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Host Plasma Proteins on the Surface of Pathogenic *Trichomonas vaginalis*

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Sodium dodecyl sulfate-gel electrophoresis and fluorography technology revealed that pathogenic *Trichomonas vaginalis* was able to acquire numerous loosely associated plasma proteins during incubation in normal human plasma. These proteins were readily removed by repeated washing of the parasite in phosphate-buffered saline. Plasma proteins avidly bound to the surface of *T. vaginalis* were also detected using a highly sensitive and specific agglutination assay with protein A-bearing *Staphylococcus aureus* pretreated with monospecific antiserum directed against individual human serum proteins. These avidly associated plasma proteins could not be removed by repeated washing in phosphate-buffered saline or by treatment of washed, live organisms with surface-modifying reagents such as trypsin and periodate. A combined radioimmunoprecipitation-gel electrophoresis-fluorography methodology indicated that parasite biosynthesis of hostlike macromolecules was not responsible for the observed agglutination and reinforced the idea of trichomonal acquisition of plasma components. Finally, incubation of trichomonads with plasma in various buffers at different pH values did not alter the agglutination patterns. These and other data suggest that specific membrane sites mediate trichomonal binding of host proteins. The biological significance of our results is discussed.

Trichomonal vaginitis, caused by *Trichomonas vaginalis*, is a sexually transmitted disease characterized by severe inflammation of the vagina, foul-smelling urogenital-vaginal discharge, severe discomfort, and tissue cytopathology (3, 16, 24). In men, the clinical picture of trichomonal urethritis remains controversial (24). Also, asymptomatic infection is known to occur in both men and women (16). The disease is accountable for significant economic loss in this country and represents the most frequently acquired protozoan infestation (32). Present knowledge of possible mechanisms of disease pathogenesis, however, is minimal. For example, the interactions between parasite and host tissue are not well defined (7, 10, 14, 15, 18, 25), and factors responsible for cellular cytopathogenicity have not been elucidated (10, 17). Biochemical characterization of the trichomonal surface has not been accomplished; therefore, no specific virulence determinants or immunogens have been identified. Equally important, information on host humoral or cellular immunological responses to infection with *T. vaginalis* remains deficient (13, 19, 27).

Parasite modulation, neutralization, or evasion (1, 3, 6, 8) of host immune mechanisms dictates that a more critical analysis of the interaction between the surface of pathogenic microorganisms and host macromolecules is necessary. Ultrastructural studies with fresh isolates of *T. vaginalis* have demonstrated a host-derived surface coat readily lost during in vitro cultivation (5, 30). Unfortunately, the nature of the material adsorbed onto the trichomonal surface remains undefined. The association of immunoglobulin with extensively washed or formaldehyde-fixed trichomonads has, however, been reported (12, 13).

Because host immune factors present in the vagina could be effectively neutralized by a surface coat of host macromolecules, these observations may be relevant to understanding some aspects of disease pathogenesis in this model system. Thus, studies which assess the binding of host tissue or serum components to pathogenic *T. vaginalis* are a prerequisite for our understanding of this host-parasite relationship and should contribute to our knowledge of the biology of this microorganism. In this report, we demonstrate the ability of pathogenic *T. vaginalis* to loosely and avidly bind numerous human plasma proteins and discuss the biological relevance of our observations.

MATERIALS AND METHODS

***T. vaginalis* organisms.** Trichomonal strains employed in this study were kindly provided by M.

Müller (29), Rockefeller University, New York, N.Y. (strains 286, 272, and IR78), or purchased from the American Type Culture Collection, Rockville, Md. (strain 30001). All were pathogenic based on lesion development after subcutaneous inoculation of 0.5 ml of medium containing 5×10^6 organisms into the hindquarters of 6- to 8-week-old BALB/c or C57BL/6J mice (16, 20).

Conditions for growth. Organisms were maintained in an air atmosphere at 37°C by daily passage in screw-capped tubes (15 by 120 mm) with Diamond (9) Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract-maltose (TYM) medium, pH 6.2, supplemented with 10% heat-inactivated (HI) horse serum (Kansas City Biologicals, Inc., Lenexa, Kans.). For outlined experiments, 10% HI, citrated human plasma (Medical Center Hospital, The University of Texas Health Science Center at San Antonio) was substituted for horse serum, and trichomonads were subcultured daily in this medium for several days before use. Organisms routinely grew to a density of 2.5×10^6 to 5.0×10^6 per ml as determined by an improved Neubauer counting chamber. Only motile parasites at the late log stage (24 h) of growth (see Fig. 1) were utilized.

