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Isolation and Differentiation of Herpes Simplex Virus and *Trichomonas vaginalis* in Cell Culture

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During the period January 1982 to January 1985, 2,234 specimens were cultured for isolation of herpes simplex virus (HSV). HSV was isolated from 23% of these, *Trichomonas vaginalis* was isolated from 1.6%, and 75.3% were negative. In 0.2% of these, HSV and *T. vaginalis* were isolated from the same specimen. Cytopathic effects produced by HSV were identified by their sensitivity to arabinosylthymine, whereas those produced by *T. vaginalis* were identified by their lack of sensitivity to arabinosylthymine and by observation of motility. Cytopathic effects produced by *T. vaginalis* were reproduced by trophozoites from axenic cultures of *T. vaginalis* as well as by lysates of *T. vaginalis* added to serum-free BHK cells.

The laboratory diagnosis of herpes simplex virus (HSV) infections typically involves examination of cell cultures for the presence of virus-specific cytopathic effect (CPE). Although HSV-specific CPEs generally have a characteristic appearance, there is sufficient variation from one isolate to the next that additional criteria are desirable. Although these often are based on identification of viral antigen, other criteria may also be used. A combination of host range and a specific anti-HSV compound, arabinosylthymine (araT), has been used to identify HSV CPE (2). By using baby hamster kidney (BHK) cells, we can exclude the only other clinically relevant herpesvirus that might be confused with HSV, varicella-zoster virus, because varicella-zoster virus does not grow in BHK cells. Any CPE observed to be resistant to araT can then be reliably excluded as not due to HSV. When our laboratory became operational, we observed a cervical specimen that produced a focal CPE similar to that of HSV but resistant to araT (Fig. 1). On closer examination we identified motile *Trichomonas vaginalis*. This report describes our subsequent experience in identifying *T. vaginalis* in clinical specimens and some preliminary experiments in which the CPEs were reproduced by using trophozoites from axenic cultures as well as cell-free lysates of *T. vaginalis*.

MATERIALS AND METHODS

Isolation of HSV and *T. vaginalis* from clinical specimens. Specimens on Bacteriologic Culturettes (Marion Scientific, Div. Marion Laboratories, Inc., Marion, Ind.) were extracted in 3 ml of viral transport medium (VTM; Flow Laboratories, Inc., McLean, Va.) to which had been added nystatin (Mycostatin; E. R. Squibb & Sons., Princeton, N.J.) (final concentration, 10 μ/ml) and amphotericin B (Fungizone) (final concentration, 5 μg/ml). The specimens were sonicated briefly (30 s, 30 kc/s, 30 W) in an ultrasonic bath (Ultrasonic Industries, Albertson, N.Y.) to disperse clumps and held at 4°C pending inoculation (within 24 h). BHK cell monolayers were prepared in 24-well Linbro plates and inoculated as described previously (11) with 0.2 ml of specimen in each of two wells. After being gently rocked at 35°C for 2 h under 100% humidity and 5% CO_2, the inocula were removed and 1 ml of Eagle minimal essential medium (EMEM) containing 5% fetal calf serum plus 2 mM glutamine, nystatin, and amphotericin B as described above, and gentamicin (final concentration, 50 μg/ml) was added to each well. The medium added to the second well (for each specimen) also contained 50 μg of araT per ml. The wells were incubated at 37°C under 100% humidity and 5% CO_2 and examined with an inverted microscope daily. A CPE typical of HSV could frequently be seen as early as 18 h postinoculation. If it was observed in the first well but not in the araT well, it was recorded as positive for HSV. Atypical CPEs were sometimes observed in both wells. These were recorded as negative for HSV. In some cases, however, motile trophozoites of *T. vaginalis* were seen in both wells, and these specimens were recorded as positive for *T. vaginalis*. Finally, on rare occasions CPEs typical of HSV were observed in the first well but not in the second, together with atypical CPE plus motile trophozoites in both wells. These were recorded as positive for both HSV and *T. vaginalis*.

