INFECTION AND IMMUNITY, Dec. 1985, p. 778–786
0019-9567/85/120778-09$02.00/0

Vol. 50, No. 3

Contact-Dependent Cytopathogenic Mechanisms of *Trichomonas vaginalis*

JOHN N. KRIEGER,[1]* JONATHAN I. RAVDIN,[2] AND MICHAEL F. REIN[2]

Department of Urology, School of Medicine, University of Washington, Seattle, Washington 98195,[1] and Department of Internal Medicine, School of Medicine, University of Virginia, Charlottesville, Virginia 22903[2]

Received 29 April 1985/Accepted 5 September 1985

The cytopathogenic mechanisms of *Trichomonas vaginalis* have been debated since the 1940s. We examined the following three proposed pathogenic mechanisms: contact-dependent extracellular killing, cytophagocytosis, and extracellular cytotoxins. Serial observations of Chinese hamster ovary (CHO) cell monolayers exposed to trichomonads revealed that (i) trichomonads form clumps, (ii) the clumps adhere to cells in culture, and (iii) monolayer destruction occurs only in areas of contact with *T. vaginalis*. Kinetic analysis of target cell killing by trichomonads revealed that the probability of CHO cell death was related to the probability of contact with *T. vaginalis*, supporting the observation by microscopy that trichomonads kill cells only by direct contact. Simultaneous studies of ^{111}indium oxine label release from CHO cells and trypan blue dye exclusion demonstrated that *T. vaginalis* kills target cells without phagocytosis. Filtrates of trichomonad cultures or from media in which trichomonads were killing CHO cells had no effect on CHO cell monolayers, indicating that trichomonads do not kill cells by a cell-free or secreted cytotoxin. The microfilament inhibitor cytochalasin D (10 μg/ml) inhibited trichomonad killing of CHO cell monolayers by 80% ($P < 0.0001$). In contrast, the microtubule inhibitor vinblastine (10^{-6} M) caused only 17% inhibition of trichomonad destruction of CHO cell monolayers ($P < 0.020$), whereas colchicine (10^{-6} M) had no effect. *T. vaginalis* kills target cells by direct contact without phagocytosis. This event requires intact trichomonad microfilament function; microtubule function appears not to be essential.

Trichomonas vaginalis is a common cause of vaginitis (7, 19, 29, 42). Trichomoniasis is associated with damage to the superficial vaginal epithelium and a profuse, acute inflammatory discharge (10, 16, 30, 31, 36). How the protozoan causes these pathologic changes is not defined and has been debated since the 1940s (16, 20, 22). Hogue first described the effect of *T. vaginalis* on cell cultures, including primary explants of human embryonic intestine, lung, and muscle (15). "Retraction" of cells was noted on exposure to living trichomonads. Production of similar changes by cell-free filtrates of old trichomonad cultures suggested that the protozoa injured cells primarily by extracellular toxins. Kotcher and Hoogasian (21) studied the adverse effects of viable *T. vaginalis* on primary chick embryo explants as well as human cell cultures. They were unable to produce corresponding changes by exposing cell cultures to protozoal filtrates and, therefore, concluded that trichomonads damage vertebrate cells by "mechanical means." Similar conclusions were drawn by Christian et al. (8), who studied pathologic changes in HeLa cells exposed to *T. vaginalis*, and by Kulda (23), who studied the effect of various trichomonad species on monkey kidney cell cultures. Honigberg and Ewalt suggested that trichomonads injure vertebrate cells both by direct contact and by extracellular cytotoxins (17). In further studies employing chicken liver cell cultures, Farris and Honigberg also suggested that phagocytosis of *T. vaginalis* by macrophages with subsequent damage to the macrophages might be important in pathogenesis (9).

