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Specific Parasitism of Purified Vaginal Epithelial Cells by *Trichomonas vaginalis*

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Human vaginal epithelial cells (VECs) from vaginal swabs obtained from normal women or from patients with trichomoniasis were purified, and VEC parasitism by *Trichomonas vaginalis* was examined. Trichomonads bound equally well to live or dead VECs, and up to 20% of VECs were parasitized. Trichomonal cytadherence of human VECs was time, temperature, and pH dependent. Saturation binding levels of live trichomonads to VECs gave ~2 organisms adherent to parasitized VEC. No differences in cytadherence levels were detected by different isolates to VECs from the same patient compared with adherenee to VECs from normal individuals. Trypsinized, live *T. vaginalis* organisms failed to recognize VECs. A ligand assay identified four adhesin candidates, and only organisms without a prominent immunogen on the surface (negative phenotype) cytadhered to VECs and synthesized the adhesins, confirming the results of a recently published report by us on adherence to HeLa cell monolayers (J. F. Alderete and G. E. Garza, Infect. Immun. 56:28–33, 1988). These data show the ability of *T. vaginalis* to parasitize human vaginal epithelial cells in a specific receptor-ligand manner.

Trichomonas vaginalis is a sexually transmitted urogenital mucosal parasite (13). Recent reports have shown the highly specific nature of the recognition of host cells by this protozoan (3, 5, 12), and at least four surface proteins have been implicated as candidates for adhesin molecules mediating host parasitism (5). In these earlier studies host cells in monolayer cultures such as HeLa, HEp-2 (3, 5), and CHO (12) cells were employed to dissect the basic features of the cell-trichomonad associations. We attempted to perform experiments examining the property of *T. vaginalis* cytadherence by using freshly derived and purified vaginal epithelial cells from normal women and patients with trichomoniasis. In this way we might relate our in vitro observations with HeLa cells to the in vivo situation (3, 5) and also begin to better understand the events of parasitism of humans and disease pathogenesis. In this report we describe a procedure for purifying vaginal epithelial cells (VECs) away from contaminating microbial flora, leukocytes, and other cell debris. Data are presented which show the specific nature of *T. vaginalis* parasitism of human vaginal epithelial cells. The properties of trichomonal VEC parasitism appear similar if not identical to those recently described for HeLa and HEp-2 epithelial cells in monolayer culture (3, 5).

MATERIALS AND METHODS

Parasites and patient materials. *T. vaginalis* NYH286, RU375, JH31A, and DD3 are long-term grown cultures which have been used in several recent studies (1–8, 15, 21). Isolates K-1 and J-1 were fresh isolates obtained from patients with trichomoniasis in Bratislava, Czechoslovakia. Approximately 20 other fresh isolates were axenized (2) during the course of this investigation for analyses as described in this report. Trichomonads were grown in Diamond Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract-maltose (TYM) medium (10) without agar with heat-inactivated fetal calf serum or horse serum as a supplement.

Parasites and VECs were from patients attending an outpatient clinic at the Institute of Parasitology, Comenius University, Bratislava (2). As before (2), the clinical presentation of patients with trichomoniasis ranged from asymptomatic carriers to symptomatic with inflammation and discharge.

Purification of human VECs. Materials from vaginal swabs were either combined or processed singly, and the volume was adjusted with phosphate-buffered saline (PBS) so that the density of VECs was 2×10^6 per ml. Volumes of 10 ml were then passed through 30-mm-diameter, 60-μm-pore-size nylon filters (Henry Simon Ltd., Cheshire, England) to remove large clumps of tissue debris and mucus. The filtrate containing most VECs was then further clarified of bacteria, inflammatory cells, and smaller tissue fragments by passage through a 30-mm-diameter, 8-μm-pore-size nylon filter type PA-8/3/SRC NYBOLT (Schweizerische Seidengazefabrik AG, Zurich, Switzerland). VECs remained trapped on the 8-μm-pore-size filter and were suspended with PBS by gentle trituration. VECs were washed twice in PBS at 400 × *g* for 5 min before use in experiments as described below. In later experiments VECs were fixed at 4°C with glutaraldehyde (2.5% final concentration) prepared in PBS and stored at 4°C. For experiments with VECs from patients with trichomoniasis, the purification procedure was carried out at 4°C to allow for dissociation of attached parasites (3).