Motile *T. vaginalis* trophozoites were removed from the tissue culture wells with a sterile, cotton-plugged Pasteur pipette and inoculated into medium TY1-S-33 as modified by Keister for the axenic growth of *Giardia lamblia* (17). The modified TY1-S-33 medium was supplemented with bovine serum and 50 μg of gentamicin or penicillin per ml. Cultures were incubated at 37°C and transferred every Monday and Friday by centrifuging the culture tubes at 200 × *g* and inoculating a portion of the pellet obtained into fresh medium. After initial culture in modified TY1-S-33 for 2 weeks, a modified medium was developed that supported better growth of *T. vaginalis* cells.

Growth of *T. vaginalis* strains in axenic cultures. The modified medium mentioned above (*T. vaginalis* medium; TVM) combines components of the Trypticase yeast extract-maltose medium of Diamond (7) and the modified TY1-S-33 medium of Keister (17). Each 100 ml of TVM contained 80 mg of K_2HPO_4, 80 mg of KH_2PO_4, 2 g of casein digest (BBL Microbiology Systems, Cockeysville, Md.), 1 g of yeast extract (BBL), 1 g of dextrose (Sigma Chemical Co., St. Louis, Mo.), 200 mg of NaCl, 200 mg of cysteine-hydrochloride monohydrate, 20 mg of ascorbic acid, 2.28 mg of ferric ammonium citrate, and 10 ml of inactivated (56°C, 30 min) bovine serum. The pH of the medium was adjusted to 6 with 0.1 N NaOH before the addition of serum. The complete (serum-containing) medium was filter sterilized as

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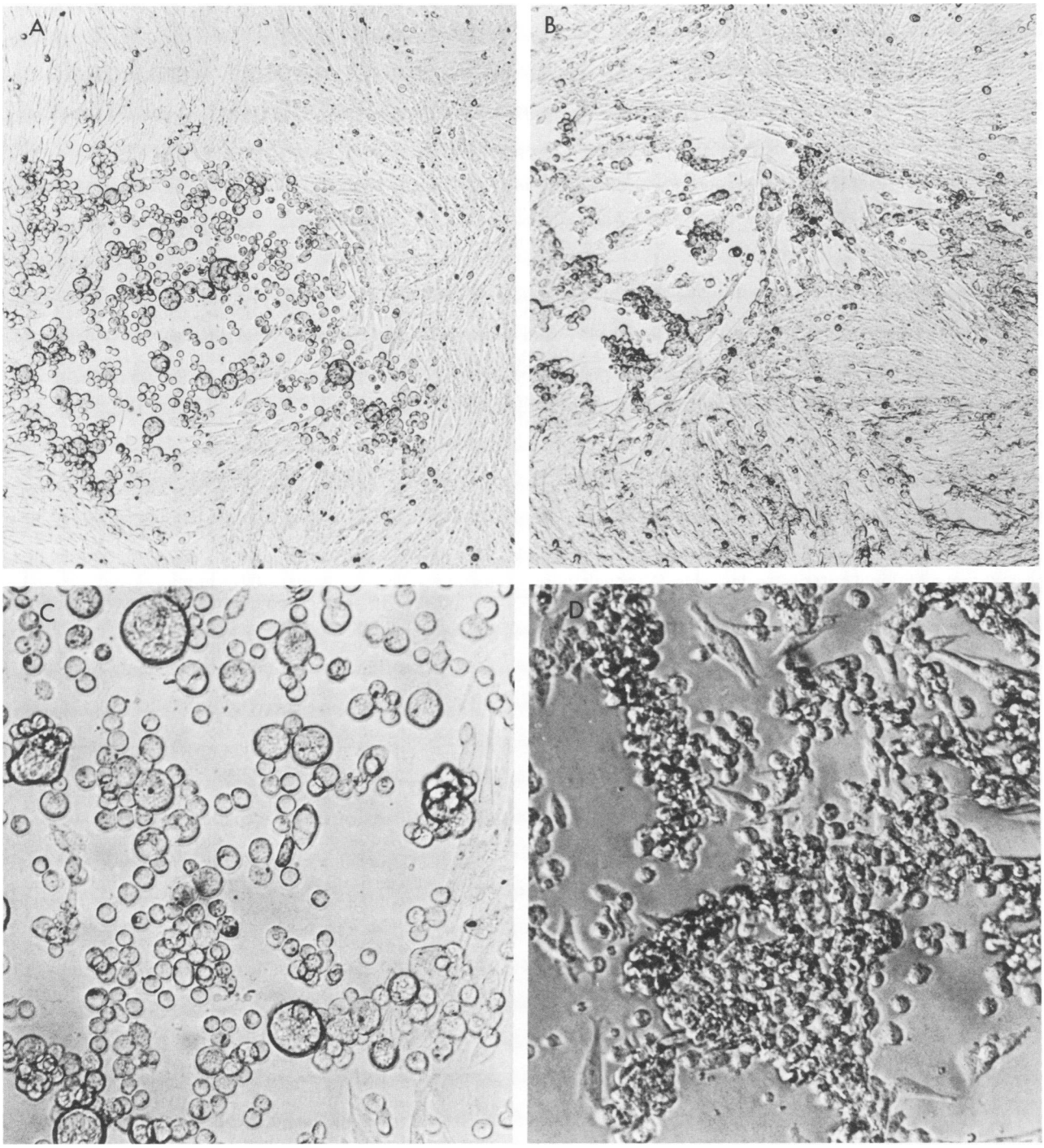

