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Association of Production of Cell-Detaching Factor with the Clinical Presentation of *Trichomonas vaginalis*

GARY E. GARBER* AND LAUREL T. LEMCHUK-FAVEL

Division of Infectious Diseases, Department of Medicine, University of Ottawa, and Ottawa General Hospital, 501 Smyth Road, Ottawa, Ontario K1H 8L6, Canada

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Recent work has shown that *Trichomonas vaginalis* produces a cell-detaching factor (CDF) that causes detachment of monolayer cells in vitro. To study the role of CDF as a pathogenic marker of disease, we studied the production of CDF in 12 clinical isolates of *T. vaginalis*. These isolates were also utilized in the mouse subcutaneous assay of Honigberg, which is the standard for pathogenicity of *T. vaginalis*. The isolates were divided into three groups based on clinical presentation (asymptomatic [*n* = 4], moderate [*n* = 4], and severe symptoms [*n* = 4]). CDF was assessed by harvesting the supernatant from the growth of *T. vaginalis* in cell culture and filtering the supernatant through a 0.45-μm-pore-size filter. The filtrate was applied in a microtiter cytotoxicity assay. The mouse subcutaneous assay did not significantly differentiate among the isolates. However, CDF was strongly associated with clinical presentation by two-way, repeated-measure analysis of variance (*P* = 0.025). Thus, CDF appears to correlate with clinical presentation and may be an important virulence marker in *T. vaginalis* pathogenesis.

Trichomonas vaginalis is a frequently encountered genital pathogen (3, 4). Despite its high prevalence, the pathogenic mechanisms by which *T. vaginalis* causes clinical vaginitis are not well defined.

The best standardized assay of pathogenesis has been the mouse subcutaneous assay of Honigberg in which the volume of the lesion size was reported to correlate with virulence (10). More recently, Honigberg et al. reported that the subcutaneous lesion did not correlate with clinical presentation (11).

We recently reported the isolation of two potential virulence factors from cell-free filtrates of *T. vaginalis* (7, 8). Cell-detaching factor (CDF) (8, 13) is a 200-kDa glycoprotein that causes detachment of monolayer cells in vitro, analogous to the vaginal epithelial cell sloughing seen in trichomoniasis (8). CDF was found to be immunogenic by immunoblotting with human sera reactive to *T. vaginalis* but not to control sera (8). The in vitro activity of CDF can also be neutralized when CDF is coincubated with anti-CDF-containing human sera.

In this study, we assessed the correlation between the clinical presentation of *T. vaginalis* and the in vitro production of CDF. We found a significant association with increased CDF activity and clinical severity.

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MATERIALS AND METHODS

Clinical isolates. Clinical isolates of *T. vaginalis* were obtained from vaginal secretions of women. Isolates were purified by repeated culture in TYI (Diamond TYI-S-33 medium [5] containing 10% heat-inactivated bovine serum) as previously described (7–9). Axenic cultures were mixed with an additional 15% bovine serum plus 10% dimethyl sulfoxide and stored at −70°C (9).

These isolates were categorized by a standard scoring scheme evaluating clinical presentation using the following criteria: symptoms (vaginal discharge, itch, dysuria, and dyspareunia), type of vaginal discharge (amount, color, and number of polymorphonuclear leukocytes) and severity of cervical, vaginal, or vulva erythema. Each of the 10 categories was scored from 0 to 3, with 3 being the most severe. On this basis (scoring from 0 to 30), isolates were classified as asymptomatic (0 to 2), moderate (3 to 20), and severe (21 to 30). Twelve clinical isolates, four isolates from each category, were randomly selected for further analysis.

CDF. (i) Production of filtrate. Filtrates of *T. vaginalis* growth were produced as described by Garber et al. (7, 8), and the procedure is detailed briefly here. Isolates were grown in axenic culture with TYI in ambient air at 37°C. The organisms were harvested by centrifugation at 500 × *g* and washed three times with phosphate-buffered saline containing 10 mM Na_2HPO_4 at pH 7.2. The pellet was suspended at a concentration of 10^5 organisms per ml in a 2:1 mixture of CMGA-TYI, pH 6.8 (7–9). Samples of 10-ml volume were added to 25-cm^2 flasks containing confluent McCoy cells (passage 13 to 20) which had been previously cultured in CMGA and then washed with phosphate-buffered saline before inoculation with *T. vaginalis*. After incubation at 37°C for 21 h, *T. vaginalis* growth was ascertained and the supernatants were harvested by centrifugation at 500 × *g* and then filtered through 0.45-μm-pore-size membranes (Millipore Corp., Bedford, Mass.). The pH of the filtrate was determined. Filtrates with pH of <6.0 were adjusted to pH 6.5 with 1 N NaOH and filtered as described above. The filtrates were aliquoted and frozen at −70°C.

