[33] Analysis for Adhesins and Specific Cytoadhesion of *Trichomonas vaginalis*

By JOHN F. ALDERETE, ROSSANA ARROYO, and MICHAEL W. LEHKER

Introduction

Trichomonas vaginalis is a protozoan parasite that is one of the most common, clinically recognized sexually transmitted infectious agents. As with other mucosal pathogens, trichomonads must be capable of surmounting the mucus barrier and must cytoadhere to the squamous epithelial cells of the vaginal epithelium in order to overcome being expelled by the continuous fluid flow of the vagina. Indeed, in some patients, a significantly elevated vaginal discharge occurs during trichomoniasis. Is it possible, then, to determine whether cytoadhesion by the parasite, either to host cells in monolayer cultures as *in vitro* models or to freshly derived human vaginal epithelial cells, is specific, and, if so, can the molecules mediating host cell recognition and binding be identified?

What follows is a description of the assays developed and employed to establish the highly specific nature of *T. vaginalis* cytoadhesion and the identity of trichomonad surface proteins involved in this property. It is noteworthy that, prerequisite to the identification of important virulence factors, like adhesins, some degree of understanding is needed of the host factors of the vagina (or at the site of infection of a given microbial pathogen) that may provide environmental signals that regulate expression of the property being examined, in this case cytoadhesion and synthesis of adhesins.

Specificity and Nature of Cytoadhesion

Adhesion Assay with HeLa Cells on Glass Coverslips

HeLa epithelial cells (American Type Culture Collection, Rockville, MD) are maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin–streptomycin or gentamicin.[1] Cultures are kept in a 7% CO_2 atmosphere at 37°, and cells in monolayer cultures on round glass cov-

[1] J. F. Alderete and E. Pearlman, *Br. J. Vener. Dis.* **60,** 99 (1984).

erslips[1,2] are passaged and used for cytoadhesion assays. Cell suspensions are seeded at a density of 2.0×10^5 to 2.5×10^5 cells onto sterile, ethanol-washed glass coverslips (diameter 15 mm) placed in individual wells of 24-well Costar culture plates (Bellco Glass, Inc., Vineland, NJ). Cells are grown for 18 to 24 hr in DMEM containing 10% FBS, at which time the monolayers have approximately 4×10^5 cells and are over 90% confluent, determined by visualization via phase-contrast microscopy.

The parasites are cultivated in a complex medium consisting of Trypticase, yeast extract, and maltose (TYM) supplemented with 10% (v/v) heat-inactivated horse serum. Organisms are radiolabeled for 18 hr with 1–10 μCi/ml [^{3}H]thymidine.[1,2] Radiolabeled trichomonads grown in TYM–serum complex medium are washed three times in phosphate-buffered saline (PBS) and suspended to the desired densities in a medium mixture of 2 parts cell culture medium (DMEM) and 1 part trichomonal medium (TYM).[1,2] The DMEM–TYM medium mixture without serum is important because it is found to support both parasites and host cells in coincubation experiments at 37° in a 7% CO_2 atmosphere. Organisms and host cells are always equilibrated in the medium mixture at 37° prior to coincubation. Experiments involving different temperatures are done without a CO_2 atmosphere; however, the pH of the medium is monitored throughout the experimental period. The ratio of parasites to host cells (*P/C*) used for the experiments is optimized to be 5:1[1] such that, for the assay, 2×10^6 trichomonads in 1 ml of DMEM–TYM are added to the cell monolayer. After the time desired (maximum cytoadhesion for trichomonads occurs by 30 min), the glass coverslips are washed by immersion no less than 10 times in a glass beaker containing sterile PBS prewarmed to 37°. The coverslips are then air-dried and placed inside vials with scintillation cocktail for measurement of adherent radioactivity.

Cytoadhesion Assay in Microtiter Wells

In the modified adhesion assay using microtiter well plates, 4×10^4 HeLa cells are seeded in each well and incubated overnight (final cell numbers in each well averaged 8×10^4). To confluent monolayers is added 100 μl containing a 4×10^5 [^{3}H]thymidine-labeled parasites (*P/C* ratio of 5) suspended in a 2:1 mixture of DMEM–TYM medium, as has been described.[1,2] After incubation for 30 min (or the time desired) at 37° in a 7% CO_2 atmosphere, unbound parasites are removed, and wells with adherent trichomonads are washed three times with temperature-equilibrated DMEM. The microtiter well plates are air-dried, and radioactivity is measured by scintillation spectroscopy.

