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Isolation of a Cell-Detaching Factor of *Trichomonas vaginalis*

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The pathogenetic role of soluble products of *Trichomonas vaginalis* growth in culture is controversial. To evaluate this role, *T. vaginalis* was grown in broth and cell culture and the cell-free filtrate was applied to fresh cell culture monolayers. When adjusted to pH 6.5, filtrates obtained from 22-h culture growth totally disrupted McCoy, HEp-2, human foreskin fibroblast, and Chinese hamster ovary cell monolayers within 6 h. These detached cells remained >90% viable. This cell-detaching factor (CDF) was heat and acid labile, with a pH optimum of 6.5. CDF has trypsinlike activity which disrupts monolayer cells, but cells do not die if the pH is controlled. CDF was purified by ethanol precipitation, ammonium sulfate fractionation, and ion-exchange and gel filtration column chromatography. A 200,000-molecular-weight glycoprotein which was also immunogenic by immunoblot with human sera reactive to *T. vaginalis* was isolated in this manner. This confirms the presence of a specific soluble CDF derived from *T. vaginalis* whose application may be important as a diagnostic tool and in further studies of pathogenesis.

Trichomonas vaginalis is a frequently encountered genital pathogen (3, 4). Despite the high prevalence of trichomoniasis, the pathogenetic mechanisms of *T. vaginalis* have not been well characterized. Previous studies have examined the behavior of *T. vaginalis* grown in cell culture (1, 5, 8, 13, 17, 19) and have shown that there is little doubt that *T. vaginalis* has a cytotoxic effect on cell culture monolayers that is dependent upon the inoculum size and length of time that *T. vaginalis* is in contact with the monolayers (1, 13, 18). However, the mechanism of this interaction has not been adequately explained. Many observers have reported that the close contact of *T. vaginalis* with the cell culture monolayer is responsible for the disruption of the monolayer (1, 5, 8, 13, 18; E. Kotcher and A. C. Hoogasin, J. Parasitol. **43**[Suppl]:102, 1957). Although products of the phagocytosis of *T. vaginalis* by macrophages have also been proposed as another pathogenic mechanism (8, 16), this would not explain the cytotoxicity seen in the absence of macrophages. Soluble products of *T. vaginalis* have been suspected as being important in the pathogenesis of this disease because although vaginitis is often diffuse, the organism may not be uniformly distributed (8, 16, 17, 22). Hogue (14) first reported the effects of *T. vaginalis* on cell cultures and noted that cell-free filtrates of *T. vaginalis* culture had similar effects. Honigberg and Ewalt (17) suggested that *T. vaginalis* pathogenicity on chick liver cell culture was caused by both direct contact and soluble cytotoxins. However, other investigations, including recent studies by Alderete and Pearlman (1) and Krieger et al. (18), were unable to detect the presence of a cell-free cytotoxin (1, 5, 18, 19). Alderete and Pearlman (1) and Krieger et al. (18) agreed with the conclusion of Christian et al. (5) that if a soluble cytotoxin is present, it is probably produced only in small quantities or is a labile substance.

In contrast to these, Pindak et al. (23) demonstrated in a recent study that cell-free filtrates of *T. vaginalis* do cause an effect, with rounding of cells and disruption of the cell culture monolayers. Our objectives were to further evaluate the monolayer-disrupting effect and determine the role of pH in cytotoxicity. We then isolated a cell-detaching factor (CDF) of the *T. vaginalis* filtrate that causes the cytopathic effect seen.

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MATERIALS AND METHODS

Strains of *T. vaginalis*. Eleven isolates of *T. vaginalis* were obtained from vaginal secretions from women and urine and urethral specimens from men. *Pentatrichomonas hominis* (ATCC 3000), a human gastrointestinal trichomonad, was purchased from the American Type Culture Collection, Rockville, Md. As previously reported (11), organisms were grown in ambient air at 37°C in glass, screw-cap tubes (16 by 125 mm) containing 13 ml of Diamond TYI-S-33 medium (6) (TYI [pH 6.2]) supplemented with 10% heat-inactivated bovine serum. Cultures routinely attained a concentration of 2×10^6 to 6×10^6 *T. vaginalis* cells per ml if an inoculum of $\geq 3 \times 10^3$ *T. vaginalis* cells per tube was used. Initial clinical isolates were cultured with penicillin (100 U/ml), streptomycin (100 μg/ml), and nystatin (100 U/ml). Cultures were passaged every 3 to 4 days, and antimicrobial agents were used only as long as needed to remove contamination. Axenic cultures were mixed with additional (final concentration, 15%) bovine serum and 10% dimethyl sulfoxide and stored in liquid nitrogen.