The final cell suspension was determined microscopically to be comprised of ~95% VECs and ~5% parabasal cells. The proportion of viable cells before and after purification ranged from 5 to 20% as determined by trypan blue staining and was similar for both cell types. For cytadherence assays, VECs were suspended to 2×10^5 per ml in TYM medium and incubated at 37°C for 15 min before the addition of *T. vaginalis*.

Cytadherence assay. Optimization of conditions for mea-

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suring VEC parasitism by *T. vaginalis* was performed in microfuge tubes containing 40,000 VECs and increasing numbers of trichomonads. The final reaction volume was 400 μl. After mixing of purified host cells and parasites, the microfuge tubes were placed in a 37°C water bath and gently shaken to keep VECs and organisms in suspension. At various time points, wet mounts were made, and the percentage of VECs with adherent trichomonads and number of parasites per cell were determined by counting no less than 50 and up to 100 total VECs in various fields. In all experiments, *T. vaginalis* were always pelleted by centrifugation and suspended in fresh TYM medium without serum just before use in cytadherence assays; however, it should be noted that under the experimental conditions employed identical results were obtained with VECs and parasites placed in PBS before coincubations. All experiments were performed no less than three times.

Ligand assay. The identification of trichomonad surface proteins which bind to VECs was performed as recently described (5).

RESULTS

Optimization and properties of human VEC parasitism by *T. vaginalis*. Human VECs were pooled from several normal individuals for initial experiments. When 4×10^4 pooled VECs were incubated with 2×10^5 live, motile trichomonads, a time-dependent attachment of VEC by trichomonads was observed (Fig. 1A). Fifteen minutes was required for maximal binding of organisms to human cells under these conditions.

We next wanted to determine whether VECs fixed with glutaraldehyde would give adherence results similar to those with unfixed VECs, since this would allow for long-term storage of VECs as well as the possible use of chemically stabilized host cells for identifying trichomonad proteins mediating cytadherence (5). Figure 1B shows the time-dependent trichomonal parasitism of pooled glutaraldehyde-fixed VECs; the results are consistent with those obtained with unfixed VECs (Fig. 1A).

Figure 2 presents data illustrating the ability to obtain saturation binding by using increasing parasite numbers incubated with 4×10^4 VECs. A ratio of 2×10^5 parasites per 4×10^4 VECs yielded saturation binding by 15 min at 37°C. Under these experimental conditions, no more than two trichomonads per individual VEC were seen. In 50 experiments performed with parasite/VEC ratios of 5:1, the percentage of VECs with adherent *T. vaginalis* ranged from 15 to 40%. This range in trichomonal cytadherence was evident when either the live or dead VECs were examined individually in the preparation of VECs used; indeed, in all of these experiments no discrimination in parasitism by trichomonads of either live or dead VECs was readily apparent.

VEC cytadherence was greatly diminished with trypsin treatment of trichomonads under previously established conditions involving *T. vaginalis* and HeLa cells (3). An 80% reduction in cytadherence levels was achieved by trypsinization of trichomonads (3) before the addition to VECs. No parasitism of VECs was detected by *T. vaginalis* at 4°C, whereas only 40 to 60% of maximal levels was observed at 22°C. Furthermore, also as recently reported (3), attachment of trichomonads to VECs was optimal between pHs 4.0 and 8.0, and maximal levels were found at pH 6.0. All of these results are very much in agreement with earlier published data, which indicate a highly specific receptor-ligand nature of trichomonal cytadherence of host cells (3, 5, 12).