FIG. 1. CPE of HSV and *T. vaginalis* in clinical specimens at 24 h after inoculation of BHK cell monolayers. (A) HSV ×175; (B) *T. vaginalis* ×175; (C) HSV ×500; (D) *T. vaginalis* (different field from [B]) ×500.

follows. The medium was prefiltered through a stainless steel filter holder (diameter, 90 mm; Millipore Corp., Bedford, Mass.) containing a thick prefilter pad and three filters (pore sizes, 1.2, 0.8, and 0.45 μm) separated from each other by mesh screens. This prefiltered medium was sterilized by being pumped through a Millistack presterilized filter (pore size, 0.2 μm; Millipore) directly into sterile borosilicate screw-cap culture tubes. The medium remained effective for more than 1 month if stored tightly capped in the refrigerator. *T. vaginalis* cells grown in this medium on a slant rack at 37°C were transferred twice weekly by gently agitating the tube to suspend free-swimming trophozoites and inoculating 0.1 to 0.01 ml of suspension into a tube of fresh medium. Strains tested to date grew better at lower inocula in this medium than in the modified TY1-S-33 (17). The bacteriological sterility of the axenic *T. vaginalis* cultures was confirmed by subculture in broth medium.

Preparation of *T. vaginalis* lysate. Trophozoites from 36- to

48-h axenic TVM cultures (logarithmic growth phase) of two *T. vaginalis* strains that had been serially subcultured in TVM 8 to 10 times were harvested by centrifugation of the culture tubes and centrifugal washing of the pooled pellets in normal saline. After two saline washes, the intact live trophozoites were washed once in EMEM without serum, centrifuged at 200 × *g*, and resuspended in an equal volume of serum-free EMEM. This 50% cell suspension was frozen (−30°F) and thawed once and then centrifuged for 30 min at 30,000 × *g*. The supernatant was used in subsequent studies as *T. vaginalis* lysate after sterilization through a syringe filter (pore size, 0.22 μm; Schleicher & Schuell, Inc., Keene, N.H.). Protein concentrations were determined by the microbiuret method of Goa (12).

Preparation of dilutions of lysate and of trophozoites for testing on monolayers of BHK cells. *T. vaginalis* trophozoites of two strains isolated from different cases of trichomoniasis were grown and harvested from axenic cultures in TVM as described for lysate preparation. The saline-washed trophozoites were counted in a hemacytometer and diluted in serum-free EMEM to a final concentration of 70,000 trophozoites per ml (14,000 per 0.2-ml tissue culture well).