Radiolabeling of trichomonads and binding of human plasma proteins. Trichomonal growth medium (100 ml) inoculated with 2.5×10^6 *T. vaginalis* organisms was radiolabeled with 5 μCi of [^{35}S]methionine (specific activity, 1,500 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml for 24 h at 37°C in an air atmosphere. Trichomonads were then dispensed into 10-ml volumes and centrifuged ($500 \times g$, 10 min), and pelleted organisms were washed twice with phosphate-buffered saline–maltose (PBS-M; 137 mM NaCl, 2.7 mM KCl, 4.6 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 15 mM maltose) before suspension in 1 ml of HI human plasma. After a 30-min incubation at 37°C and continuous gentle shaking, organisms were pelleted and the supernatant was discarded. Parasites were then processed for gel electrophoresis as described below. Duplicate tubes handled identically but without organisms were also included as controls for these studies and showed no nonspecific binding of plasma proteins to tube walls. Human plasma did not adversely affect parasite motility or decrease parasite density in our reaction vials. Additionally, no antitrichomonal activity based on trypan blue exclusion was detected.

Sodium dodecyl sulfate-gel electrophoresis and fluorography. Approximately 2.5×10^7 *T. vaginalis* organisms utilized for plasma protein-binding studies were centrifuged at $500 \times g$ for 10 min, and the pellets were suspended in cold PBS. Trichloroacetic acid was added to a 10% final concentration, and the suspension was incubated at 4°C for not less than 4 h. The acid-precipitated material was washed twice with cold PBS and dissolved in 200 μl of solubilizing buffer consisting of 60.5 mM Tris-hydrochloride (pH 6.8), 2% β-mercaptoethanol, 10% glycerol, and 2% sodium dodecyl sulfate. Bromophenol blue was included as the tracking dye, and samples were boiled for 3 min. Undissolved residues were removed by centrifugation before application of 30-μl volumes to individual wells. Stacking and separating slab gels consisted of 3 and 7.5% acrylamide, respectively (Bio-Rad Laboratories, Richmond, Calif.), and electrophoresis (26) was carried out (Protean Dual 16-cm Slab Cell Apparatus) (Bio-Rad Laboratories) with a constant current of 15 mA per gel. The current was increased to 30 mA per gel after penetration of the tracking dye into the separating gel. The total time for electrophoresis was 4 to 6 h for 12-cm slab gels. Gels were stained with Coomassie brilliant blue (Sigma Chemical Co., St. Louis, Mo.), destained, and processed for fluorography by the dimethyl sulfoxide-diphenyloxazole (Fisher Scientific Co., Houston, Tex.) procedure previously described (1, 4). X-ray film (X-Omat, AR5; Eastman Kodak Co., Rochester, N.Y.) was then exposed to the gels for various lengths of time at −70°C before development.

Antiserum reagents used in agglutination assays. Commercially available normal rabbit, goat, and horse sera were used as controls in these experiments. High-titer monospecific antisera were obtained from animals hyperimmunized to specific serum proteins (Cappel Laboratories, Cochranville, Pa.). Immunoelectrophoresis of each antiserum was performed by Cappel Laboratories and in our laboratory, and each antiserum reacted monospecifically with the respective host protein and fresh human serum. Sera against transferrin and lipoproteins A and B were rabbit anti-human; sera against α_2-macroglobulin, fibronectin, fibrinogen, immunoglobulin M (IgM), and α_1-antitrypsin were goat anti-human; and sera against IgG and IgA were horse anti-human. No reactivity of these monospecific serum reagents with *T. vaginalis* antigens was detected using Ouchterlony double diffusion and radioimmunoprecipitation technology (1, 2, 22; K. M. Peterson and J. F. Alderete, submitted for publication).

