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Beta-Hemolytic Activity of *Trichomonas vaginalis* Correlates with Virulence

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The reasons that some women develop symptomatic trichomonal vaginitis, whereas many other infected women remain asymptomatic, are unclear, but it has been suggested that *Trichomonas vaginalis* strains vary in their intrinsic virulence. We describe beta-hemolytic activity in *T. vaginalis* which correlates with virulence in patients as well as in an animal model and in tissue culture. Fresh *T. vaginalis* isolates from four women with severe, symptomatic trichomoniasis had high-level (86.3 ± 6.6%) hemolytic activity, whereas isolates from three completely asymptomatic women had low-level (45.3 ± 8.4%) hemolytic activity ($P < 0.001$). Hemolytic activity also correlated with the production of subcutaneous abscesses in mice ($r = 0.74$) and with destruction of CHO cell monolayers ($r = 0.94$). All of the 20 clinical isolates of *T. vaginalis* tested possessed hemolytic activity. The beta-hemolysin may be a virulence factor for *T. vaginalis*.

Trichomonas vaginalis is one of the most common sexually transmitted urogenital pathogens. Many women infected with *T. vaginalis* develop florid vaginitis with considerable morbidity (8, 13, 18, 28). In contrast, other women are asymptomatic carriers (28). Factors responsible for this marked clinical variation are unknown (12, 13, 28).

There is some circumstantial evidence that *T. vaginalis* strains differ in their intrinsic virulence, in that strains vary in their ability to produce abscesses after inoculation in animals (13, 18). These findings correlate imperfectly with the assessment of virulence in patients (17). Moreover, there are no well-documented, physiological, or biochemical correlates of trichomonad virulence (13, 18). A number of workers have suggested morphological associations, namely, that more virulent trichomonads may be more rounded, smaller, and more ameoboid than less virulent isolates (9, 17, 19, 22, 23, 27). In culture, virulent strains may have long generation times, and prolonged laboratory passage has been associated with loss of virulence (12, 14, 16, 23). At present, however, there is no uniform system for classifying *T. vaginalis* strains with respect to virulence (13, 18).

During our studies on nutritional requirements of trichomonads, we serendipidously demonstrated that a strain of *T. vaginalis* possessed beta-hemolytic activity. We have investigated the possibility that hemolytic activity in *T. vaginalis* might be related to virulence by quantitating the beta-hemolytic activity and comparing it to virulence in patients, in an animal model, and in a tissue culture system.

(This study was presented at the annual meeting of the American Federation for Clinical Research, Washington, D.C., 7 to 10 May 1982.)

MATERIALS AND METHODS

Patients. Women attending the Albemarle County Venereal Disease Clinic underwent standardized evaluation by a single investigator. Approximately 50 women attending the clinic during the study interval had vaginal cultures positive for *T. vaginalis*. Twenty random isolates were assayed for hemolytic activity without regard to clinical findings. Isolates from seven women were subjected to more intensive study; four from women with symptomatic infection and three from asymptomatic carriers. The patients represented extremes of clinical manifestations, the organisms were propagated in the laboratory, and cultures for *Neisseria gonorrhoeae*, *Candida albicans*, and *G. vaginalis* were negative. The seven thus do not represent the full spectrum of clinical disease.

The three asymptomatic patients had no genitourinary symptoms and presented because they were partners of men with sexually transmitted diseases. Physical examination revealed normal appearing labia and vaginal walls without clinically apparent excessive vaginal discharge. The four symptomatic women noted change in character or amount of vaginal discharge (4 of 4), vulvovaginal irritation (4 of 4), dyspareunia (2 of 4), or dysuria (2 of 4). Signs of vaginitis included excessive purulent vaginal discharge (4 of 4), erythema

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of the vaginal walls (4 of 4), granular vaginitis with punctate hemorrhages (2 of 4), and vaginal tenderness during the examination (4 of 4). Wet mount preparations of vaginal discharge revealed motile trichomonads in both symptomatic and asymptomatic patients.

All patients were subsequently treated with a single oral administration of 2 g of metronidazole with complete resolution of symptoms and signs and negative posttreatment cultures for *T. vaginalis*.