Growth of *T. vaginalis* in cell culture. McCoy cells were grown in CMGA medium (11) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories–Life Technologies Inc., Chagrin Falls, Ohio). A 1-ml portion of a CMGA suspension containing 1.5×10^5 McCoy cells per ml was inoculated into glass MLB polyshell snap-cap vials (15 by 45 mm) (John's Scientific, Toronto, Ontario, Canada). After 48 h of incubation at 37°C, confluent monolayers resulted, with an average of 3×10^5 to 5×10^5 McCoy cells per vial. HEp-2, human foreskin fibroblast (HFF) passage 3 to 8, and Chinese hamster ovary (CHO) cells were also

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grown in CMGA and inoculated into vials, as was done with the McCoy cells. As previously described (10, 11), isolates of *T. vaginalis* were incubated at 37°C in TYI medium for 48 to 72 h to mid-log growth. The culture was then centrifuged at 500 × *g* for 5 min, and the pellet was washed three times by suspension and centrifugation in phosphate-buffered saline (PBS; pH 7.2). The pellet was then suspended in a combined medium of CMGA-TYI at a ratio of 2:1 (pH 6.8). Motile *T. vaginalis* cells were counted by using a hemacytometer, and appropriate dilutions were made in CMGA-TYI. Confluent McCoy monolayers in vials were washed three times with PBS, inoculated with 1.0 ml of *T. vaginalis* diluted in CMGA-TYI, and incubated overnight at 37°C.

Effects of pH on McCoy cell monolayers. To assess the effect of pH changes on McCoy cell monolayers, portions of CMGA-TYI were adjusted with 1 N HCl and 1 N NaOH so that their pHs ranged from 4.0 to 7.0 in increments of 0.5. At each pH, three vials containing McCoy cell monolayers were used. Unadjusted CMGA-TYI (pH 6.8) and CMGA (pH 7.2) were used as controls. All media were filter sterilized through 0.22-μm filters (Millipore Corp., Bedford, Mass.) before use.

Preparation of cell-free filtrates. Supernatants from *T. vaginalis* grown on McCoy cell monolayers were filtered through 0.22-μm cellulose acetate filters (Millipore). The cell cultures were inoculated with 10^3 to 10^5 *T. vaginalis* cells per ml, and the supernatants were sampled at various time intervals. *T. vaginalis* cells grown to early and late log phase in TYI broth culture were similarly filtered through 0.22-μm filters, and this filtrate was mixed with 2 volumes of CMGA. If indicated for an experiment, the broth filtrate was adjusted to pH 6.5 with 1 N NaOH and filter sterilized. Filtrates from uninoculated McCoy cell monolayers grown in CMGA-TYI for up to 2 weeks and filtrates of *P. hominis* similarly grown in cell culture were used as controls. A 1-ml sample of undiluted filtrate or filtrate diluted with CMGA-TYI was placed on fresh McCoy cell monolayers and incubated at 37°C. Filtrates were tested at least in triplicate and in repeated experiments to assess their effects on monolayer confluence and cell viability.

To evaluate the effect of pH on filtrate activity, we used a modification of the microdilution cytotoxicity assay as described by Lushbaugh et al. (21). McCoy cells at 4×10^5/ml in CMGA were inoculated in 0.1-ml aliquots into each well of 96-well, flat-bottom microdilution plates (Costar, Cambridge, Mass.). After 22 h in 5% CO_2 at 37°C, confluent monolayers resulted. The CMGA was then aspirated, and *T. vaginalis* filtrate was added in 0.1-ml aliquots, in triplicate, to the initial column of wells. After serial twofold dilutions in CMGA-TYI were made, the plates were incubated for 22 h in 5% CO_2 at 37°C. The monolayers were assessed as described below.

Assessment of monolayers. For assays in which monolayers in glass vials were used, each monolayer was assessed visually for the presence of CDF by using inverted light microscopy. Each monolayer was scanned at low power (×5) and then at ×20. A minimum of 20 fields were screened. Confluence was estimated visually (0, 10, 25, 50, 75, 90, and 100%), and when sections of the same monolayer differed in confluence, an average confluence for the monolayer was calculated. When using a microdilution assay, the monolayers were assessed as above for percent confluence and by being stained with crystal violet by the method of Alderete and Pearlman (1). A Bio-Tek 310 autoplate reader was used to measure the optical density of individual wells at 540 nm.