(ii) Cytotoxicity assay. CDF preparations from 12 isolates were concurrently tested for cell-detaching effects on McCoy cell monolayers, using a microtiter cytotoxicity assay (8). In this microtiter assay, 4×10^4 McCoy cells in 50 μl of CMGA were coincubated with 50 μl of filtrate. The assay was incubated for 24 h at 37°C in 5% CO_2. Filtrates were assayed in triplicate and repeated two times. Monolayers

* Corresponding author.

were assessed for percent confluence by staining the monolayer with crystal violet (2). Absorbance of individual wells was read at an optical density at 540 nm (OD_{540}), using a Bio-Tek 310 autoplate reader. The percentage of attached monolayers was calculated by dividing the OD of the test wells with the average OD of six control wells and multiplying by 100. The percentage detachment was obtained by subtracting this value from 100%. The controls contained filtrate from uninoculated CMGA-TYI incubated on McCoy cell monolayers for 21 h.

Subcutaneous assay. *T. vaginalis*, grown axenically in TYI at 37°C, was harvested by centrifugation at 500 × *g* for 10 min, washed three times with phosphate-buffered saline, and suspended in bacto fluid thioglycolate medium (Difco Laboratories, Detroit, Mich.) at 1.8×10^6 *T. vaginalis* per ml. A subcutaneous injection of 0.5 ml was performed on each shaved flank of 22- to 24-g female BALB/c mice (Charles River, Montreal, Quebec, Canada). Each *T. vaginalis* isolate was inoculated into both flanks of three mice, for a total of six lesions. Control mice received identical treatment (0.5 ml of prewarmed fluid thioglycolate media without *T. vaginalis*). Six days postinoculation, the mice were sacrificed and the length, width, and height of the lesions were measured with calipers and a metric ruler. The total volumes of the abscess was determined by length × width × height × 0.5236 (10), and the mean of the six lesions was expressed in cubic millimeters.

To determine whether the lesion size was in response to live organisms or to the antigen, control assays with two killed isolates (severe symptomatic category) were performed. The isolates were killed by overgrowth in culture and the resulting low pH (<4.0). These killed cultures were exposed overnight to UV light to prevent bacterial contamination. The pelleted, killed *T. vaginalis* isolates were suspended in fluid thioglycolate broth and injected as described above. The entire procedure was performed in duplicate, for a total of 12 lesions for each isolate. Any resulting nodules were excised and cultured in TYI and thioglycolate broth.

Statistical analysis. For all isolates, results of CDF production and subcutaneous abscess formation were each analyzed for correlation with clinical presentation by two-way repeated measure analysis of variance.

RESULTS

Mouse subcutaneous assay. Using the subcutaneous assay of Honigberg (10), we determined that killed *T. vaginalis* produced either no detectable lesions at 6 days postinoculation or at most a lesion size of approximately 25 mm^3. In these cases, the excised and cultured nodules exhibited no growth, in contrast to the excised lesions of active *T. vaginalis*. The mean lesion size from live isolates of *T. vaginalis* ranged from 91.8 to 254.2 mm^3. Thus, the subcutaneous lesions were associated with live, actively metabolizing organisms and were not merely a delayed-type hypersensitivity reaction to *T. vaginalis* antigen.

Table 1 shows the results of the subcutaneous assays; each value is the mean of six subcutaneous lesions (three mice, each receiving an injection into both flanks). Isolates from asymptomatic individuals yielded a mean volume (in cubic millimeters) of 151.0 ± 48.3 compared with 193.1 ± 68.3 for moderate and 205.7 ± 54.6 for severely symptomatic individuals. By analysis of variance, no significance was determined ($P = 0.26$) between clinical presentation and lesion size.

CDF. After 21 h of incubation, the mean number of *T. vaginalis* was 6.5×10^5 *T. vaginalis* per ml (range, 5×10^5 to 9×10^5 *T. vaginalis* per ml). The mean pH of the filtrates was 6.23 (range, 6.1 to 6.5). Protein concentration of the filtrate was 4.16 ± 0.34 mg/ml. There were no significant differences in either growth, pH, or protein concentration among the three clinical presentations.