Several recent morphologic studies have examined the interaction between *T. vaginalis* and epithelial cells. Nielsen and Nielsen found clusters of protozoa overlying mucosal microulcerations in biopsies from 4 of 11 women with trichomoniasis (24). Since trichomonads were not present in every area of epithelial disruption, the authors concluded that most cytopathology resulted from cell-free cytotoxins and that contact-dependent destruction of epithelial cells was a secondary phenomenon. Ovcinikov et al. described a change in trichomonad morphology from spherical to ameboid associated with development of surface projection directed toward the epithelial cells (25). These observations were interpreted as active phagocytosis of epithelial cells by *T. vaginalis* (11, 25). Heath studied the effect of trichomonads on rabbit kidney tubule cell monolayers (13). Destruction of the monolayer was observed below adherent clumps of parasites, suggesting that "contact between parasites and epithelial cells was an important factor in pathogenesis." This conclusion was supported by Alderete and Pearlman, who investigated the destruction of various cell monolayers by living trichomonads (1). Absence of cytotoxicity in the presence of fixed trichomonads or fractions of culture filtrate strongly implicated contact-dependent mechanisms. Thus, the following three types of pathogenic mechanisms have been proposed to explain the destruction of epithelial cells by *T. vaginalis*: extracellular cytotoxins, cytophagocytosis, and contact-dependent extracellular killing.

Our present studies support and extend previous observations that *T. vaginalis* exerts a cytolethal effect only on direct contact with a target cell. We employed recent clinical isolates and a different mammalian tissue culture cell line, the Chinese hamster ovary (CHO-K1) cell line. Systems using CHO cells have proven useful for elucidation of pathogenic mechanisms in other protozoa because it is possible to investigate the killing of target cells in both monolayers and pellets (28). Mathematical analysis of the interaction between trichomonads and target cells in pellets was useful because such studies were independent of trichomonad motility and permitted better control of the

* Corresponding author.

organism/target cell ratio than is possible with monolayer systems. Investigations employing specific pharmacologic inhibitors suggested that motility and changes in the parasite cytoskeleton were important in the contact-dependent killing of target cells. The potential role of phagocytosis was dissected out by investigating the killing of radiolabeled target cells by trichomonads.

MATERIALS AND METHODS

Isolation and cultivation of *T. vaginalis*. The three strains of *T. vaginalis*, i.e., CHAR-1, CHAR-2, and CHAR-3, used in these studies were isolated from women with symptomatic vaginitis attending the Albemarle County Sexually Transmitted Disease Clinic (located in central Virginia). Concomitant cultures for other genital pathogens were negative. Trichomonads were grown in modified Feinberg-Whittington (FW) liquid medium (31) and incubated in anaerobic jars (GasPak, BBL Microbiology Systems, Cockeysville, Md.) at 37°C. Isolates were subcultured every 3 to 4 days (31). The protozoa were evaluated between 2 and 10 weeks after initial isolation.

Trichomonads used in experiments were in the logarithmic phase of growth and within 48 h of subculture, and ≥95% exhibited normal morphology and characteristic motility. Before being used, protozoa were washed three times by centrifugation at 250 × *g* for 20 min in Hanks balanced salt solution. Organisms were counted with a hemacytometer and then diluted to the appropriate concentration in serum-free FW medium adjusted to pH 7.0.

Cultivation and harvesting of CHO cells. CHO-K1 cell cultures (ATCC CCL 61) were grown in F-12 medium (GIBCO Laboratories, Grand Island, N.Y.) which was supplemented with 10% fetal calf serum (GIBCO), penicillin (100 U/ml), and streptomycin (100 μg/ml). For monolayer experiments, CHO cells were grown to confluency in 24-well, flat-bottom cell culture plates (no. 756-033-05; Linbro Chemical Co., Hamden, Conn.). After removal of the growth medium, the monolayers were washed twice before the study. For pellet experiments, trypsinized CHO cells (28) were suspended in FW, washed twice by centrifugation at 250 × *g* for 5 min, and adjusted to 2.0×10^5 cells in 1.0 ml of FW per polystyrene tube (12 by 75 mm; Becton Dickinson Labware, Oxnard, Calif.).

Studies of target CHO cell killing by *T. vaginalis*. (i) CHO cell monolayer experiments. Trichomonads (2×10^5) suspended in 1 ml of serum-free FW were added to confluent CHO cell monolayers and incubated at 37°C in 5% CO_2. At desired times the monolayers were evaluated with a Nikon inverted microscope with phase contrast. At each interval, three random observations of each monolayer were made, and a 0 to 4 scale was used for 0 to 100% of the monolayer remaining intact (28). Gradations of 0.25 (7.5% intact monolayer) were recorded.