Trichomonads. Vaginal vault specimens were obtained from both symptomatic and asymptomatic patients with sterile, cotton-tipped applicator sticks and inoculated immediately into 4.5 ml of Feinberg-Whittington liquid medium (5) containing 1,000 U of penicillin per ml, 100 μg of gentamicin per ml, and 2.4 μg of amphotericin B per ml. Cultures were incubated under anaerobic conditions with hydrogen and carbon dioxide generator envelopes in brewer jars (GasPak anaerobic systems, BBL Microbiology Systems, Cockeysville, Md.; Becton, Dickinson & Co., Rutherford, N.J.) at 37°C. *T. vaginalis* isolates were identified by their characteristic morphology and motility. Isolates were subcultured every 3 to 4 days. In all, 20 vaginal isolates were studied for hemolytic activity.

Erythrocytes. Fresh human blood was mixed with an equal volume of a solution of 10 U of heparin per ml and 3% dextran in physiological saline. The mixture was allowed to sediment for 1 h on a 45° slant rack. After the supernatant and buffy coat layers were discarded, the erythrocytes were washed with an equal volume of Hanks balanced salt solution (HBSS) containing 10 U of heparin per ml. The supernatant was discarded after centrifugation at 250 × *g* for 10 min.

Qualitative hemolysis assay. A 0.5-ml amount of Feinberg-Whittington liquid medium containing 25 to 50 trophozoites was inoculated into each of three 60 by 15 mm polystrene petri plates (Becton, Dickinson Labware, Oxnard, Calif.). A 4.5-ml amount of Diamond's medium containing 0.5% purified agar (15) (KC Biologicals, Inc., Lenexa, Kans.) and 2% washed, fresh erythrocytes were then added to each plate. Hardened pour plates were incubated anaerobically for 5 days at 37°C, and zones of hemolysis were examined with a dissecting microscope.

Quantitative hemolysis assay. Washed, fresh erythrocytes (0.05 ml) were mixed with 2.5 ml of HBSS containing a total of 10^6 trophozoites. After 24 h of incubation in a carbon dioxide incubator at 37°C, the culture was centrifuged at 250 × *g* for 10 min. Absorbance of the supernatant, measured at 412 nm with a spectrometer (model 2400; Gilford Instrument Laboratories, Inc., Oberlin, Ohio), was compared to a standard curve prepared by osmotic lysis of erythrocytes and dilution of hemoglobin in HBSS. Standard tubes were incubated along with those containing trichomonads, as were tubes containing erythrocytes and no trichomonads to control for spontaneous hemolysis.

Mouse inoculation. We tested virulence in animals by using a modification of the mouse subcutaneous abscess assay (7, 12, 14). Trichomonads were harvested from 48-h Feinberg-Whittington cultures by centrifugation at 250 × *g* for 10 min. Organisms were washed in HBSS and resuspended at a final concentration of 2 × 10^5 to 2 × 10^6 organisms per ml. Six-week-old male C3H Dublin mice (Flow Laboratories, Inc., Hamden, Conn.) were injected subcutaneously with 0.5 ml of *T. vaginalis* suspension in each flank. Abscess formation was determined by palpation 6 to 7 days after injection, and the proportion of injections resulting in abscesses was noted for each *T. vaginalis* isolate. Representative lesions were examined microscopically and cultured for *T. vaginalis*, aerobic and anaerobic bacteria, and fungi. Control injections of HBSS did not result in abscess formation.

Tissue culture assay. CHO K1 cell monolayers (ATCC CCL 61) were grown to confluency in Ham's F-12 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum in 24-well polystyrene tissue culture plates (76-033-05; Linbro Division, Flow Laboratories) (24). After removal of the growth medium, the monolayers were washed with Feinberg-Whittington medium adjusted to pH 7.0 and with 10% heat-inactivated fetal calf serum substituted for heat-inactivated fresh human serum. A 1-ml amount of Feinberg-Whittington medium was then added to each well.