The viability of monolayer cells was determined by trypan blue exclusion. A minimum of 100 cells were counted by using a hemacytometer, and viability was expressed as a percentage of the total cells counted.

Preliminary physicochemical properties of the filtrate. Aliquots of cell-free filtrates of *T. vaginalis* were either tested immediately or stored at −70, −20, 4, or 20°C. The filtrate was also subjected to heating at 59°C for 30 min in a water bath. For evaluation of the effect of pH on filtrate CDF activity, filtrate samples (pH 6.5) were adjusted to pH 3.5 to 6.0 with 1 N HCl or to pH 7.0 to 8.5 with 1 N NaOH. The filtrates were then filter sterilized and left at room temperature or 4°C for 16 to 18 h. The pH of all filtrates was then readjusted to 6.5, the filtrates were applied to the microdilution plates and incubated for 22 h in 5% CO_2 at 37°C, and the monolayers were examined as above.

Filtrate purification. (i) Ethanol precipitation. Cell-free filtrate at 4°C was added to 2 volumes of ice-cold ethanol (−20°C), immediately mixed, and placed on ice for 2 h. The resulting precipitate was removed by centrifugation at 7,000 × *g* for 30 min, and then dissolved in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid)–10 mM NaCl (pH 6.0; HEPES buffer).

(ii) Fractionation with ammonium sulfate. Solid ammonium sulfate was added to the dissolved precipitate to 30% of saturation (176 g/liter), and the mixture was stirred for 1 h on ice. Precipitated protein was removed by centrifugation at 10,000 × *g* for 30 min. The supernatant at 30% saturation was brought to 65% saturation by addition of 235 g of ammonium sulfate per liter, stirred for 1 h on ice, and centrifuged at 10,000 × *g* for 30 min. The supernatant was dialyzed at 4°C against three changes of HEPES buffer over a period of 40 h. The dialyzed supernatant was then concentrated to 20 to 30 ml by ultrafiltration through a CX-10 cellulose acetate filter (Millipore).

(iii) Ion-exchange chromatography. The concentrated supernatant was purified at a flow rate of 42 ml/h over a column containing at 15-cm bed of DEAE Sephacel (no. K16×20; Pharmacia, Uppsala, Sweden) equilibrated with HEPES buffer. Fractions were eluted from the column by increasing ionic strength, beginning with 10 mM NaCl, then 100, 300, and 500 mM NaCl. Column fractions were dialyzed against three changes of HEPES buffer and monitored for CDF activity by using the microdilution system described above. Fractions containing CDF were pooled and concentrated to 2.0 ml by Millipore CX-10 ultrafiltration.

(iv) Gel filtration chromatography. The concentrated samples from the ion-exchange column were pumped at a flow rate of 9 ml/h over a gel filtration column (1.5 by 120 cm) containing a 120-cm bed of Sephacryl S-300 HR (Pharmacia) equilibrated with HEPES buffer. Column fractions (2 ml) were eluted with HEPES buffer. CDF activity was monitored by the microdilution assay described above. Active fractions were concentrated by ultrafiltration prior to gel electrophoresis.

Protein determination. The protein concentration was determined by the method of Bradford (2), using the commercially available kit from Bio-Rad Laboratories, Richmond, Calif., in which bovine serum albumin is used as the protein standard.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the procedure of Laemmli (20), with 7% acrylamide mini-Protean II dual slab gels (Bio-Rad). Samples to be applied were adjusted to 0.5 M Tris (pH 6.8), 2% SDS, 10% glycerol, 0.025% bromophenol blue, and 2% 2-mercaptoethanol and then heated to 100°C for 5 min before being applied to the

gel. Electrophoresis was carried out at 150 V until the dye band reached the bottom of the gel. Proteins were then visualized by silver staining (Bio-Rad) or by immunoblot (see below).