The methods for dilution of *T. vaginalis* lysate and trophozoites of two strains and their assay for CPEs have been described earlier for the assay of similar substances from *Entamoeba histolytica* (22, 25). BHK cells were grown to confluency at 37°C under 5% CO_2 in 96-well tissue culture plates containing 0.2 ml of complete EMEM with serum. Before the assay, the confluent monolayers were washed with three changes of serum-free EMEM. The last change was completely drained from the plate and replaced in successive wells with serial twofold dilutions of *T. vaginalis* lysates or trophozoites suspended in serum-free EMEM, serum-free EMEM plus araT (50 μg/ml), complete EMEM (with 5% serum), or complete EMEM plus araT (50 μg/ml). Inoculated plates were incubated (at 37°C under 5% CO_2) and read at 12-h intervals for 3 days. Endpoints (when the CPE affected 50% of the cells) were determined by phase-contrast microscopy and were expressed as the reciprocal of dilution or micrograms of protein per well.

RESULTS

Isolation of HSV and *T. vaginalis* from patient specimens. Of a total of 2,234 specimens obtained from various sites from patients of both sexes, 511 (23%) were positive for HSV, 35 (1.6%) were positive for *T. vaginalis*, and 5 (0.2%) were positive for both (Table 1). All of the specimens from which *T. vaginalis* cells were isolated were from female genital tracts (cervical or vaginal swabs).

TABLE 1. Isolation of HSV and *T. vaginalis* from patient specimens

Specimen	No. of specimens in:			
	1982	1983	1984	1982–1984
Total specimens	231	865	1,138	2,234
Positive for HSV	37 (16%)	231 (27%)	243 (21%)	511 (23%)
Positive for *T. vaginalis*	2 (0.9%)	6 (0.7%)	27 (2.4%)	35 (1.6%)
Positive for HSV and *T. vaginalis*	1 (0.4%)	1 (0.1%)	3 (0.3%)	5 (0.2%)

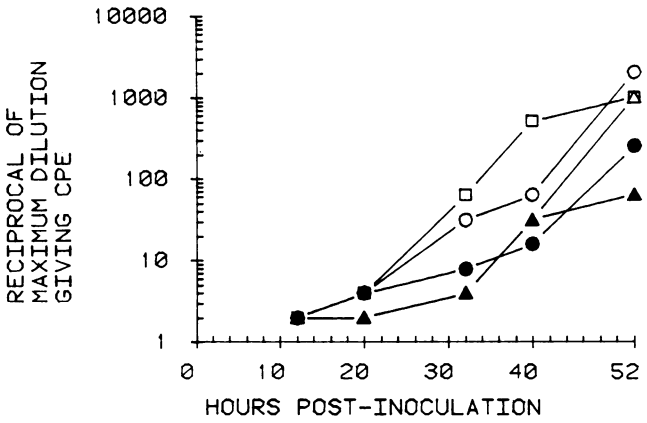

FIG. 2. Production of progressive CPEs over a 52-h incubation under 5% CO_2 at 37°C by trophozoites of *T. vaginalis* TV1 and TV2. *T. vaginalis* trophozoites were incubated in EMEM supplemented with antibiotics, with or without serum, with or without araT (an antiviral drug) over confluent monolayers of BHK cells in tissue culture plates as described in the text. The curves for strains TV1 and TV2 in the absence of serum were identical except at 52 h. Symbols: ○, EMEM plus serum (strain TV1); ●, EMEM plus serum (strain TV2); □, EMEM with serum and araT (strain TV1); △, EMEM without serum (strain TV1); ▲, EMEM without serum (strain TV2).