[2] J. F. Alderete and G. E. Garza, *Infect. Immun.* **50,** 701 (1985).

All experiments are performed in quadruplicate, and one sample is always visualized by phase-contrast microscopy to ensure that the results from radioactivity measurements represent *bona fide* radiolabeled organisms adherent to host cells. Parasite viability and motility are always monitored throughout the experiment.

Cytoadhesion Assay Using Vaginal Epithelial Cells

Using cells from the site of infection affirms the results derived from using HeLa cells in monolayer cultures as the *in vitro* model. The preparation of vaginal epithelial cells (VECs) is done through sequential filtration to remove debris and other host cells and to enrich for VECs.[3] Swabs from the vagina of control, uninfected women as well as of patients with trichomoniasis are immersed singly or combined in a 10-ml volume of PBS for processing. The VEC preparation is then passed through a 60-μm pore-size nylon filter, which removes the very large debris while allowing the VECs, bacteria, and other small diameter debris to pass. The flow-through is then filtered with an 8-μm pore-size nylon filter. The VECs are retained in this filter and are then gently suspended and washed twice in PBS. Once it became clear that glutaraldehyde fixation of VECs still allows for trichomonal parasitism under the same conditions, as established with HeLa cells in monolayer cultures,[3,4] VECs are routinely fixed at 4° with glutaraldehyde at a 2.5% (v/v) final concentration prepared in PBS and stored at 4°.[3] Cells from patients used for cytoadhesion experiments are purified at 4°, which allows for detachment of the adherent parasites from the host cells, and trichomonads are then removed from VECs during the filtrations.

The results from employment of the adhesion assays have been reported in a series of articles that, collectively, show the highly specific nature of *T. vaginalis* recognition and binding to host cells.[1–7] Furthermore, the cytoadhesion findings reinforce the idea that trichomonad surface proteins are mediators of specific host cell attachment.

Identification of Adhesins and Other Considerations

Ligand Assay

A method has been developed by which specific microbial surface proteins involved in cytoadhesion are identified. A detergent extract of radiola-

[3] J. F. Alderete, P. Demes, A. Gombosova, M. Valent, A. Janoska, J. Stefanovic, and R. Arroyo, *Infect. Immun.* **56,** 2258 (1988).
[4] J. F. Alderete and G. E. Garza, *Infect. Immun.* **56,** 28 (1988).
[5] R. Arroyo and J. F. Alderete, *Infect. Immun.* **57,** 2991 (1989).
[6] R. Arroyo, J. Engbring, and J. F. Alderete, *Mol. Microbiol.* **6,** 853 (1992).
[7] M. Lehker, R. Arroyo, and J. F. Alderete, *J. Exp. Med.* **174,** 311 (1991).

beled microorganisms is incubated with chemically stabilized host cells.[8,9] The assay has been employed successfully to identify the fibronectin-binding proteins of *Treponema pallidum*[8] and the adhesin of *Mycoplasma pneumoniae*.[9] The earlier work had showed that fixation of host cells still preserved the adhesin-binding receptors and, equally important, that detergents could be used which allowed for functionality of the receptor-binding epitopes of the adhesins. Clearly, the specificity by which *T. vaginalis* organisms recognize and bind to HeLa cells and VECs and especially the implication that surface proteins are mediating cytoadhesion prompted the use of the ligand assay for identifying the putative trichomonad adhesin proteins.