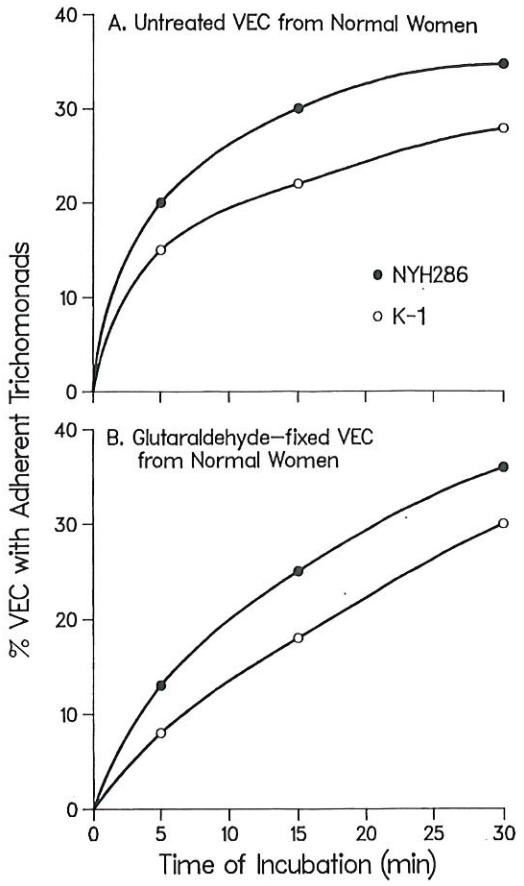

FIG. 1. Representative experiment showing time-dependent parasitism by *T. vaginalis* NYH286 and K-1 of purified VECs pooled from five normal women. VECs from individual vaginal swabs were suspended in PBS, pooled, and purified as described in Materials and Methods. VECs were either used immediately after isolation (A) or chemically stabilized with glutaraldehyde (B) and stored at 4°C overnight before washing and incubation with trichomonads. For this experiments, 4×10^4 VECs were suspended in 200 μl of TYM medium and placed at 37°C for at least 15 min before the addition of an equal volume of TYM medium containing 2×10^5 parasites also preequilibrated to 37°C. No more than two trichomonads per cell were seen for the 15- and 30-min time points.

Finally, cytadherence levels of individual preparations of VECs from each of 10 normal women were compared with those in the pooled preparation of VECs used for experiments for which results are shown in Fig. 1 and 2. Also, VECs of normal women ranging from 15 to 60 years of age were evaluated for any variations in levels of cytadherence. Data not shown indicated no variability among VECs of different women and of different age groups for susceptibility to parasitism by *T. vaginalis*.

Similar parasitism levels among VECs from normal or infected women and lack of relationships between cytadherence levels and symptomology. We then compared pooled VECs from normal women with VECs from individual patients for cytadherence by *T. vaginalis* organisms obtained from that same patient. Figure 3 shows representative results of trichomonad cytadherence to VECs from seven patients. Similar VEC parasitism was seen when the *T. vaginalis* isolates from individual patients were incubated with homologous versus heterologous VECs. All reactions were repeated no less than three times; no clear-cut distinc-

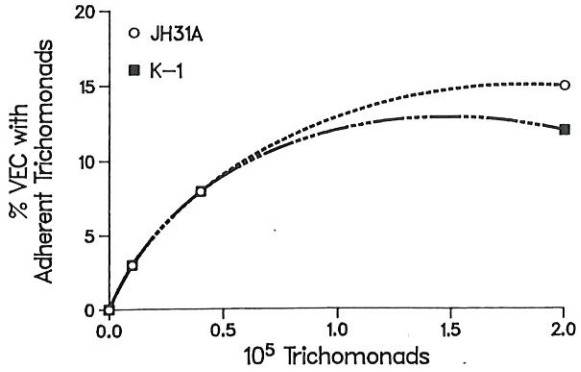

FIG. 2. Representative experiment showing saturation cytadherence kinetics of *T. vaginalis* JH31A and K-1 to VECs. The procedure was as described in the legend to Fig. 1 except that increasing numbers of organisms were used. Maximal levels of *T. vaginalis* parasitism to VECs at 15 min were achieved with 2×10^5 trichomonads, which yielded one to two organisms attached per VEC.

TABLE 1. Representative data from 8 of 60 patients showing no relationship between symptomology and levels of VEC parasitism by *T. vaginalis*

Patient no.	Symptomology		Menstrual cycle time (days)	% of VECs parasitized[a]
	Inflammation	Discharge		
1	+	+	26	8 (2)
2	+	+	13	14 (2)
3	NA[b]	NA	Menstruating	16 (2)
4	NA	NA	Menstruating	6 (2)
5	−	+	3	12 (2)
6	+	+	27	15 (2)
7	−	+	8	8 (2)
8	+	+	12	15 (5)

[a] Percentage of VECs with adherent trichomonads in wet-mount preparations as a measure of the extent of parasitism. VECs were isolated from patients by cotton-tipped swabs and purified at 4°C as described in Materials and Methods. Numbers within parentheses indicate organisms observed per VEC.

[b] NA, Not applicable.

tions were evident between the ability of respective isolates to parasitize VECs from either normal women or patients.

An attempt was made to determine whether any relationship could be established between cytadherence levels of VECs from individual patients and their symptomology. Of interest was also whether VECs collected at different times during the menstrual cycle resulted in different levels of VEC parasitism. No obvious associations were evident between these variables and levels of trichomonal binding to VECs (Table 1).