(ii) Pellet experiments. Cellular pellets were prepared by combining 10^6 washed trichomonads and 2.0×10^5 washed CHO cells (trophozoite/CHO cell ratio of 5:1) in 1.0 ml of serum-free FW and centrifuging them at 250 × *g* for 5 min. After incubation at 37°C in 5% CO_2 for the desired intervals, 0.1 ml of a 4% aqueous tryphan blue dye was added to each tube. The pellets were dispersed by vortexing, and the total numbers of CHO cells excluding or taking up dye were counted in a hemacytometer (minimum of 100 cells counted). Trichomonads were distinguished from CHO cells by their characteristic morphology and motility.

(iii) Evaluation of CHO cell killing by trichomonads by the one-hit hypothesis. In a series of pellet experiments, the number of trichomonads was varied from 2.5×10^4 to 5.0×10^6 while the number of target CHO cells was kept constant at 2.0×10^5 (trichomonad/CHO cell ratio, 1:8 to 25:1).

By analogy with the contact-dependent cytopathogenicity of lymphocytes (3, 4), the one-hit hypothesis may be described as follows. Let t be the initial number of target (CHO) cells, x be the number of trichomonads, and n be the fraction of trichomonads which are effector cells. Furthermore, let y be the corrected, specific CHO cell killing by trichomonads (Tv); that is, y = [(% dead CHO cells with Tv added) − (% dead CHO cells in control tube)]/[100 − (% dead CHO cells in control tube)].

If killing of CHO cells depends on direct contact by a single trichomonad, then the fraction of CHO cells surviving $(1 - y)$ should equal the probability that a target cell was not contracted by a trichomonad (effector cell). This probability is described by the Poisson distribution as

$$\begin{aligned} 1 - y &= e^{-nx/t} \\ \ln(1 - y) &= -nx/t \\ \ln[1/(1 - y)] &= nx/t \\ \log \ln[1/(1 - y)] &= \log(nx/t) \\ \log \ln[1/(1 - y)] &= \log(n/t) + \log(x) \end{aligned}$$

If n and t are kept constant, then $\log \ln [1/(1 - y)] = c + (\log x)$. The constant, c, will shift the curve up or down without changing its shape or slope. Thus, if killing of target cells occurs with one-hit kinetics, that it, if the death of a CHO cell results from a single contact by a trichomonad, then $\log \ln[1/(1 - y)]$ should be directly proportional to $\log x$, and a plot of the function should be a straight line with a slope of 1.0.

(iv) ^{111}InOx label studies. Indium-111 oxine (^{111}InOx) in its complete chelated form containing 1 mCi with 0.05 ng of oxine in 0.05 ml of ethanol was obtained from Mediphysics, Inc., South Plainfield, N.J. For each experiment, 4×10^6 CHO cells in a confluent monolayer were labeled by adding 300 μCi of ^{111}InOx in 4.5 ml of F-12 medium with 10% fetal calf serum for 15 min at 37°C in 5% CO_2 (28). Approximately one-third of the ^{111}InOx label was taken up by the CHO cells. The washed monolayer was then trypsinized, and the cells were suspended in serum-free FW in 5-ml polystyrene tubes. The cells were washed once by centrifugation at 250 × *g* for 5 min, counted in a hemacytometer, and adjusted to 2×10^5 CHO cells per tube in 1 ml of serum-free FW. Less than 1% of the ^{111}InOx was lost during the labeling and washing procedure. CHO cell viability immediately after labeling was 97% of trypan blue dye exclusion. The activity in each tube of CHO cells was measured with a Beckman Gamma Counter (Beckman Instruments, Inc., Fullerton, Calif.). Trichomonads were washed twice in Hanks balanced salt solution and then added to the CHO cells. After centrifugation at 250 × *g* for 5 min, the trichomonad-CHO cell pellets were incubated in 5% CO_2 at 37°C. At desired times the supernatants were decanted. Pellets and supernatants were counted separately for gamma activity, with background and decay (half life of ^{111}In is 67.2 h) corrected for. Previous studies demonstrated that 70% of the ^{111}InOx label was released from CHO cells after 500 μg of amphotericin B (28) per ml was added. This result approximates the 80% of ^{111}InOx released from other tissue culture cell lines in freeze-thaw experiments (41).