Immunoblot. The Bio-Rad technique, based on the method of Towbin et al. (25), was used for the immunoblot analysis. The nitrocellulose was hydrated in transfer buffer (20% [vol/vol] methanol, 25 mM Tris, 192 mM glycine [pH 8.3]) at 4°C and blotted overnight at 30 V. It was placed in a blocking solution of PBS–2% skim milk (Blotto) and incubated at 37°C for 1 h in a rocking apparatus. It was then washed five times with PBS–0.1% Tween 20 (PBS-Tween). The first antibody solution, consisting of a 1:300 dilution of a high-titer anti-*T. vaginalis* human serum in Blotto was incubated overnight at 20°C. After being washed, the blot was incubated for 1 h at 37°C with a 1:300 dilution of sheep anti-human immunoglobulin G biotinylated (Amersham Corp., Arlington Heights, Ill.) in Blotto. After being washed as above, the nitrocellulose was incubated for 30 min at 20°C with 1:300 streptavidin biotinylated horseradish peroxidase complex (Amersham) in Blotto. After being washed, the bands were developed with 3-amino-9-ethyl carbazole (3AEC) (Sigma Chemical Co., St. Louis, Mo.). 3AEC was prepared by mixing 180 mg of 3AEC in 45 ml of *N*,*N*-dimethylformamide and then adding 105 ml of 0.05 M acetate–acetic acid buffer (pH 5.0). The solution was mixed, filtered through a 0.22-μm filter, and stored at −20°C in the dark. Immediately prior to use, 1.0 μl of 30% H_2O_2 per ml of 3AEC substrate was added.

Serum samples from uninfected patients and nonreactive by *T. vaginalis* enzyme-linked immunosorbent assay (ELISA) (10) were used as controls in the immunoblot. As a further control to test the specificity of the second biotinylated antibody, the incubation with human serum was omitted from the immunoblot staining procedure. The pattern of transferred protein from gel to nitrocellulose was confirmed by staining the blotted nitrocellulose with 0.2% Ponceau Red in 0.3% trichloroacetic acid. The blotted gel was also checked for residual untransferred protein by silver stain.

Characterization of CDF. CDF, purified by gel filtration as described above, was incubated with proteinase K and sodium periodate. Ten microliters of proteinase K (500 μg/ml) and 90 μl of CDF were mixed and incubated at 37°C for 3 h. Ten microliters of 0.4 M $NaIO_4$ and 90 μl of CDF were mixed and similarly incubated. After incubation, the treated CDF preparations were dialyzed against HEPES buffer and examined by SDS-PAGE in which they were compared with controls diluted similarly with water, incubated, and dialyzed as described above.

Purified CDF was adjusted to pH 4.5 with 0.1 N HCl and incubated overnight at 20°C. SDS-PAGE was used to compare this acid-treated CDF with CDF at pH 6.5, similarly incubated, as well as with control CDF.

Statistical methods. Statistical analysis was performed by the paired *t* test and by the correlation coefficient method.

RESULTS

CDF activity on monolayers. Coincubation of confluent McCoy cell monolayers with cell-free filtrates from *T. vaginalis* growth resulted in detachment of the monolayer cells from the vial surface. The monolayer exhibited rounding of cells, with focal areas of sloughing which enlarged over time until the entire monolayer could be detached with one swirl of the vial. This cell-detaching effect is similar to that seen when *T. vaginalis* is in contact with monolayer cells.

The initial viability of the detached cells, as determined by visual inspection of trypan blue exclusion, was 95%, decreasing to 75% after 48 h of exposure to the filtrate. If washed and seeded to new vials, these detached cells grew and produced new confluent monolayers without alteration of growth kinetics.

Neither CMGA or CMGA-TYI had any effect on the McCoy monolayer when incubated as above. Filtrates derived from McCoy monolayers maintained in CMGA-TYI for 2 weeks (monolayers uplifting) had no CDF properties when applied to new McCoy monolayers for 6 days. When dead *T. vaginalis* (1.6×10^5 *T. vaginalis* cells obtained from overgrown broth culture) were used as inocula for filtrate preparation, the resulting filtrate had no CDF properties. Interestingly, filtrate prepared from inoculating 1.0×10^5 live *T. vaginalis* cells onto dead McCoy monolayers (monolayers rendered nonviable by exposure to CMGA [pH 4.5] for 24 h) did exhibit a similar cell-detaching effect to that obtained from *T. vaginalis* growth on live McCoy cells. Secretion of CDF appears to be dependent on the viability of *T. vaginalis* cells and independent of McCoy cell viability.