Table 2 shows CDF activity; each value is the mean of three experiments done in triplicate. The four isolates derived from asymptomatic women had a mean CDF activity of 32.5% ± 6.6% destruction compared with 38.7% ± 2.8% for women with moderate symptoms and 45.7% ± 10.7% for isolates causing severe symptoms. By analysis of variance, we found a strong statistical correlation between clinical presentation and CDF activity ($P = 0.025$). Increasing production of CDF was associated with increased severity of

TABLE 1. Subcutaneous assay of 12 clinical isolates of *T. vaginalis*

Type[a] and isolate	Mean lesion size[b] (mm^3)
Asymptomatic	
364	91.8 ± 28.4
386	140.8 ± 35.3
387	163.7 ± 47.3
396	207.7 ± 57.8
Moderate	
202	254.2 ± 138.2
256	161.0 ± 51.9
263	244.8 ± 49.0
335	112.2 ± 62.9
Severe	
002	140.5 ± 51.8
277	180.7 ± 85.1
330	247.5 ± 66.7
86-2	254.0 ± 119.8

[a] Classification of isolates was based on a standard scoring scheme evaluating clinical presentation as described in text.

[b] Volume of each lesion was determined by multiplying length × width × height × 0.5236. Each isolate was assayed on both flanks of three female BALB/c mice.

TABLE 2. CDF activity[a] of 12 clinical isolates of *T. vaginalis*

Type[b] and isolate	Mean isolate detachment[c] (%)
Asymptomatic	
364	40.5 ± 8.2
386	33.1 ± 13.6
387	24.3 ± 12.9
396	32.1 ± 16.3
Moderate	
202	41.0 ± 10.7
256	41.1 ± 13.5
263	37.0 ± 18.9
335	35.7 ± 7.2
Severe	
002	50.3 ± 10.5
277	54.7 ± 14.6
330	30.3 ± 12.7
86-2	47.4 ± 5.3

[a] CDF activity was assessed by a cytotoxicity assay as described in the text.

[b] Classification of isolates was based on a standard scoring scheme evaluating clinical presentation as described in the text.

[c] Calculated by dividing the test OD_{540} with the mean OD_{540} of the six controls and multiplying by 100. The percentage detachment was obtained by subtracting this value from 100%. Each isolate was assayed three times in triplicate.

clinical disease. Among the three experiments, no statistically significant run-to-run variability was seen.

DISCUSSION

The pathogenic mechanisms of *T. vaginalis* have puzzled investigators for over 40 years of active study. Recently, two possible mechanisms have been studied in detail. Alderete and coworkers (1) have studied *T. vaginalis* cytotoxicity and found an association with adherence to epithelial cells, including vaginal epithelial cells. Although many studies rejected the possibility of a soluble cytotoxin, Pindak et al. (13) demonstrated that cell-free filtrates of *T. vaginalis* do cause cell rounding and disruption of monolayer cells. We expanded upon this work (8) and isolated CDF, a 200-kDa glycoprotein which causes cell detachment in vitro and is acid and heat labile.

In this study, we determined that production of CDF is associated with the clinical presentation of trichomonal vaginitis. CDF may be a virulence marker of disease. CDF activity in vitro is quenched by immunogenic human serum (8). In chronic trichomoniasis, the inflammatory reaction seen on examination appears to diminish, although the organism is not eradicated (10). Local vaginal antibodies may decrease local CDF effects, resulting in decreased inflammation and symptoms.

All *T. vaginalis* isolates tested produce CDF as determined by immunoblotting (G. E. Garber and L. T. Lemchuk-Favel, Proc. 16th Int. Congr. Chemother., p. 598.1–598.2, 1989). In these experiments, differences in CDF activity were not caused by differences in *T. vaginalis* growth, as all isolates grew to similar concentrations at 21 h. The phenomenon of some individuals being asymptomatic may be explained by decreased CDF production in some instances. In others, over time, one-third of asymptomatic individuals will develop clinical symptoms (10, 12). Whether this phenomenon is caused by regulation of CDF production, immune response to infection, or other environmental factors is speculative. A normal vaginal pH of 4.5 would inhibit CDF activity. CDF is active only at a pH of >5.0 (8), which is the pH usually found in cases of *T. vaginalis* vaginitis (6).

There has been controversy over the role of the subcutaneous assay. It was initially found to differentiate pathogenic from nonpathogenic strains but recently was shown not to differentiate among clinical isolates based on severity of presentation (11). Our work has shown that the subcutaneous reaction is dependent on live organisms, is not due to an immunologic reaction to *T. vaginalis* antigen, and did not differentiate among clinical isolates.

CDF activity did correlate significantly with clinical presentation in the 12 isolates tested. The pattern of results was consistent from experiment to experiment, with no significant variability seen. CDF appears to be an important virulence marker and may be useful as a sensitive diagnostic tool and in further studies of pathogenesis.

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