Relationship between trichomonal immunogen expression and VEC adherence. In earlier studies, isolates of *T. vaginalis* were defined on the basis of surface expression of a repertoire of immunogens as determined by immunofluorescence reactions with a monoclonal antibody (C20A3) or sera from patients with trichomoniasis (1, 6, 8, 9). Parasites without detectable surface immunogens (negative phenotype) (5, 6) but not positive-phenotype trichomonads were capable of HeLa cell parasitism (1, 5, 6). Negative-phenotype organisms synthesized at least four HeLa cell-binding proteins (5). Therefore, it was important to test whether organisms expressing the C20A3-reactive immunogen failed to recognize and attach to VECs as was previously demonstrated with HeLa cells (5). A subpopulation of positive-phenotype trichomonads derived from the heterogeneous parent NYH286 isolate failed to bind to VECs (Fig. 4). Cytadherence to VECs was always observed with populations of parasites containing negative-phenotype organisms.

Identification of VEC-binding proteins of *T. vaginalis*. Recently, a ligand assay was employed to identify trichomonad adhesin proteins which recognize surfaces of fixed HeLa cells (5). It appeared possible to perform the ligand assay by utilizing the glutaraldehyde-fixed VECs used earlier for cytadherence studies (Fig. 1B). Detergent extracts of extrinsically labeled *T. vaginalis* were incubated with fixed VECs and HeLa cells. The same four adhesin candidates recently identified using HeLa cells (5) were also detected with

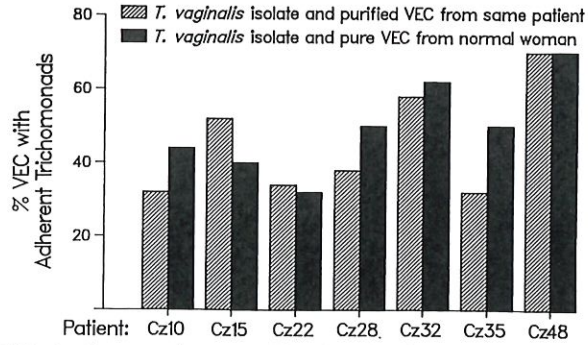

FIG. 3. Comparative parasitism levels of seven individual *T. vaginalis* isolates with purified VECs from the same patient (▨) and with pooled VECs from normal women (■). For these experiments the VECs from patients and normal women were obtained at the same time and fixed with glutaraldehyde for use 2 to 3 days later after axenization of trichomonads. To ensure no dramatic alteration of the VECs due to fixation, freshly harvested and purified VECs were also examined for cytadherence with *T. vaginalis* NYH286 and K-1; results were always consistent with those presented in Fig. 1A for the 15-min time point.

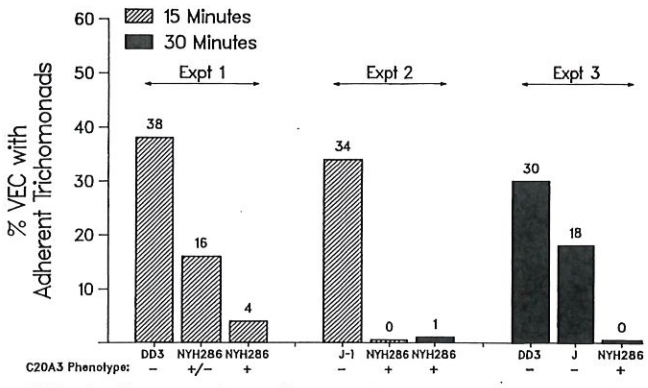

FIG. 4. Absence of cytadherence by *T. vaginalis* expressing immunogens. Monoclonal antibody C20A3 recognized an immunogen which undergoes phenotypic variation (6), and organisms expressing this and other surface immunogens are deficient in their ability to attach to HeLa cells (1, 5). Live trichomonads of several isolates were evaluated by indirect immunofluorescence with C20A3 (1, 5, 6, 7). *T. vaginalis* isolates were either heterogeneous (+/−), comprised of both fluorescent and nonfluorescent trichomonads, or were homogeneous nonfluorescent (−). Fluorescent trichomonads (+) were derived from the NYH286 parent by fluorescence-activated cell sorting (6) and used simultaneously in duplicate experiments. Numbers above bar graphs indicate percentages of VECs that were parasitized.