As another control, filtrates were prepared from *P. hominis* similarly grown in cell culture. Suspensions of *P. hominis* in CMGA-TYI were inoculated into vials containing McCoy monolayers. An inoculum of 1.0×10^5 *P. hominis* cells per ml grew to 1.0×10^6 in 40 h (pH 6.8). The monolayers remained >95% intact and viable. Filtrates of *P. hominis* were prepared at 16 and 40 h and applied to fresh McCoy monolayers. After 5 days of incubation, the monolayers remained >90% intact and viable, and no rounding or disrupting effects were seen.

Effects of pH on McCoy monolayer. The effect of pH on McCoy monolayers in vials was examined to determine the role of pH as a possible cause of CDF activity.

The pH of CMGA-TYI had a direct effect on cell viability, since exposure of McCoy cells to pH < 6.0 resulted in rapid cell death. At 24 h, 98% of McCoy cells in CMGA-TYI at pH 6.7 ± 0.1 remained viable compared with 89% at pH 6.2 ± 0.2 ($P < 0.025$), 55% at pH 5.7 ± 0.2 ($P < 0.005$ versus pH 6.2 or 6.7), and 5% at pH 5.2 ± 0.2 and 4.5 ± 0.05 ($P < 0.005$ versus pH 5.7, 6.2, 6.7).

pH had no effect on monolayer confluence. Although at pH < 6.0, the monolayer cells were rounded and rapidly nonviable, monolayer confluence was maintained for 5 days. At 7 days, all monolayers in vials showed decreased confluence regardless of pH. Therefore, when pH was examined independently, confluence was not affected, even though cell death occurred at pH < 6.0. This is in direct contrast to the CDF properties of monolayer disruption without cell death.

Physical properties of CDF. Analysis of the CDF preparation procedure revealed that the quantity of CDF in filtrates prepared as described above varied with three factors: duration of *T. vaginalis* growth prior to filtrate preparation, initial inoculum of *T. vaginalis*, and pH of the filtrate at the time of harvesting.

To demonstrate the increased formation of CDF over time, multiple vials containing McCoy monolayers were inoculated with 1.0×10^5 *T. vaginalis* cells as described above. Filtrates were prepared at 0, 1, 2, 4, 8, 12, and 22 h and placed on fresh monolayers at the following dilutions in CMGA-TYI: undiluted, 1:2 dilution, and 1:10 dilution. Experiments were done in triplicate and read by visual inspection. In Fig. 1 and 2, the points represent mean values of triplicate samples with standard errors. In Fig. 1, each curve represents a different time (postinoculation) of preparation of the filtrate and effect of that filtrate (1:2 dilution) on

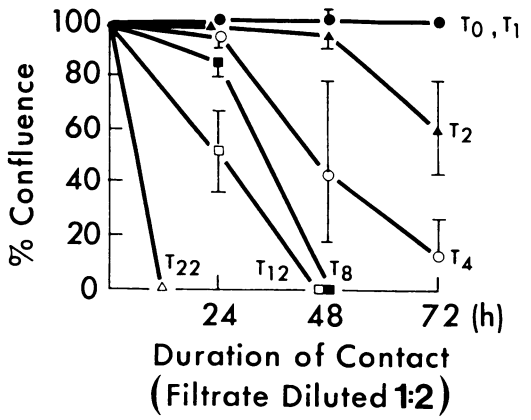

FIG. 1. Filtrates were prepared from cell culture vials inoculated with 1×10^5 *T. vaginalis* cells per ml at different times after inoculation (in hours) as represented by the curves (T_0, T_1, T_2, T_4, T_8, T_{12}, and T_{22}, where the subscript gives the number of hours). The effect on fresh monolayers of the filtrate diluted 1:2 with CMGA-TYI was observed over 72 h and is expressed as the percent confluence of the cell culture monolayer.

McCoy monolayers over a 72-h period. With increasing incubation time and growth of *T. vaginalis* in cell culture, the resulting filtrates caused more monolayer disruption (Fig. 1).

The detaching effect of the filtrate could be titrated out, with more dilute filtrates requiring longer incubations to demonstrate an effect. Filtrate was obtained from a 22-h growth of *T. vaginalis* in cell culture, and serial twofold dilutions (undiluted to 1:64) were inoculated onto McCoy monolayers in triplicate. In Fig. 2, each curve represents observations of the monolayers at 0, 3, 6, 12, and 24 h after addition of the filtrates. At 0 and 3 h (Fig. 2, T_0, T_3), no effect on the monolayer was seen. By 6 h, undiluted filtrate disrupted the monolayer, whereas at all other dilutions the monolayer remained confluent. At 12 h, there was a significant correlation between the dilution of the filtrate and the effect over time on the confluence of the monolayer (r = 0.95, $P < 0.01$). With more dilute filtrates, the disruptive effect was seen, but it occurred more slowly.