Parasites are radiolabeled intrinsically with [^{35}S]methionine or extrinsically through radioiodination using standard protocols.[4,6,7] After three washes in PBS, approximately 2×10^7 trichomonads are then incubated for 10 min at 4° in 0.5 ml of NET buffer [50 m*M* Tris-HCl, pH 7.4, 15 m*M* NaCl, and 5 m*M* ethylenediaminetetraacetic acid (EDTA)] containing 1 m*M* *p*-tosyl-L-lysine chloromethyl ketone (TLCK; a cysteine proteinase inhibitor).[5,6] Parasites are then solubilized by the addition of 1% (w/v) deoxycholate and trituration. To the extract is added 0.5 ml of TDSET buffer [10 m*M* Tris-HCl, pH 7.8, 0.2% (w/v) deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 10 m*M* EDTA, and 1% (v/v) Triton X-100] containing 1 m*M* TLCK, and the mixture is triturated gently.The detergent extract is then centrifuged at 100,000 *g* through a 10% (w/v) sucrose–phenol red cushion to remove any insoluble debris, and the supernatant (~1 ml) is added to a pellet of 10^6 HeLa cells or 10^6 VECs previously fixed with glutaraldehyde.[4,6] After an 18-hr incubation at 4°, the fixed host cells are then centrifuged at 600 *g* and washed three times with TDSET buffer containing 1 m*M* TLCK. To elute any specifically, avidly bound proteins, fixed host cells are finally boiled in electrophoresis dissolving buffer[10] for 3 min, then microcentrifuged to remove host cells, and released, radiolabeled trichomonad proteins are subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using 7.5 or 10% (w/v) separating acrylamide gels.[6,7,11,12]

For chemical stabilization of host cells used in the ligand assay, cells are washed in PBS and suspended to a density of 5×10^6 cells/ml in PBS. Then the cells are fixed with 2.5% (v/v) glutaraldehyde in PBS for 30 min to 1 hr and gently stirred at 4°. Cells are monitored continuously and triturated to avoid clumping. Fixed cells are then washed extensively at least five times with ice-cold PBS and incubated with 0.2 *M* glycine in PBS

[8] J. B. Baseman and E. C. Hayes, *J. Exp. Med.* **151,** 573 (1980).
[9] D. C. Krause and J. B. Baseman, *Infect. Immun.* **37,** 382 (1982).
[10] U. K. Laemmli, *Nature (London)* **227,** 680 (1970).
[11] J. F. Alderete, *Infect. Immun.* **39,** 1041 (1983).
[12] J. F. Alderete, *Infect. Immun.* **40,** 284 (1983)

for at least 1 hr at 37°. The cells are then washed five times with TDSET and suspended to the desired density in individual microcentrifuge tubes for immediate use. Fixed cells are also kept at 4° for several days prior to use in the ligand assay.

Other Considerations

At least three considerations were important in the successful employment of the ligand assay for identification of putative adhesins. First, the ligand assay was employed using extracts of radiolabeled *T. vaginalis* organisms solubilized with different detergents. Zwitterionic detergents were initially favored because of efficient solubilization of trichomonad membrane protein immunogens, as evidenced by the successful utilization of radioimmunoprecipitation assays.[11-14] Extracts of *T. vaginalis* made with Zwittergent 3–12 detergent and several other detergents did not allow for detection of any trichomonad proteins specifically and avidly bound onto host cells. Only experiments performed with deoxycholate gave reproducible binding of four proteins which could be resolved by SDS–PAGE and autoradiography.[4] The detergent and conditions required for efficient solubilization of the adhesins with preservation of the receptor-recognition epitopes have since been modified, as described above, but still incorporate the essential feature of exposure of trichomonads to deoxycholate before addition of TDSET buffer.

Next, early attempts to identify the adhesins resulted in autoradiograms with very diffuse protein bands in four gel areas.[4] The diffuse nature of the proteins was subsequently determined to be due to trichomonal proteinases released during detergent solubilization. Although the early ligand assays incorporated inhibitors of proteinases, the inhibitors were without effect owing to the complexity and specificity of the proteinases of *T. vaginalis*.[15,16] Inclusion of the correct inhibitors during the detergent solubilization allowed for resolution of four proteins, as has been reported.[7] The analysis of the trichomonad cysteine proteinases[16] then prompted further experiments which showed the exquisitely sensitive nature of the adhesins to the proteinases.[6]