Antiserum against total normal human plasma proteins was generated in New Zealand white rabbits injected subcutaneously and intramuscularly with a mixture of 0.5 ml of normal human plasma and 0.5 ml of Freund complete adjuvant (GIBCO Laboratories, Grand Island, N.Y.) followed by booster injections 2 and 4 weeks later with equal amounts of normal human plasma in Freund incomplete adjuvant. Rabbits were bled 14 days post-booster immunization, and the sera were stored at −70°C until use. Control prebled rabbit serum was tested for parasite reactivity before use in the agglutination assay.

Specific *S. aureus*-mediated agglutination of live trichomonads. Samples (1 ml each) containing 5×10^6 *Trichomonas vaginalis* organisms grown in TYM medium in the presence of HI human plasma and washed twice in PBS-M were incubated with a 35-μl 10% (vol/vol) suspension of formaldehyde-fixed protein A-bearing *Staphylococcus aureus* (22) pretreated with serum reagent. After a 10-min incubation at 37°C, samples of pellets containing agglutinated parasites as well as supernatants consisting of individual motile trichomonads were evaluated using dark-field microscopy. The extent and specificity of trichomonal agglutination due to monospecific antiserum against individual plasma proteins was assessed by measuring nonagglutinated organisms remaining in the supernatant after this time period. Therefore, the agglutination index was measured as follows: 4+, 75 to 100% agglutination of motile trichomonads; 3+, 50 to 75% agglutination; 2+, 25 to 50% agglutination; 1+, 0 to 25% agglutination; and 0, no measurable specific agglutination. Dark-field optics on a Leitz Ortholux II microscope were used for all microscopic observations.

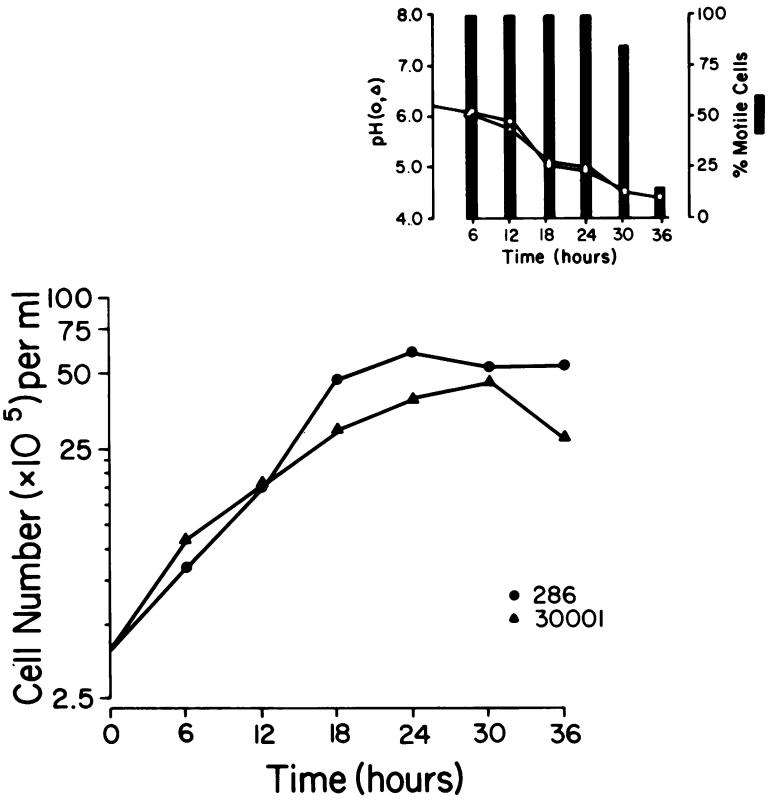

FIG. 1. Representative growth characteristics of two strains of *T. vaginalis* grown in TYM medium supplemented with HI horse serum or HI human plasma. The insert shows the decrease in motility correlated with trichomonal death. Results were similar for both strains.

Formaldehyde-fixed protein A-bearing *S. aureus* was prepared as previously described (22) and washed numerous times in an NaCl–EDTA–Tris-hydrochloride (NET) buffer, pH 7.4 (1), before use. A pellet representing 100 μl of a 10% (vol/vol) suspension of protein A-bearing *S. aureus* was suspended in 250 μl of control or individual antiserum. After a 30-min incubation at 37°C, IgG-coated protein A-bearing *S. aureus* was washed twice in NET buffer and suspended to 100 μl in PBS-M. Inhibition of agglutination was accomplished by prior treatment of IgG-coated protein A-bearing *S. aureus* with 1 mg of purified protein preparations (Sigma Chemical Co.) in 100 μl of PBS. Duplicate samples were employed for each experiment, and each experiment was repeated at least three times.