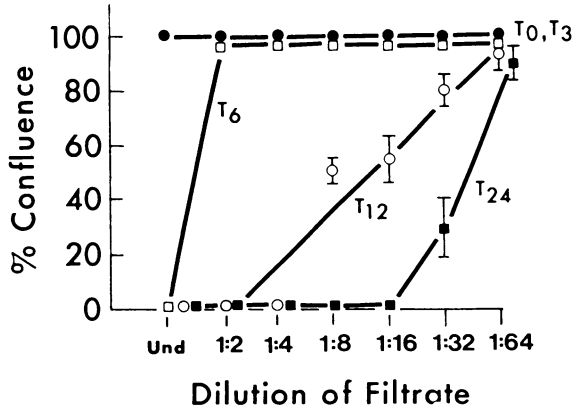

FIG. 2. Filtrates were prepared from the supernatant fluid of vials containing McCoy cell monolayers inoculated with 1×10^5 *T. vaginalis* cells per ml and grown for 22 h. Undiluted filtrate and filtrate diluted with CMGA-TYI were added to fresh monolayers. The curves represent observation of the monolayers at 0, 3, 6, 12, and 24 h after addition of the filtrates (curves T_0, T_3, T_6, T_{12}, and T_{24}, respectively). At 12 h, there was a significant correlation between the dilution of the filtrate and the effect on monolayer confluence (r = 0.95, $P < 0.01$).

To investigate the effect of initial inoculum size on CDF activity, we used three inocula of *T. vaginalis* (3.0×10^3, 3.0×10^4, and 4.0×10^5 *T. vaginalis* cells per ml) in a 22-h filtrate preparation. CDF activity was greater in the filtrate prepared from 3.0×10^4 *T. vaginalis* cells than in the filtrate prepared from 3.0×10^3 cells (pH of both filtrates 6.5). Initially, no CDF effect could be seen with the filtrate 4.0×10^5 prepared from cells (pH 4.7), although rounding of the McCoy cells and 100% cell death were observed. When the pH of this 22-h filtrate was adjusted to 6.5 from 4.7, the CDF effect was restored and fresh McCoy monolayers were disrupted after 6 h. If the incubation of this culture containing 4.0×10^5 *T. vaginalis* cells was extended to 72 h (pH 4.0), the CDF activity was irreversibly destroyed, since even after adjustment to pH 6.5, the filtrate had no disrupting effect on McCoy monolayers.

Filtrates obtained from *T. vaginalis* grown in TYI broth (without cell culture coincubation) also showed CDF activity. Filtrate was prepared by using an inoculum of 1.0×10^5 *T. vaginalis* cells per ml and a 22-h incubation, and 9.0×10^5 *T. vaginalis* cells per ml (pH 6.2) resulted. When this filtrate was mixed with 2 volumes of CMGA and applied to McCoy monolayers, disruption occurred by 48 h. If the filtrate was prepared from a similar culture incubated for 48 h to a concentration of 5.0×10^6 cells per ml (pH 4.5), no CDF activity was seen even after adjustment to pH 6.5. These results are similar to the pH effect observed above after 72 h of *T. vaginalis* growth in cell culture.

CDF effect on other cell lines and with other *T. vaginalis* isolates. Filtrates of *T. vaginalis* stored for 1 week at −70°C were quickly warmed and placed on McCoy, HEp-2, HFF, and CHO monolayers. At 6 h, 1 ml of undiluted filtrate disrupted all four monolayers. HFF and CHO cells appeared more sensitive than the others to the filtrate effect, because 1:2 dilutions in CMGA-TYI disrupted the HFF and CHO monolayers by 6 h, whereas the other two cell lines were intact. By 24 h, all four cell lines were destroyed by a 1:10 dilution. The above findings are all from *T. vaginalis* isolate 2 (10). Ten additional isolates were grown in cell culture, and their filtrates also showed CDF activity.

Physicochemical properties of CDF. Filtrates were prepared from 22-h culture growth, and the pH was adjusted to 6.5. The CDF activity of undiluted filtrates was preserved by storage at −70 or −20°C for 6 months and at 4°C for at least 7 weeks. Storage at room temperature for 5 days caused a delay of disruption of McCoy monolayers to 24 h as compared with 6 h for storage at 4°C. CMGA-TYI control filtrates (no *T. vaginalis*) remained negative for CDF activity throughout the various storage periods under different conditions.