After determination of ^{111}In activity, the pellets were suspended in 1.0 ml of fresh FW, 0.1 ml of 4% aqueous trypan blue dye was added, and the tubes were vortexed

gently. The remaining viable and nonviable CHO cells were counted with a hemacytometer (27).

(v) Filtrate and sonic extract studies. Trichomonad suspensions containing 10^5 to 10^7 organisms per ml of serum-free FW medium were incubated for 2 h at 37°C in 5% CO_2; trichomonad motility remained >90% after centrifugation at 250 × *g* for 5 min. The supernatants were decanted, passed through a filter (Acrodisc; 45-μm pore diameter; Gelman Sciences, Inc., Ann Arbor, Mich.), and used as trichomonad filtrate. Cell-free filtrates of 10^5 to 10^7 *T. vaginalis* organisms actively destroying CHO cell monolayers were prepared in the same manner. Undiluted filtrates from protozoa and from protozoa plus CHO cells were added to CHO cell monolayers.

Organisms were washed three times in Hanks balanced salt solution, counted, and then diluted in serum-free FW to 10^5 to 10^7 organisms per ml. Suspensions were sonicated on ice with a Branson Sonifier (model no. 5125; Heat Systems Co., Melville, N.Y.) equipped with microtip and set at six, for six bursts of 20-s duration each. Sonic extracts were examined microscopically to ensure disruption of all trichomonads. Sonic extracts were added to intact CHO cell monolayers, and the monolayers were observed as described previously.

To decrease the possibility of cytotoxin inactivation, experiments were repeated in the presence of the serine protease inhibitor, phenylmethylsulfonyl fluoride (38). As described above, 10^8 protozoa were sonicated on ice, and 5% (vol/vol) phenylmethylsulfonyl fluoride solution (6 mg/ml in 95% ethanol) was added to the sonic extract. The treated sonic extracts were then added to CHO cell monolayers.

Studies with pharmacologic inhibitors of microfilament and microtubule functions. Cytochalasin D (cyto D) (Sigma Chemical Co., St. Louis, Mo.) was diluted in dimethyl sulfoxide (Sigma) to a concentration of 500 μg/ml and stored at −20°C (28). The stock solution was further diluted in serum-free FW to either 1.0 or 10 μg/ml prior to use. Washed protozoa were added to the cyto D solution to achieve a final concentration of 10^6 organisms per ml, and the mixtures were then added to CHO cell monolayers or used in pellet studies.

Colchicine (Sigma) was diluted to a concentration of 10^{-4} M in Puck saline F and stored at −20°C (28). For use, the colchicine stock solution was diluted to 10^{-6} M in serum-free FW medium. Doubly washed trichomonads were added at a concentration of 10^6 organisms per ml, and the suspension was incubated at 37°C in 5% CO_2 for 2 h (26). One milliliter of the colchicine-treated trichomonads was then added to confluent CHO cell monolayers or trypsinized CHO cells for pellet experiments.

Vinblastine sulfate (Eli Lilly & Co., Indianapolis, Inc.) was diluted in normal saline to 10^{-3} M and stored at −20°C (28). Prior to use, the stock solution was diluted to 10^{-6} M in serum-free FW. Trichomonads were washed and suspended in the vinblastine solution at 10^6 organisms per ml for monolayer and pellet experiments.

Destruction of monolayers and of cells in pellets was analyzed by the Student's *t* test (32).

RESULTS

Studies of CHO cell killing by *T. vaginalis*. (i) Monolayer studies. A distinct sequence of events was noted on direct microscopic examination when trichomonads were added to intact CHO cell monolayers (Fig. 1A). Clusters of organisms were not apparent on inspection of the monolayers immediately after the addition of trichomonads. At 1 h, organisms had aggregated into multiple clumps (Fig. 1B) over the intact monolayer. Destruction of the CHO cell monolayers first occurred beneath the clumps of trichomonads (Fig. 1C) and by 5 h had proceeded in centrifugal fashion until the monolayer was disrupted completely (Fig. 1D). Increasing the number of trichomonads in the inoculum over a range of 1.6×10^4 to 1.6×10^6 organisms per ml (Fig. 2) increased the rate of CHO cell monolayer destruction ($P < 0.001$).