Trichomonads from 48-h cultures were harvested by centrifugation, washed in HBSS, and then resuspended in Feinberg-Whittington medium at a concentration of 10^7/ml. Each of six isolates was tested by inoculation of six monolayers with 0.1 ml of the trichomonad suspension yielding a final concentration of 10^6 trichomonads per ml; approximately six trichomonads per CHO cell. Different *T. vaginalis* isolates were studied in parallel. Monolayers were examined hourly with an inverted microscope with phase contrast. Cytopathic effect at each time period was evaluated by examination of three microscopic fields per monolayer. CHO cell confluency within each field was scored as 0, 25, 50, 75, or 100%, and readings were averaged for each monolayer at each interval.

Clinical evaluation, hemolytic assays, and tissue culture assays were each carried out by a separate investigator in blinded fashion.

Statistical analysis. The significance of the association between clinical vaginitis and hemolytic activity or monolayer destruction was evaluated by the Student *t* test, two-tailed. The association between vaginitis and abscess formation was evaluated by the Chi-square test with Yates correction.

RESULTS

Hemolytic activity. After 5 days of incubation in pour plates, *T. vaginalis* colonies ranged from 0.2 to 2.0 mm in diameter. Typical colonies were subsurface, circular, and dull cream colored. Colonies from all fresh clinical isolates were surrounded by 1.0 to 2.0 mm of clear zones from which erythrocytes were completely absent (Fig. 1). Trichomonads were not present in the erythrocyte-free zone, which implies that the absence of red cells was not due to erythrophagocytosis. Hemolytic activity was maintained after serial passage in axenic culture for 6 months. Trichomonads hemolyzed all human blood groups as well as sheep, rabbit, and chicken erythrocytes. In the quantitative assay, trichomonads from the four symptomatic women hemolyzed 86.3 ± 6.6% of erythrocytes,

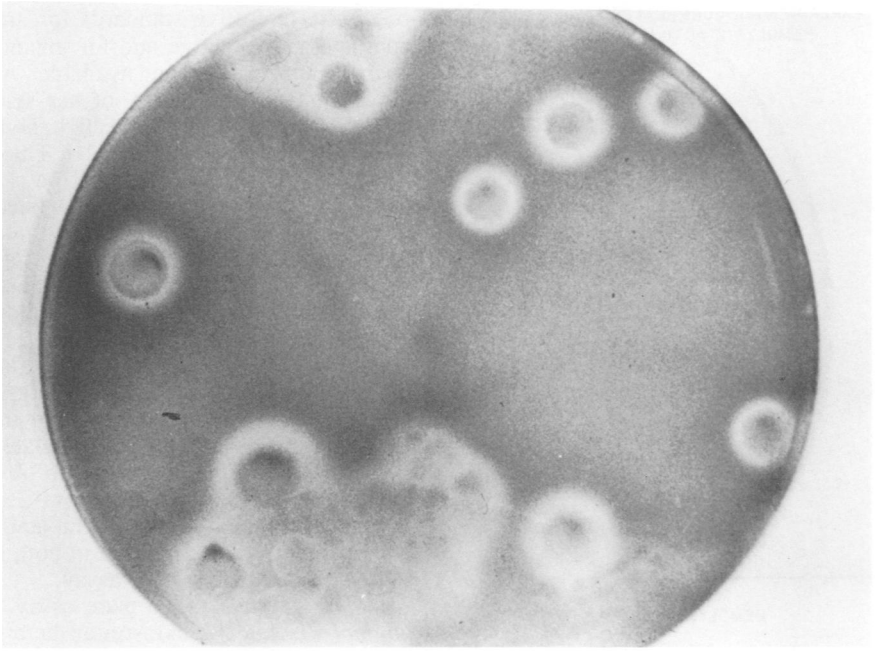

FIG. 1. Beta-hemolytic activity of colonies of *T. vaginalis* is demonstrated in a pour plate containing 2% human erthrocytes after 5 days of anaerobic incubation.

whereas trichomonads from the three asymptomatic women hemolyzed only 45.3 ± 8.4% of erythrocytes (Table 1). These differences were significant ($P < 0.001$).