CDF activity was transferrable from one monolayer to another, as shown when filtrate that disrupted new monolayers within 24 h was removed and refiltered and then still disrupted new monolayers within 24 h.

Heating the filtrate to 59°C for 30 min destroyed the CDF activity. The acid lability of the CDF was also investigated by using serial twofold dilutions in a 96-well microdilution plate system. CDF activity was expressed as the highest dilution of filtrate producing total loss of confluence upon gentle agitation of the monolayer after 18 h of incubation. Exposure of the filtrate to pH 3.5 to 4.5 totally inactivated CDF activity. CDF activity was present from pH 5.0 to 8.5, with peak activity at pH 6.5.

Purification of the CDF. The majority of the recoverable

TABLE 1. Purification summary

Purification stage	Amt of protein (mg)	Activity (U)[a]	Sp act (U/mg)	Recovery (%)[b]
Cell-free filtrate	73.50	152,800	2,079	100
Ammonium sulfate fractionation	15.54	47,360	3,048	31.0
Ion-exchange chromatography	2.67	19,840	7,431	13.0
Gel filtration	0.065	720	11,077	0.4

[a] One unit of CDF activity is defined as the reciprocal of the last dilution with 50% or less confluency in the microdilution cytotoxicity assay.
[b] Based on CDF activity.

activity from the unpurified cell-free filtrate was found in the 65% supernatant fraction following ethanol precipitation and ammonium sulfate fractionation, resulting in a 47% increase of specific activity (Table 1). The CDF activity was bound by DEAE-Sephacel in the subsequent purification step, and analysis of the column fractions indicated that the activity eluted with 100 mM NaCl and represented a 144% increase in specific activity. When these active fractions were pooled, dialyzed, concentrated, and applied to a Sephacryl S-300 gel filtration column and the resulting active fractions were analyzed by SDS-PAGE, a single band with a molecular size of 200 kilodaltons (kDa) was observed (Fig. 3).

When this purified CDF was incubated with either proteinase K or sodium periodate, degradation of the 200-kDa band was observed by SDS-PAGE. These degraded products showed no cytotoxicity on McCoy monolayers. Furthermore, total inactivation of the CDF activity occurred when the band was incubated at pH 4.5 overnight at room temperature, although no changes could be observed by SDS-PAGE.

Immunoblot analysis of the 200-kDa band and the unpurified filtrate with ELISA high-titer anti-*T. vaginalis* human sera (10) showed a specific staining of the 200-kDa band with immune sera (Fig. 3) which was not seen when using nonimmune sera.

DISCUSSION

Although the potential importance of a cell-free cytotoxin has been postulated in the pathogenesis of *T. vaginalis* infection (8, 13, 16, 17), recent observers, with the exception of Pindak et al. (23), have been unable to demonstrate a cytotoxic effect on cell culture monolayers by using cell-free filtrates of *T. vaginalis* (1, 18). In our experiments, we clearly and reproducibly showed that cell-free filtrates of all 11 isolates of *T. vaginalis* tested produced a monolayer-detaching effect. The effect was not produced by *P. hominis*. It was more easily produced by *T. vaginalis* grown in cell culture, but could also be obtained from filtrates of *T. vaginalis* grown in TYI medium without McCoy cells.

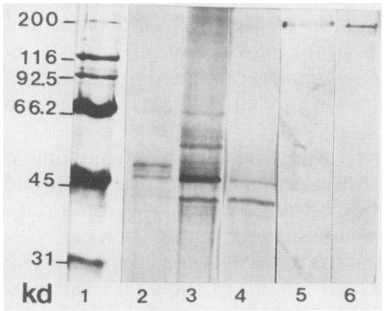

FIG. 3. Lanes 1 to 5 show SDS-PAGE of CDF active fractions during purification. Lanes: 1, molecular size standards (200 kDa, myosin; 116 kDa, β-galactosidase; 92.5 kDa, phosphorylase *b*; 66.2 kDa, bovine serum albumin; 45 kDa, ovalbumin; 31 kDa, carbonic anhydrase); 2, unpurified filtrate; 3, after ammonium sulfate fractionation; 4, after ion-exchange chromatography; 5, after gel filtration. Lane 6 shows an immunoblot of the 200-kDa purified CDF with ELISA-positive human serum.