Enzyme and periodate treatment. A 1-ml suspension containing 2.5×10^7 trichomonads grown in HI human plasma and washed twice in PBS was treated with 10 mg of trypsin (Sigma type XI, diphenylcarbomylchloride treated, 7,500 U of α-*N*-benzoyl-L-arginine ethyl ester per mg; Sigma Chemical Co.) for various lengths of time at 37°C. After trypsinization, the organisms were again washed twice with PBS, and suspensions of trichomonads were dispensed for use in the agglutination assay. Trypsin activity was assessed using a protease detection kit with casein as the substrate (Bio-Rad Laboratories). Furthermore, washed parasite preparations were treated with 1 ml of 10 mM sodium meta-periodate (Sigma Chemical Co.) under conditions optimal for other biological systems (2, 31). Reactions never exceeded 30 min at 37°C. Loss of motility or cell death was not observed with these treatment regimens.

RESULTS

Growth characteristics. Significant self-agglutination and settling out of organisms occurred after 24 h of in vitro growth in either HI horse serum or HI human plasma and correlated with a rapid decline in total motile cells (Fig. 1). Stabilization of pH during growth, adjustment of pH to initial values, or changing of growth medium did not prevent parasite self-agglutination and death based on trypan blue exclusion. Thus, because physicochemical changes of the trichomonal surface during growth may be responsible for these phenomena, it was important to evaluate growth characteristics for all strains and to insure utilization of parasites before possible alterations in trichomonal surface properties. Only cultures between 18 and 24 h of growth were therefore employed in subsequent studies. Re-

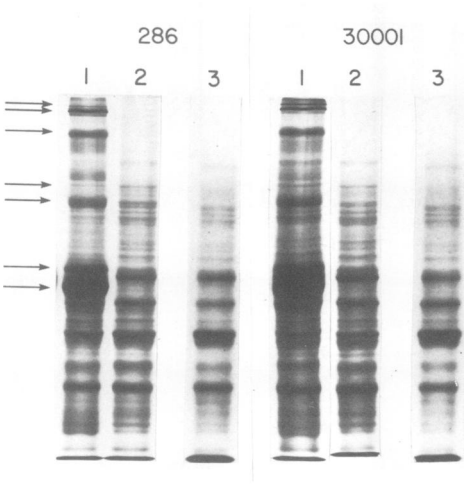

FIG. 2. Coomassie brilliant blue stained profiles and fluorograms of *T. vaginalis* 286 and 30001. (Lane 1) Stained protein patterns of trichomonads incubated with normal human plasma; (lane 2) trichomonads incubated with normal human plasma and washed at least twice in PBS-M before electrophoresis and staining; (lane 3) fluorogram showing [^{35}S]methionine-labeled trichomonal proteins.

sults for *T. vaginalis* 286 and 30001 are representative of all strains employed, and similar observations were obtained for all trichomonal strains unless otherwise stated.

Loosely associated host proteins. The Coomassie brilliant blue-stained profiles and fluorograms of *T. vaginalis* 286 and 30001 are presented in Fig. 2. The stained protein patterns of trichomonads incubated with normal human plasma are shown in lane 1. Lane 2 is of trichomonads incubated with human plasma but washed twice in PBS-M before electrophoresis and staining of protein bands. In each case, the corresponding fluorogram (lane 3) was identical and suggests that numerous high-molecular-weight proteins (arrows) as well as the intensely stained area in the middle of the gel were of host origin. Of interest was the reacquisition of the same comigrating host plasma proteins by washed organisms after reincubation with human plasma (lane 1). These observations suggest the presence of specific low-affinity sites on the trichomonal membrane for the selective acquisition of these plasma proteins.

Staphylococcus-mediated agglutination of intact *T. vaginalis*. To determine whether plasma proteins not detectable by gel electrophoretic analysis remain associated with washed trichomonads, it was necessary to develop a highly sensitive and specific method for detecting host-derived material. An agglutination assay was employed using formaldehyde-fixed protein A-bearing *S. aureus* as an adsorbent for IgG antibody directed against individual host proteins. Antibody-coated protein A-bearing *S. aureus* then mediated agglutination of washed motile organisms.