Finally, evaluation of fresh *T. vaginalis* isolates, for the cytoadhesion and ligand assays, as compared to laboratory strains that were being examined, immediately revealed dramatic differences in the levels of cytoadhesion

[13] J. F. Alderete, L. Suprun-Brown, L. Kasmala, J. Smith, and M. Spence, *Infect. Immun.* **49,** 463 (1985).
[14] J. F. Alderete, L. Suprun-Brown, and L. Kasmala, *Infect. Immun.* **52,** 70 (1986).
[15] K. A. Neale and J. F. Alderete, *Infect. Immun.* **58,** 157 (1990).
[16] G. H. Coombs, D. T. Hart, and J. Capaldo, *Parasitology* **86,** 1 (1983).

(Table I).[6,7] All fresh isolates yielded higher overall levels of cytoadhesion. The range of percent diferences in cytoadhesion between organisms grown in high- versus low-iron medium ranged from 14 to 67%. Some long-term grown isolates, like IR 78 and JH 31A, were relatively unresponsive to iron supplementing the medium. Clearly, isolates passaged *in vitro* for extended periods were no longer representative of wild-type, infecting *T. vaginalis* parasites in the overall level of cytoadhesion.

Elevated levels of cytoadhesion (Table I) and upregulation of adhesin synthesis (Fig. 1) occurred under iron-replete medium conditions.[7] All isolates seemed capable of responding to iron added to the medium. Four trichomonad proteins designated AP65, AP51, AP33, and AP23 were iden-

TABLE I
EXAMINATION OF *Trichomonas vaginalis* ISOLATES GROWN IN HIGH- AND LOW-IRON MEDIUM FOR LEVELS OF CYTOADHESION

Isolate[a]	Relative adhesion value[b]		
	High-iron	Low-iron	Reduction (%)
Fresh			
T038	4.96 ± 0.39	2.08 ± 0.23	58.0
T048	6.53 ± 0.84	2.16 ± 0.32	66.9
T023	2.31 ± 0.21	1.58 ± 0.17	31.6
T036	2.75 ± 0.31	1.82 ± 0.16	33.8
Long-term grown			
NYH 286	1.19 ± 0.14	0.58 ± 0.04	51.0
T005	1.46 ± 0.21	0.72 ± 0.12	50.6
T003	1.52 ± 0.14	0.96 ± 0.06	37.0
RU 375	1.50 ± 0.11	1.10 ± 0.09	26.0
IR 78	0.73 ± 0.08	0.60 ± 0.12	14.2
JH 31A	0.95 ± 0.12	0.78 ± 0.15	17.8

[a] Isolates grown for more than 30 days *in vitro* were considered long-term grown. Fresh clinical isolates were grown for less than 30 days *in vitro*.[6,7]

[b] *Trichomonas vaginalis* organisms were grown in high- or low-iron medium as described[7] in the presence of 2 μCi/ml [^{3}H]thymidine. At the late exponential growth phase, which occurred between 24 and 48 hr of growth, parasites were washed twice and added to confluent HeLa cell monolayers in microtiter wells, as described in the text.[7] Nonadherent trichomonads were removed by washing and associated radioactivity of the wells counted by scintillation spectroscopy. Adhesion of NYH 286 grown in normal complex growth medium was set as 1 for comparative purposes, as has been done before,[6,7] to show the greater levels of cytoadhesion by fresh isolates. Reproduced from the *Journal of Experimental Medicine*, 1991, *174*, 311–318, by copyright permission of the Rockefeller University Press.