Animal virulence. The same seven *T. vaginalis* isolates were evaluated for the ability to produce subcutaneous abscesses in mice (Table 1; Fig. 2). Two isolates did not produce any abscesses. The other five isolates caused abscess formation after 5 to 71% of the injections. Excised abscesses contained motile trichomonads, and cultures from four animals were positive for *T. vaginalis* but negative for bacteria or fungi. Hemolytic activity of the isolates correlated with production of subcutaneous abscesses ($r = 0.74$). The four isolates from symptomatic patients (Table 1) caused significantly more abscesses (mean 40%) than the three isolates from asymptomatic patients (mean 1.9%; $P < 0.001$).

Tissue culture virulence. Six isolates were evaluated for cytopathic effects on CHO cells. All isolates destroyed the monolayers. The kinetics of monolayer destruction, however, varied considerably among the isolates. Such differences were maximal after 4 h of exposure of the monolayers to the trichomonads, at which time monolayer destruction varied from 22.6 ± 2.3% to 86.2 ± 4.7% (Fig. 2). Monolayer destruction after 4 h of incubation correlated closely with hemolytic activity ($r = 0.94$). The differences in monolayer destruction between the four isolates from symptomatic patients (mean 65.8 ± 17.0%) and two isolates from asymptomatic patients (mean 26.7 ± 5.8%) were significant ($P = 0.019$).

TABLE 1. Correlations of hemolytic activity in *T. vaginalis* with virulence

Clinical vaginitis[a]	No. of isolates	Hemolytic activity (mean % ± SD)[b]	Monolayer destruction (mean % ± SD)[c]	No. of abscesses/ no. of injections (%)[d]
+	4	86.3 ± 6.6	65.8 ± 17.0	18/45 (40)
−	3	45.3 ± 8.4	26.7 ± 5.8	1/54 (1.9)
Significance		$P < 0.001$	$P < 0.020$	$P < 0.001$

[a] + indicates the presence of symptoms and signs of vaginitis described in the text.
[b] Two to three spectrophotometric determinations per isolate.
[c] Six CHO cell monolayers evaluated for each of four isolates from symptomatic women and two isolates from asymptomatic women. Monolayer destruction was quantitated after a 4-h exposure to trichomonads.
[d] Abscess production 6 days after subcutaneous inoculation of trichomonads in mice.

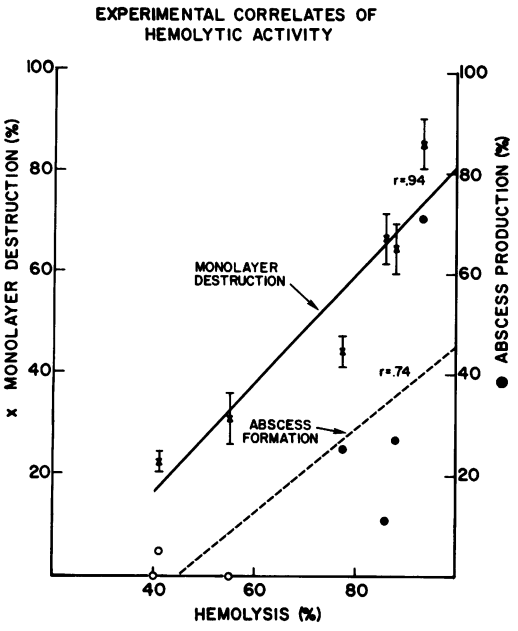

FIG. 2. Monolayer destruction by *T. vaginalis* isolates from symptomatic (X) and asymptomatic (⊗) patients; and subcutaneous abscess formation by *T. vaginalis* isolates from symptmatic (●) and asymptomatic (○) patients as functions of hemolytic activity. Data points for monolayer destruction represent the mean ± the standard error of the mean of six monolayers. Subcutaneous abscess formation was evaluated in 3 to 30 mice per isolate.

DISCUSSION

Trichomoniasis is a common disease causing considerable morbidity in an estimated 3 million American women annually (1, 13). The reasons that some women have clinical disease, whereas others are asymptomatic carriers have remained unclear (13, 18). Efforts to define virulence factors and mechanisms in *T. vaginalis* have been especially hindered by the lack of objective and reproducible measures of virulence in either animal or tissue culture systems (14, 24). Some investigators have used stock isolates maintained for years by axenic cultivation (13, 18), but such laboratory strains may bear little resemblance to fresh clinical isolates (16, 17). To date, there has been no clear-cut biochemical or physiological marker of virulence in *T. vaginalis* (13, 18).