CPEs of *T. vaginalis* trophozoites. Trophozoites of two strains produced progressive CPEs that included additional wells of affected cells at higher dilutions of inoculum as the incubation progressed (Fig. 2). Both strains were capable of producing CPEs in the presence or absence of serum, although there was a longer lag when serum was absent from the incubation medium. Strain TV1 attained higher final CPE titers than did strain TV2, although starting inocula were comparable (7.1×10^4 versus 6.5×10^4 per ml, or about 14,000 per well). The presence of araT had no effect on the cytopathogenicity of strain TV1 trophozoites.

CPEs of *T. vaginalis* lysates. During the first 40 h of incubation, no CPE was produced by lysates on BHK cells in the presence of serum. In the absence of serum, lysates produced progressive CPEs, from a titer of 4 after 12 h of incubation to a titer of 16 after 32 h of incubation (Fig. 3). After 32 h of incubation there was no further progression in the titer of BHK cells affected by *T. vaginalis* lysates in the absence of serum. After 40 h of incubation, slight changes in the appearance of the BHK cells were noted at the highest concentration of lysate (1:4 = 6 or 4 μg of protein per well) incubated in the presence of serum. At the later observation period, more than 50% of the cells in one well were affected by the highest concentration of lysate tested in the presence of serum. In the absence of serum, 4 to 6 μg of *T. vaginalis* lysate protein per well caused CPEs after 12 h of incubation, and as little as 1 μg of *T. vaginalis* lysate protein per well produced CPEs after 32 h of incubation.

DISCUSSION

Differentiating the CPEs of HSV and *T. vaginalis*. Although the ability of *T. vaginalis* to induce cytopathic changes in cell cultures has been known for some time, there is to the best of our knowledge only one report (in 1985) of the isolation of *T. vaginalis* as a by-product of culturing for HSV (29). In that brief report it is suggested that *T. vaginalis* and HSV can be differentiated on the basis of CPE and the observation of

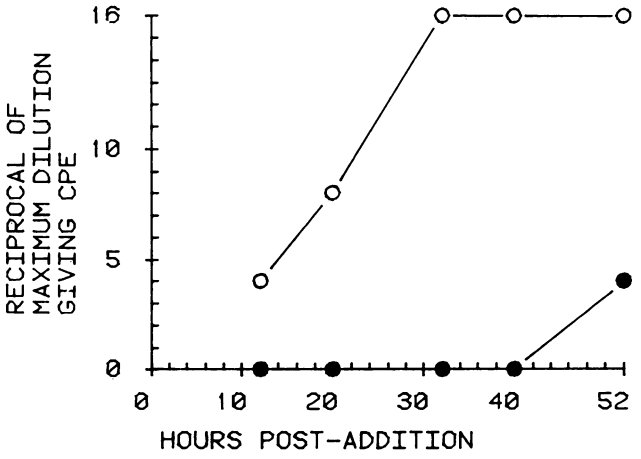

FIG. 3. Production of progressive CPEs by cell-free lysates prepared from trophozoites of two strains of *T. vaginalis* grown in axenic cultures. *T. vaginalis* lysate was serially diluted in serum-free or serum-containing EMEM and added to washed, confluent BHK monolayers as described in the text. In the first 40 h of incubation (5% CO_2, 37°C), CPE was observed only in the absence of serum (with or without the antiviral drug araT). Results from both strains were identical. Symbols: ●, EMEM plus serum, with or without araT; ○, EMEM without serum, with or without araT.

motility. We agree, but suggest that if motility is absent, the inexperienced observer may have some difficulty (Fig. 1). In our hands the sensitivity of the CPE to araT immediately rules out *T. vaginalis*, whereas resistance of the CPE to araT rules out HSV and indicates a thorough search for motile trophozoites. This use of araT appears to be quite reliable, because in the 2,234 specimens reported here there was not a single instance in which CPEs typical of HSV were found to be resistant to araT. This further suggests that in our patient population, drug-resistant mutants of HSV are presently nonexistent. In addition, there was no instance in which *T. vaginalis* cells were susceptible to araT. The other advantage of using araT is that when an atypical CPE appears early (15 to 18 h), it is often possible to reach a conclusion without having to wait for more extensive and obvious changes (although for a diagnosis of *T. vaginalis* motility must be observed at some point).