(ii) Pellet studies. Trichomonal killing of target CHO cells in cellular pellets was evaluated (Fig. 3). After 4 h, trichomonads killed significant numbers of CHO cells ($P < 0.0001$). The total number of viable plus nonviable but intact CHO cells was less than 100% ($P < 0.05$), indicating either extracellular lysis or phagocytosis of intact CHO cells by trichomonads.

In a serum-free system, we evaluated the killing of CHO cells by trichomonads in pellets, varying the concentration of protozoa over a 2.3-log range while maintaining a constant number of target cells. There was a linear relationship between the log ln of corrected CHO cell killing and the log of trichomonad concentration, with a slope of 0.94 and a correlation coefficient of 0.97 (Fig. 4), indicating that in the pellet system one trichomonad kills one target CHO cell and only upon direct contact.

(iii) Studies of trichomonad killing of ^{111}InOx-labeled CHO cells. A comparison of microscopic cell counts with trypan blue dye with the simultaneous release of ^{111}InOx label from target CHO cells revealed killing of CHO cells by protozoa at 2, 4, and 6 h ($P < 0.01$) (Fig. 5). Significantly more CHO cells took up trypan blue in the presence of trichomonads than in control tubes at 2, 4, and 6 h ($P < 0.0001$). After 6 h of incubation, only 21.5% of the CHO cells were alive by trypan blue exclusion, and 33% of the ^{111}InOx label remained in the pellet.

(iv) Studies of trichomonad filtrates and sonic extracts. Filtrates of 10^5 to 10^7 trichomonads per ml and filtrates from trichomonads actively destroying CHO cells had no effect on CHO cell monolayers. The addition of filtrates from trichomonads (10^7/ml) actively destroying CHO cells did not increase the rate of CHO cell monolayer destruction by fresh trichomonads (10^3 to 10^5/ml). Sonic extracts of trichomonads (10^7/ml) with or without the protease inhibitor phenylmethylsulfonyl fluoride had no effect on CHO cell monolayers after 24 h at 37°C.

Studies with pharmacologic inhibitors of microfilament and microtubule functions. The specific microfilament inhibitor cyto D (12, 35) reduced target cell killing by trichomonads in both monolayer and pellet systems. Cyto D (1.0 or 10.0 μg/ml) inhibited the destruction of CHO cell monolayers by trichomonads ($P < 0.0001$) (Fig. 6). Cyto D at 10 μg/ml inhibited target cell killing in pellets (Fig. 3) by 80% ($P < 0.0001$), but cyto D at 1 μg/ml had no effect. Cyto D at 1 or 10 μg/ml in serum-free FW was nontoxic to CHO cells, and trichomonad viability remained >95% for 24 h. On microscopic examination, the protozoa retained flagellar motion at both cyto D concentrations.

Colchicine and vinblastine bind to different sites in mammalian cells, resulting in inhibition of microtubule function (5, 26, 40). Neither drug was toxic to CHO cells or trichomonads under the experimental conditions. Colchicine (10^{-6} M) did not inhibit protozoal killing of CHO cells in confluent monolayers (Fig. 7) or in pellets (Fig. 3). Vinblastine (10^{-6} M) caused a small (16.7%) but statistically significant inhibition of trichomonad-mediated CHO cell monolayer destruction (Fig. 6) at 4 and 5 h ($P < 0.02$). In