Alderete and Pearlman (1) failed to show cytotoxic effects with lyophilized cell-free filtrates prepared from *T. vaginalis* grown in TYI medium. Unfortunately, no data were given about the inoculum or duration of growth of *T. vaginalis* before filtering. They concluded that pH was not the cause of the monolayer disruption seen in *T. vaginalis* grown in cell culture, because if they maintained the pH, monolayers were still disrupted. Our data are consistent with their data, and we also do not believe that pH causes the monolayer disruption. On the contrary, pH < 5.0 will inactivate the CDF on the monolayer.

Krieger et al. (18) made filtrates of 10^5 to 10^7 *T. vaginalis* cells per ml both from 2-h cultures in serum-free medium and from cultures of *T. vaginalis* actively destroying CHO monolayers. We have shown that filtrates of a 2-h inoculation of 10^5 *T. vaginalis* cells show little disrupting effect. Also, large inocula of *T. vaginalis* often have pH < 5.0, which could prevent the manifestation of CDF.

Krieger et al. (18) also studied the effects of filtered extracts of sonicated *T. vaginalis* cells and failed to detect a cytotoxic effect. This is in contrast to the findings of Gentry et al. (12), who demonstrated a cytopathic effect of *T. vaginalis* lysates. Gentry et al. (12) also found that the cytopathic effect of the lysates was inhibited by serum and was dependent on the amount of protein present in the lysate. In the presence of less protein, a longer incubation time was needed to demonstrate the same cytopathogenicity. This is similar to our results with cell-free filtrates, in which the effect was dependent on the inoculum size and the duration of growth of *T. vaginalis* prior to filtration. With longer growth in cell culture, the effect of the filtrate was seen more rapidly and with more dilute filtrate.

Our findings confirm the data of Pindak et al. (23), who recently reported a CDF derived from *T. vaginalis* cells grown in cell culture. However, we have also shown that the CDF can be derived from broth cultures if the pH is appropriately controlled to prevent inactivation and from growth of *T. vaginalis* cells on dead McCoy cell monolayers. In this way, we have shown that the CDF is conclusively derived from *T. vaginalis* and not from the monolayer cells.

We have also isolated and characterized the CDF. It is a 200-kDa band in SDS-PAGE that is destroyed by coincubation with both proteinase K and periodate, suggesting that it is a glycoprotein. It is pH and heat labile, the pH lability probably explaining why other investigators have been unable to demonstrate its presence.

The isolated glycoprotein has the same detaching properties as seen in the unpurified filtrate. It acts similarly to trypsin in that it causes detachment of monolayer cells without affecting cell viability. Immunoblots using three ELISA-positive anti-*T. vaginalis* human sera all showed specific staining of the 200-kDa CDF in both unpurified and purified fractions, which was not seen with nonimmune sera. This substance is present in very small quantities in *T. vaginalis* cells, because when trichloroacetic acid precipitates of these cells were analyzed by silver stain and immunoblot

(10), only the highly sensitive immunoblot method was able to demonstrate the 200-kDa band, whereas silver staining was negative.

The pathogenic role of the CDF is not known. It may explain the clinical findings of erythema seen in the vaginal mucosa during acute *T. vaginalis* infection (24). These clinical findings are analogous to the cell sloughing seen in vitro. The antibody response to *T. vaginalis* does not appear to eradicate the disease. However, our preliminary experiments do show that filtrate coincubated with ELISA-reactive sera markedly reduced the detachment of the monolayer cells compared with the control (nonreactive sera). This may explain the state of the vagina in chronic *T. vaginalis* infection (15, 16), in which the infection is still present but the inflammatory response has diminished substantially.

The fact that low pH inactivates the CDF does not mean that the effect is clinically irrelevant, because, in contrast to the normal vaginal pH of <4.5 (9, 15, 24), in the presence of *T. vaginalis* infection the vaginal pH is usually >5.0 (9, 15). It is at these higher pHs that the CDF is active in vitro.

In summary, the published literature has questioned the presence of a *T. vaginalis* cytotoxin or CDF. Our work has confirmed the presence of a soluble substance that causes detachment of monolayer cells (in vitro). The CDF is immunogenic, as demonstrated by immunoblot, and is inactivated by human sera reactive by ELISA to *T. vaginalis*. Thus, CDF may be a useful tool in diagnosis and in further studies of *T. vaginalis* pathogenesis.

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