Our findings support the concept that variation in intrinsic virulence of *T. vaginalis* strains is one factor influencing the severity of trichomoniasis in women. In particular, the level of beta-hemolytic activity in fresh *T. vaginalis* isolates was correlated with clinical virulence. Clinical severity of disease is difficult to quantitate precisely. Objective standards for amount and character of discharge and for inflammation of vaginal mucosae are not available. A patient's perception of the severity of her symptoms is also modified by highly subjective, nonquantifiable, psychological factors (3, 21). Thus, correlation with clinical severity must be regarded as soft. Our subjects were chosen because they manifested extremes of the spectrum of clinical disease and could be easily placed into the two groups. We suspect that correlations with disease of intermediate severity would be more difficult.

Vaginal factors also influence the clinical expression of disease. Symptoms of trichomoniasis may be related to variation of the vaginal epithelium, vaginal pH, or associated vaginal flora. It is therefore reassuring to observe that the level of hemolytic activity in *T. vaginalis* isolates correlated with experimental measures of virulence as demonstrated in both an animal model and a tissue culture assay.

In a qualitative, pour plate assay, all 20 *T. vaginalis* isolates demonstrated beta-hemolytic activity which was maintained on repeated subculture. In the quantitative, spectrophotometric assay, however, high-level beta-hemolytic activity was associated with clinical disease in four patients. In contrast, low-level beta-hemolytic activity was associated with asymptomatic carriage in three patients ($P < 0.001$). Thus, quantitative determination of beta-hemolytic activity appears to be a potentially useful biochemical correlate of virulence in *T. vaginalis*. To evaluate further the significance of this finding, we compared hemolytic activity with other experimental measures of virulence.

Various animal models have been developed to investigate *T. vaginalis* virulence. The mouse subcutaneous abscess model, employed in this study, has been used most widely (13, 14, 19). Unfortunately, abscess production does not correspond exactly with the severity of trichomoniasis in patients (16, 17). In the present series, abscess formation was significantly associated with clinical signs and symptoms ($P < 0.001$), although one isolate from an asymptomatic patient did produce abscesses in mice. This model has obvious differences from natural routes or sites of infection. Several genital models have been proposed, but they employ extensive hormonal manipulations and, therefore, are difficult to relate to events in humans (13). Thus, although the standard subcutaneous abscess assay of trichomonad virulence correlated with quantitative hemolytic activity ($r = 0.74$), we employed a second experimental measure of virulence.

The CHO cell culture assay has several advantages over previous systems. Tissue culture

is much less complex than an animal model and permits control of many more experimental variables (10). Unlike organ culture systems employed by previous workers (2, 4, 11), there is only a single cell type. It is possible to distinguish among *T. vaginalis* isolates by following the kinetics of CHO cell monolayer destruction. Isolates from symptomatic patients destroyed monolayers significantly faster than isolates from asymptomatic patients ($P < 0.020$). Hemolytic activity of *T. vaginalis* isolates correlated more closely with CHO cell monolayer destruction than with production of subcutaneous abscesses in mice. These findings provide a quantitative framework for classifying *T. vaginalis* strains according to multiple characteristics that relate to clinical virulence.

A specific role for hemolytic activity in *T. vaginalis* ecology and pathogenicity is undefined. Hemolysins have been described in bacteria such as staphylococci, streptococci, clostridia, vibrios, escherichia, and aeromonas, and correlate with virulence in many species (6). Hemolysins have also been reported in protozoa other than trichomonads, such as trypanosomes and amoebae, although their relationship to virulence is unclear (20, 25, 26). Hemolysis may be important in providing nutrients from lysed erythrocytes (M. Lambert and M. F. Rein, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 828, 1982), and the clinical observation that trichomoniasis is frequently exacerbated by the menses may be related to increased substrate. Hemolytic activity might also inhibit cellular aspects of the immune response of the host. Alternatively, the hemolysin may act as a direct cytotoxin destroying vaginal epithelial cells.

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