The extensive agglutination of strains 286 and 30001 resulting from protein A-bearing *S. aureus* pretreated with antiserum against total plasma proteins compared with incubation of protein A-bearing *S. aureus* with control, prebled serum is shown in Fig. 3. Furthermore, agglutination was neutralized by treatment of IgG-coated protein A-bearing *S. aureus* with whole plasma before mixture with the trichomonal suspension.

Additionally, monospecific antiserum against certain plasma proteins also resulted in pronounced protein A-bearing *S. aureus*-mediated trichomonal agglutination (Fig. 3 and Table 1). Specificity of the assay was established by lack of agglutination with protein A-bearing *S. aureus* alone or with staphylococci pretreated with control and several antiserum reagents (Table 1). In contrast, monospecific antiserum against human α_2-macroglobulin, α_1-antitrypsin, fibronectin, and IgM gave greater than 90% agglutination, whereas the extent of agglutination with anti-IgG antiserum was approximately 60%. In all cases, only the respective homologous plasma protein neutralized the protein A-bearing *S. aureus* reactivity with the parasite surface (Table 1). Treatment of IgG-coated staphylococci with albumin or proteins not reactive with appropriate antiserum did not alter the agglutination index (Table 1). Combined radioimmunoprecipitation-electrophoresis-fluorography technology (1) showed that the various antisera yielding extensive agglutination did not possess antitrichomonal antibody (Peterson and Alderete, submitted for publication). These data reinforce the idea of specific trichomonal acquisition of plasma macromolecules.

Effect of pH and enzyme treatment on avidly associated proteins. Because the pH of the vagina (pH 4.5) is less than that used for the agglutination assay, it was important to assess plasma protein acquisition in various buffers at different pH values. Therefore, *T. vaginalis* incubated with whole plasma at buffered pH values ranging from 4.0 to 7.5 followed by extensive washing in PBS-M was employed in the agglutination assay. The same level of agglutination was observed. These data suggest that the interactions which mediate parasite binding of host proteins are stable under these experimental conditions. The type of interactions responsible for trichomonal membrane binding of plasma proteins remains undefined.

We then attempted to implicate surface structures involved in host protein binding by adversely affecting the extent of agglutination