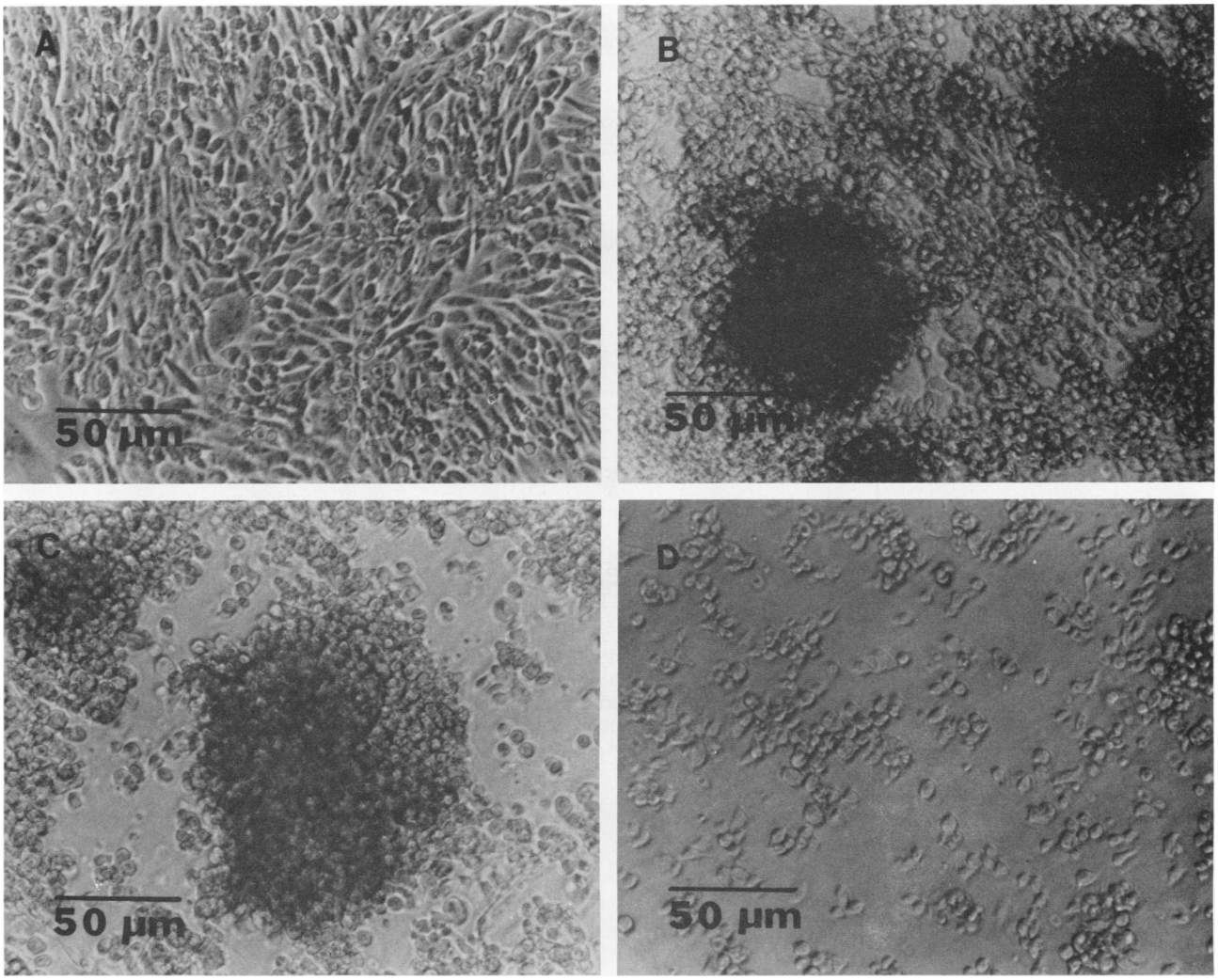

FIG. 1. CHO cell monolayer destruction by virulent *T. vaginalis* at a concentration of 10^6 trichomonads per ml in serum-free FW medium. (A) Intact confluent monolayer prior to addition of trichomonads. (B) After 1 h, the protozoa have formed clumps which adhere to the monolayer. (C) Monolayer disruption occurs only in areas in contact with trichomonads. (D) After 5 h, there is >75% monolayer destruction by the trichomonads. Similar observations were made in over 30 different experiments.

pellet studies, neither colchicine nor vinblastine inhibited the killing of CHO cells by trichomonads (Fig. 4).

DISCUSSION

We have determined that viable *T. vaginalis* isolated from women with symptomatic vaginitis kills target CHO cells exclusively on direct contact. In agreement with the microscopy findings of previous workers (11, 13), we observed that trichomonads formed clumps, the clumps then adhered to cell culture monolayers, and monolayer destruction occurred only in areas in contact with protozoa. Neighboring cells not in contact with protozoa remained intact. We propose that motile *T. vaginalis* may move away from epithelium they have already damaged, thus explaining the clinical observation that cytopathology is observed in epithelium apparently free of protozoa (24). Because motile trichomonads were not distributed uniformly over the monolayers, it was important to examine the observation of contract-dependent killing of cells in culture by using a system in which parasite motility was not a factor.