The mechanism by which araT differentiates between HSV depends primarily on its selective phosphorylation to 1-β-D-arabinofuranosylthymine monophosphate by the HSV-coded deoxythymidine kinase (2). Because the growth of *T. vaginalis* is not inhibited by araT, it seems clear that if *T. vaginalis* possesses a deoxythymidine kinase, it resembles the human deoxythymidine kinase in its inability to phosphorylate araT. On the other hand, it may, like some other organisms, use alternative pathways of thymine salvage such as nucleoside phosphotransferase or thymine-7-hydroxylase (10). Indeed, the related *Tritrichomonas foetus* depends on a nucleoside phosphotransferase for thymine salvage (30), and we consider it likely that *T. vaginalis* also uses this mechanism. It is also clear that other anti-HSV compounds that depend on the viral-coded deoxythymidine kinase should work as well as araT, provided that they are shown not to inhibit *T. vaginalis*. Acycloguanosine is probably the best alternative candidate; bromovinyldeoxyuridine would not be useful by itself because it does not inhibit HSV-2 (6). Indeed, we have on occasion used it to differentiate between HSV-1 and HSV-2 on primary isolation (data not shown); in this case a third well, containing bromovinyldeoxyuridine, was used.

Interaction of *T. vaginalis* with BHK monolayers. As the number of *T. vaginalis* trophozoites inoculated per well decreased, the production of CPEs took progressively longer. As few as 7 to 14 trophozoites (dilution 1:1,024) were capable of producing CPEs if incubated long enough. Initial production of CPEs by trophozoites took 12 to 24 h longer in the absence of serum. Trophozoites of either strain, with or without serum, produced CPE titers after incubation for 52 h. The TV2 strain produced a CPE less rapidly than did strain TV1, reflecting either slower growth under assay conditions or comparably less ability to produce a CPE on BHK monolayers than that of TV1. The rate of CPE production by trophozoites in this assay system was correlated with the size of the inoculum. Growing *T. vaginalis* trophozoites of a certain *T. vaginalis* population size may be required for CPEs to be produced.

Since Hogue (14) first reported the CPE of *T. vaginalis* trophozoites on tissue cultures in 1943, a number of workers have studied the growth of these protozoa in tissue cultures (1, 3, 4, 8, 9, 13, 15, 19, 20; E. Kotcher and A. C. Hoogasian, *J. Parasitol.* **43**:39, 1957). Kulda (19) reported that several species of nonpathogenic trichomonads had no effect on cell monolayers in culture, whereas pathogenic species destroyed the monolayer. Farris and Honigberg (8) studied a pathogenic and a mild strain of *T. vaginalis*, noting that in addition to toxic products released by *T. vaginalis* trophozoites, intimate contact between parasites and target cells was important in the formation of CPEs. Contact-dependent cytopathogenicity of *T. vaginalis* trophozoites is also supported by recent scanning and transmission electron microscopic studies (13) and the development of a contact-mediated assay of *T. vaginalis* CPEs (1). Higher trophozoite densities on tissue culture could contribute to the rapidity of CPE production whether the mechanism of causation was mediated by contact or soluble toxin. Recently, Brasseur and Savel (3) suggested that the rapidity of CPE production in cell cultures might serve as an adequate assay of *T. vaginalis* virulence. Neither the importance of serum to the rapid initial production of CPE by *T. vaginalis* trophozoites in vitro nor the growth of trichomonads on cell monolayers in the absence of serum has been previously reported.