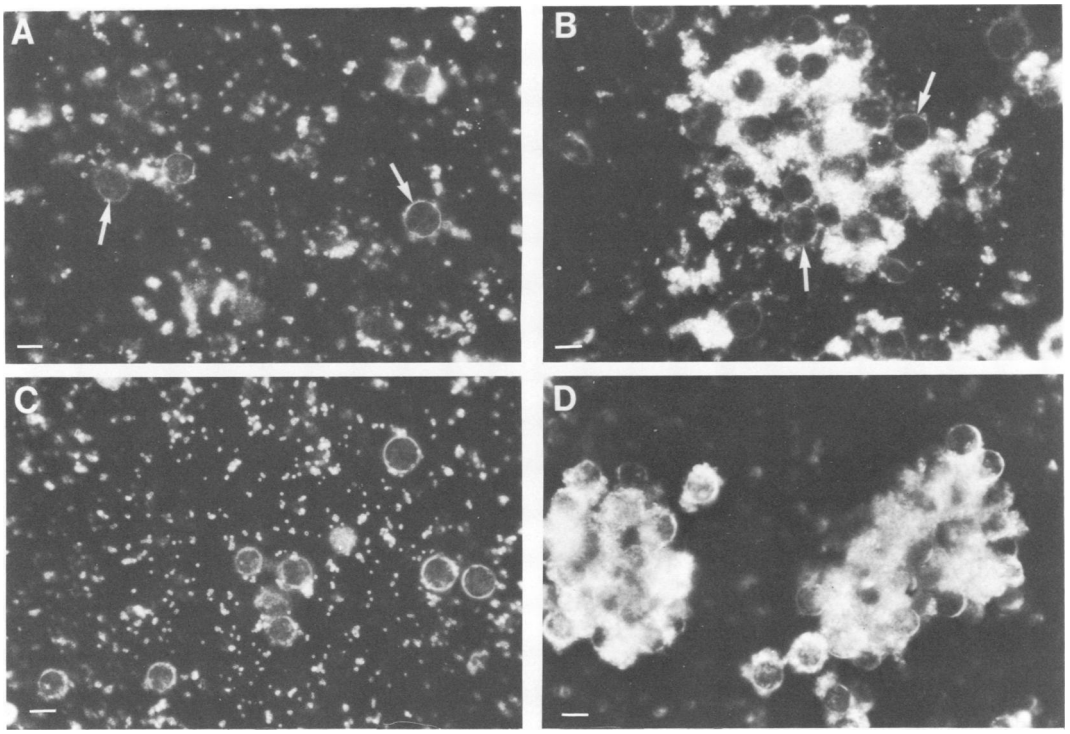

FIG. 3. Specific protein A-bearing *S. aureus*-mediated agglutination of live, motile *T. vaginalis* 286 (A and B) and 30001 (C and D). Extensive agglutination was observed after incubation of protein A-bearing *S. aureus* with antisera against whole human plasma or monospecific antiserum directed against individual host proteins (B and D). Pretreatment of protein A-bearing *S. aureus* with control serum resulted in no detectable trichomonal agglutination for each strain (A and C). Duplicates of IgG-coated protein A-bearing *S. aureus* producing pronounced agglutination (B and D) were incubated with whole plasma or homologous protein preparations and resulted in little or no parasite agglutination as in photographs (A) and (C). Bars = 15 μm.

through treatment of *T. vaginalis* with surface modifying reagents before incubation with IgG-coated staphylococci. We reasoned that treatment with protease might either cause release of acquired plasma proteins (1) or modify bound host proteins, resulting in a decrease in the agglutination index. Table 1 shows that treatment with either 10 mg of trypsin per ml or 10 mM periodate for 15 min did not decrease the extent of agglutination. Proteinase K, pronase, and neuraminidase also did not affect protein A-bearing *S. aureus*-IgG-parasite agglutination (data not shown). No loss in cell viability was detected with either protease or periodate treatment based on parasite motility and exclusion of trypan blue.

DISCUSSION

Trichomonal vaginitis is a sexually transmitted disease responsible for significant morbidity in both men and women (16, 17, 32). The causative agent, *T. vaginalis*, is the most frequently acquired protozoan infestation in this country; yet few data are available on key areas of this host-parasite interaction which would yield information on a possible mechanism(s) of disease pathogenesis. Because of our interest in using up-to-date technology to study the biology of this parasite and identify virulence determinants useful as future vaccinogen or immunodiagnostic reagents, our initial strategy was to examine the interaction between trichomonads and host macromolecules.

These studies were designed to demonstrate the acquisition of specific host proteins by live motile *T. vaginalis*. Pathogenic trichomonads possessed the ability to adsorb plasma proteins readily removed by repeated washings (Fig. 2). The nature of the interaction between loosely bound host proteins and trichomonal membranes is not known, although the enrichment for these proteins from plasma may be indicative of specific low-affinity sites present on trichomonads. Coating of parasite surfaces with host molecules may represent a mechanism by which immune factors in the vagina are effectively neutralized, allowing for survival in an otherwise hostile environment early in infection or

TABLE 1. Presence of human serum proteins on pathogenic *T. vaginalis* 286 and 30001

Serum reagent[a]	Agglutination index[b]	Agglutination index after treatment of organisms[b]	
		Trypsin	Periodate
Protein A-bearing *S. aureus* alone	0	ND[c]	ND
Normal serum control	0	0	ND
Anti-α_2-macroglobulin	4+	4+	4+
Anti-α_2-macroglobulin + α_2-macroglobulin	0	ND	ND
Anti-α_2-macroglobulin + albumin	4+	4+	4+
Anti-α_1-antitrypsin	4+	4+	4+
Anti-α_1-antitrypsin + α_1-antitrypsin	0	ND	ND
Anti-α_1-antitrypsin + albumin	4+	ND	ND
Anti-fibronectin	4+	4+	4+
Anti-fibronectin + fibronectin	1+	ND	ND
Anti-fibronectin + albumin	4+	ND	ND
Anti-IgM	4+	4+	4+
Anti-IgM + IgM	0	ND	ND
Anti-IgM + albumin	4+	ND	ND
Anti-IgG	2+	2+	2+
Anti-IgG + IgG	0	ND	ND
Anti-IgA	0	ND	ND
Anti-apolipoproteins A and B	0	ND	ND
Anti-fibrinogen	0	ND	ND