VECs. Additional supporting evidence that these VEC-binding proteins might be putative adhesins was provided by demonstrating that antiserum to the HeLa cell-binding proteins (5) also inhibited the extent of trichomonal cytadherence. In this case, the addition of 100 μg of immunoglobulin G antibody per ml to individual adhesins gave ~60% decreased *T. vaginalis* bound to VEC with parasite/VEC ratios of 2:1 (Fig. 2). These data show the antigenic cross-reactivity between trichomonad surface proteins that recognize HeLa cells (3, 5) and VECs.

DISCUSSION

Prior work has utilized a variety of cells in tissue cultures, including cells of cervical or vaginal origin, as models (3, 5, 7, 12, 19) to study the recognition and binding of *T. vaginalis* organisms to mammalian cells. In our laboratory we have been involved in characterizing trichomonal cytadherence mechanisms with HeLa cells (3, 5). More recently, for example, specific trichomonad surface proteins were implicated as the adhesins (5) mediating the receptor-ligand type reactions between HeLa cells and parasites (3, 12). Because no animal model of human trichomoniasis exists, it has been often difficult to relate observations made with in vitro systems to the in vivo situation. We have attempted to determine whether results generated with HeLa cells were indeed relevant to *T. vaginalis* cytadherence of human VECs. Data presented herein show that the pathogenic human trichomonads readily recognize and attach to VECs in a fashion similar if not identical to that described with HeLa cells (5). These and previous data (5) strengthen the argument favoring the biofunctionality of at least four trichomonad surface proteins in human parasitism events. In addition, we recently showed that only parasites without surface immunogens as defined by the absence of antibody reactivity (1, 5, 6) expressed the adhesins; this was also demonstrated for VECs (Fig. 4). Therefore, this work shows that alternating orchestrated differential expression among repertoires of surface proteins is also relevant to VECs (1a). These data illustrate the validity of using cells in monolayer culture as models for studying cytadherence properties of *T. vaginalis*.

Seldom were fewer than two parasites seen adherent to individual VECs with a ratio of 5 trichomonads added per VEC. This result is identical to that enumerated earlier with HeLa cells with similar multiplicities of infection (3). It was possible to increase the overall percentage of parasitized VECs up to levels approaching 80% when ~50:1 ratios of trichomonads to VECs were used, suggesting that most, if not all, VECs are equally susceptible to recognition and binding by live parasites. If, in fact, all VECs of women are equally susceptible to infection with *T. vaginalis*, then relative resistance to infection may be due to other variables such as availability of host macromolecules required for growth and multiplication of trichomonads (15–18) or to host immunologic considerations (20). Although antibody to *T. vaginalis* has been detected in vaginal fluids (20), nothing is known regarding acquired immunity or whether mucosal antibody can indeed recognize and eliminate live trichomonads.

The fact that *T. vaginalis* organisms may parasitize dead VECs to the same extent and effectiveness as live VECs indicates that de novo receptor synthesis by host cells is not prerequisite for trichomonal parasitism. Indeed, attachment of trichomonads to dead VECs possibly predominating on the surface of vaginal epithelium would ensure a successful infection, since metabolic integrity of host cells would not be required. Furthermore, if dead squamous VECs on the vaginal wall are parasitized during infection as has been reported by other investigators (14), then the contact-dependent killing of cells as has been described before (7, 12, 19) would not necessarily be responsible for the pathobiochemistry of the disease. Alternative host cells, therefore, would represent targets for any possible cytolytic factors produced by *T. vaginalis*.

No correlation was observed between several variables, including the extent of inflammation, fluid discharge, and menstrual cycle time of VEC isolation (Table 1) with cytadherence of *T. vaginalis*. However, all isolates which have been examined by us (1–9), including the fresh isolates used here for evaluating VEC parasitism, possess the adhesin proteins (5) (data not shown), parasitize VECs, and produce contact-dependent killing of HeLa cells (1–4, 6, 7). These data support the idea that based on this aspect of the host-parasite relationship, i.e. cytadherence, the broad spectrum of symptomology among patients with trichomoniasis is not due to different pathogenicity levels of trichomonads (11), since every isolate of *T. vaginalis* contains trichomonads capable of host infection and disease pathogenesis (1–9).

This absence of clear-cut correlations, therefore, serves to illustrate the complexity of the interrelationship between the human host and *T. vaginalis*. Furthermore, it emphasizes the need for continued research defining further the biology of this parasite along with the identification of precise virulence factors involved in disease pathogenesis.

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