CPE of cell-free lysate prepared from axenically grown *T. vaginalis* cells. *T. vaginalis* lysate prepared from trophozoites of either strain was effective in producing a CPE only on serum-free BHK monolayers. Although 4 to 2 μg of *T. vaginalis* lysate protein per well produced a CPE in 12 h, less protein required more contact time with the cells to produce the same effects. A 1-μg portion of *T. vaginalis* lysate protein per well produced a detectable CPE in 32 h. When serum was present in the incubation medium, the lysate produced no CPE on BHK cells after 32 h of incubation. The antiviral agent araT had no effect on the expression of the cytopathic activity of *T. vaginalis* lysates. We conclude that the in vitro CPE of *T. vaginalis* lysates is not due to a latent viral agent carried by the protozoan but is perhaps due to proteolytic enzymes released from the hydrogenosomes (27) of *T. vaginalis* during freeze-thawing. These proteases may be inhibited in vitro by binding to alpha globulins in serum, as was reported for cytopathic enzymes in lysates of *E. histolytica* (24). The appearance of a CPE after incubation for 40 h with lysate and serum could indicate that alpha globulin binding of *T. vaginalis* proteases is reversible, perhaps by autolysis of enzyme-globulin complexes, or that the cell monolayer was becoming senescent after this period.

The expression of cytopathic activity in the lysates of the two strains was serum sensitive and time and dose dependent. Although two strains isolated from symptomatic patients (and therefore presumably virulent) were used in this study, it would be particularly interesting to compare the cytopathogenicity of additional lysates prepared from avirulent strains or other *Trichomonas* spp. to determine whether hydrolytic enzyme content is related to pathogenicity, as has been reported for azocasein-hydrolyzing cysteine proteinase content among pathogenic strains of the dysentery amoeba *E. histolytica* (23). The amount of amoebal cathepsin B-like activity per trophozoite was related to the reported virulence of the trophozoites from which the lysates were prepared (W. B. Lushbaugh, A. F. Hofbauer, and F. E. Pittman, Exp. Parasitol., in press). A cathepsin B-like enzyme has been isolated and purified from *Tritrichomonas foetus*, a pathogenic, sexually transmitted trichimonad of cattle (26), and several cysteine proteases with activity on azocasein and denatured hemoglobin are present in *T. vaginalis* lysates (5, 21). Furthermore, it has been reported that more virulent strains of *T. vaginalis* have higher beta-hemolytic activity (18). The beta hemolysin of pathogenic *T. vaginalis* might be a cytotoxic cysteine proteinase similar to the one we observed in *E. histolytica* and suggest as a possible mechanism of the cytopathic activity of *T. vaginalis* lysates in vitro.

Early studies of in vitro *T. vaginalis* cytopathogenicity proposed both the effects of "toxins" released by the flagellates and parasite contact as a mechanism of pathogenicity. Subsequently, some workers were unable to demonstrate cytopathogenic activity in *T. vaginalis* culture supernatants. Although we have shown that *T. vaginalis* lysates produced a CPE on serum-free monolayers and that this effect was inhibited by serum, we have not shown that *T. vaginalis* trophozoites normally release a serum-inhibitable CPE-producing factor under growth conditions, nor have we demonstrated a release mechanism or mode of action for such a putative toxic factor. A possible reason that a *T. vaginalis* toxin has been difficult to demonstrate in culture supernatants is that it is bound to serum proteins; the cytotoxic cathepsin of *E. histolytica* has been shown to be inactivated thus (24). Recent studies have linked the contact of trophozoites of *E. histolytica* (28) or *T. vaginalis* (1) with cytolysis of target cells in vitro. Contact cytolytic events between *E. histolytica* and target cells in vitro may be related to surface membrane-associated phospholipase A enzyme activity (S. A. Long-Krug, K. J. Fischer, and J. I. Rardin, Proc. Joint Meet. R. Am. Soc. Trop. Med. Hyg. 1984, no. 146, p. 120). Phospholipase activity has not yet been studied in *T. vaginalis*. Unlike *E. histolytica* infection, *T. vaginalis* is usually not associated with extensive ulceration but may often be characterized by frankly dysplastic or neoplastic changes in vaginal cytology (16). Such changes in vaginal cytology during trichomoniasis suggest that *T. vaginalis* may be present without causing cytolysis and that the role of a secreted toxin in this disease might deserve further exploration.

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