[a] Protein A-bearing *S. aureus* pretreated with control or monospecific antiserum reagent directed against a human serum protein-mediated trichomonal agglutination. Live, motile organisms were washed at least twice before incubation with protein A-bearing *S. aureus*. Duplicate samples were mixed with protein A-bearing *S. aureus* coated with monospecific antiserum and treated with a 1-mg preparation of commercially available serum proteins before incubation with *T. vaginalis* as described in the text.

[b] As described in the text.

[c] ND, Not done.

during menstruation when other specific as well as nonspecific immune factors are abundant. For example, complement-mediated lysis of *T. vaginalis* (12) could be circumvented by masking of both parasite and host components essential for functional reactions of complement factors.

Host proteins may also influence the extent and nature of tissue parasitism by trichomonads. The presence of fibronectin or other tissue substances in vaginal secretions may provide a mechanism for adherence of these parasites to host cell surfaces (Table 1). Vaginal bleeding resulting from trichomonal infection (16) would provide *T. vaginalis* with a source of plasma proteins. Alternately, trichomonads parasitizing vaginal epithelium could be protected from host immunological factors as discussed above, allowing for tissue cytopathological reactions (7, 10, 15, 17).

The presence of human plasma proteins not detectable by gel electrophoretic analysis and which could not be removed by repeated washing of organisms was evaluated with a highly sensitive agglutination assay. Fixed protein A-bearing *S. aureus* pretreated with antiserum monospecific against individual plasma proteins or antiserum against whole plasma mediated agglutination of trichomonads (Fig. 3). The ability to avidly bind plasma proteins may reflect a mechanism by which pathogenic trichomonads sequester biologically important host macromolecules and may have important implications for this host-parasite relationship. For example, foci of infection might alter biochemical processes in hormone-responsive tissue due to the concentration of cellular growth-promoting substances on parasite surfaces. Hyperplasia of vaginal epithelium after trichomonal infection (10, 16, 17, 23) has been reported. Alternately, parasite membrane integrity or modulation of membrane fluidity (11) may be influenced by host-derived substances. Of interest, therefore, would be

research directed toward elucidating the biological function of plasma or tissue components preferentially acquired by *T. vaginalis*.

Controversy exists in the literature about the nature and extent of tissue cytopathology in infected individuals (7, 10, 14, 15, 17, 18, 25). Whereas an intimate association between vaginal epithelium and trichomonads has been described previously (14, 25, 30), factors potentially responsible for observed cellular pathophysiology have not been identified. We believe that complexes between the surface of pathogenic microorganisms and host macromolecules may induce the formation of novel immunodeterminants with the potential for eliciting autoimmune reactions. Additionally, these host-parasite protein complexes could be responsible for local or generalized tissue inflammatory responses.

Avidly associated plasma proteins were not displaced from the trichomonal surface by protease or periodate treatment (Table 1). Neuraminidase-like substances elaborated by pathogenic trichomonads have been reported previously (17) and the lack of decreased agglutination after periodate treatment may reflect an absence of susceptible carbohydrate moieties on the parasite membrane. Alternately, gross modification of acidic carbohydrate residues on the cell surface without affecting agglutination may suggest noninvolvement of sugars in plasma protein binding. Of interest was the inability of proteases at relatively high concentrations to adversely affect agglutination. No modification of the total trichomonal protein profile was detected after trypsin treatment as determined by Coomassie brilliant blue staining and fluorography (Fig. 2, lanes 2 and 3). Trichomonal membrane components or host-derived substances such as α_2-macroglobulin and α_1-antitrypsin (Table 1) may be effective inhibitors of serum proteases (21, 28). Identification of tissue or serum macromolecules bound to surfaces of pathogenic trichomonads, therefore, might allow elucidation of macromolecules which contribute to microbial survival through inactivation of host protective factors (proteases) which reside in mucous secretions. This paper represents the foundation for continued studies in our laboratory aimed at biochemical analysis of parasite acquisition of host macromolecules.

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