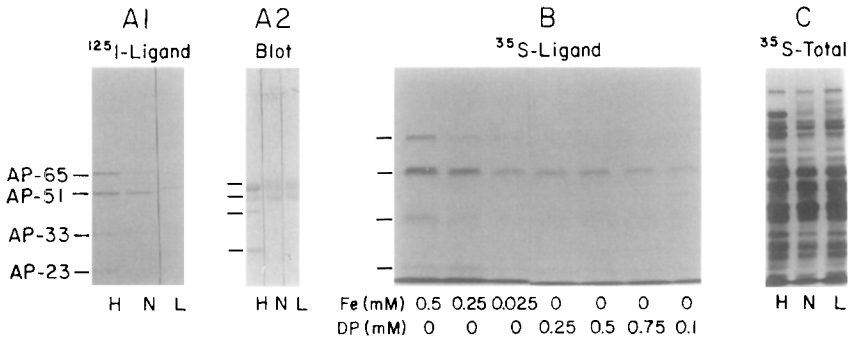

FIG. 1. Iron modulation of surface expression and synthesis of the four trichomonad adhesins.[7] *Trichomonas vaginalis* T048 cells were grown in high- (H) or low- (L) iron medium or in normal growth medium (N) as detailed.[7] Parasites were then used for a ligand assay to identify the adhesins as described in the text,[7] and acrylamide gels were analyzed by autoradiography using ^{125}I surface-labeled parasites (A1) and by fluorography using [^{35}S]methionine-labeled trichomonads (B) grown in TYM–serum medium at different iron concentrations. Duplicate gels of the experiment performed in (A1) were also blotted and probed using a pooled preparation of monospecific antiadhesin serum (A2). The fluorogram of total proteins of parasites grown in high-, normal-, and low-iron medium is shown in (C). Reproduced from the *Journal of Experimental Medicine,* 1991, **174,** 311–318, by copyright permission of the Rockefeller University Press.

tified as the trichomonad adhesins,[4,6,7] as evidenced by the autoradiographic profiles obtained from a ligand assay in which a detergent extract of surface-labeled parasites was incubated with fixed HeLa cells (Fig. 1, A1).[7] The four iodinated adhesins were present in larger amounts when the ligand assay was performed with organisms grown in a high-iron medium (lane H) when compared to parasites grown in either a normal growth medium (lane N) or a low-iron medium (lane L). Visualization of iodinated adhesins in the normal and low-iron grown trichomonads required a longer exposure of the gel to X-ray film for this ligand assay.

Immunoblot experiments were performed simultaneously on the adhesins from a ligand assay using antiadhesin antibodies known to inhibit cytoadhesion by *T. vaginalis*[6] (Fig. 1, A2). After their isolation from the fixed cells, electrophoresis, and blotting, adhesins on nitrocellulose were probed with a pooled preparation of each monospecific antiadhesin serum.[6] The four adhesins were readily detected in parasites grown in high-iron medium (Fig. 1, A2, lane H) but not in blots with adhesins from organisms grown in normal (lane N) or low-iron medium (lane L), confirming that the host cell-bound proteins that were upregulated with iron were the adhesins.

Iron influenced overall adhesin synthesis and not just surface expression of adhesins (Fig. 1B). Total synthesis of the adhesins was monitored in a ligand assay using detergent extracts of [^{35}S]methionine-labeled trichomonads. Synthesis of adhesins was dependent on the concentration of iron, and again organisms from a high-iron medium (lane H, Fig. 1B) had the most intense bands on fluorograms, indicative of the highest amounts of adhesins. Because fluorograms of total proteins were equally complex and comparable for both high-iron and low-iron medium grown organisms (Fig. 1C), the data show the regulation by iron of only the adhesins, and not all proteins. It was noteworthy that the routinely used complex TYM–serum medium employed for growing the parasites was deficient in amounts of iron needed for optimal adhesin synthesis.

Finally, when isolates and media were obtained from different laboratories, a large variation in levels of cytoadhesion and amounts of adhesins were obtained. In all cases, however, the same isolates grown in TYM–serum supplemented with iron exhibited enhanced synthesis of adhesins with correspondingly elevated levels of cytoadhesion. The significance of the latter results cannot be underscored, however, because the precise environmental cues responsible for expression of virulence factors must be understood in order for results among different laboratories to be reproducible.

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[34] Adhesion of Fungi

By KEVIN C. HAZEN and PATI M. GLEE

Introduction

Adhesion of a fungus to an appropriate surface precipitates a variety of biological events that could influence fungal survival and could ultimately jeopardize viability or integrity of the host surface. Following adhesion, fungal spores will elaborate vegetative growth structures or will produce structures that penetrate the host surface. Within the human host, conversion of mycelial elements or conidia to yeast cells and subsequent growth may result in destruction of host tissue and dissemination to vital organs.