dependent (9, 20), and any inhibition of cytadherence might be expected to concomitantly diminish cytotoxicity. Indeed, it was possible to greatly reduce cellular destruction with the same molar concentrations of cysteine proteinase inhibitors used in the cytadherence assays. The fact that TLCK protected host cells more effectively than did leupeptin from trichomonad-mediated host cell killing (Table 3) may be helpful in functional discrimination among the many *T. vaginalis* proteinases (22) to determine more precisely those involved in cytadherence or cytolytic events. The reduction in *T. vaginalis* parasitism of epithelial cells by cysteine proteinase inhibitors was observed in all isolates examined to date (5). This is not surprising, since all isolates have been found to adhere to cells, albeit to different levels (4, 5, 7). The fact that all isolates are affected by the same inhibitor in a key step during infection is an important observation in view of the potential for pharmacologic intervention (14). Our results point toward approaches other than identification of molecules for vaccine development as reagents for control of this sexually transmitted infectious agent.

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