We evaluated the kinetics of the killing of CHO cells by trichomonads in pellets by varying the concentration of trichomonads while maintaining a constant number of target cells. In these experiments, the trichomonads were prevented from clumping, indeed from moving, thus facilitating more rigid control of target/effector cell ratios than is possible in monolayer studies. As the number of organisms was increased over a 2.3-log range while the number of target cells was held constant, the fraction of target cells killed was directly proportional to the probability of their making direct contact with trichomonads. If target cell death were mediated by diffusible substances, such linear proportionality would not be expected (3, 4). The slope of the regression line in Fig. 4 should be proportional to the number of direct contacts necessary to kill a target cell. The slope of 0.94 thus suggests that contact with a single trichomonad is lethal for a target cell. This analysis supports the visual observation that trichomonads kill cells by direct contact (4, 14).

Studies with ^{111} InOxine-labeled CHO cells and simultaneous trypan blue dye exclusion confirmed that trichomonads kill the target cells in culture by an extracellular process. After 6 h, 33% of the initial ^{111}In label remained in the

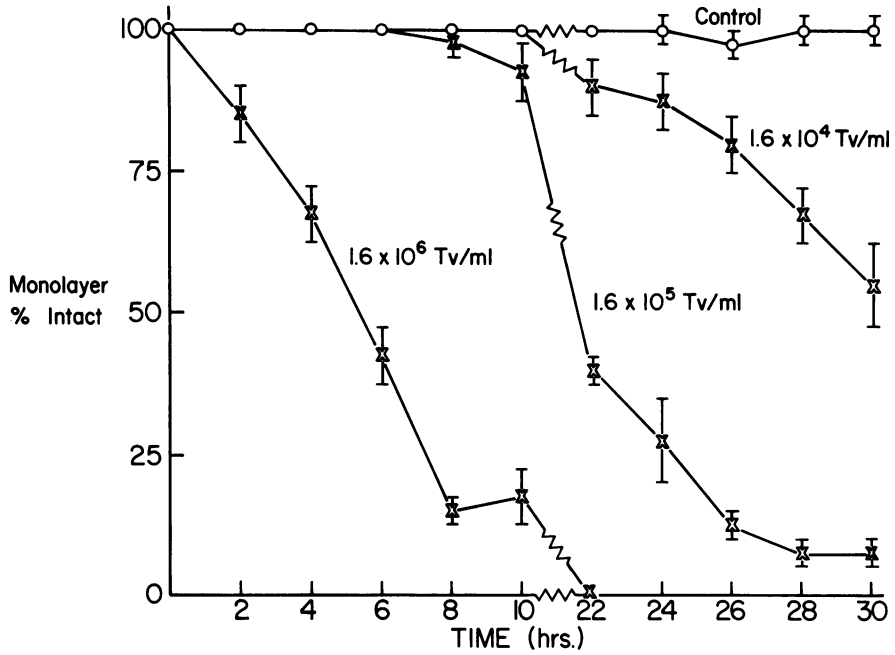

FIG. 2. CHO cell monolayer destruction by a single *T. vaginalis* strain at different inoculum concentrations. Increasing the inoculum concentration of trichomonads from 1.6×10^4/ml to 1.6×10^6/ml significantly increased the rate of monolayer destruction. Each point represents the mean of six monolayers ± the standard error of the mean (SEM). Six separate experiments were done with three different isolates. A single *T. vaginalis* isolate (CHAR-1) was used in these experiments. Similar dose-response relationships were observed with other isolates, although the rate of monolayer destruction by similar numbers of different isolates varied as a function of the intrinsic virulence of the isolates. Control monolayers incubated in similar medium without organisms remained intact.

cellular pellet. This amount represented the sum of activity in viable CHO cells, retained label in nonviable intact CHO cells, and ^{111}InOx in CHO cells phagocytized by protozoa. Based on direct counts of viable CHO cells and dead intact CHO cells (which retain 20% of their label) (25, 31), 30% of the initial label would be expected to remain in the pellet. If, however, significant numbers of living CHO cells had been phagocytized by trichomonads, then the trichomonads would be expected to contain radiolabel, and the pellet should manifest higher total counts. Because all of the gamma activity in the pellet could be accounted for by directly visualized living and intact dead CHO cells, none of the radioactivity in the pellet could be attributed to phagocytized CHO cells (28). We conclude that *T. vaginalis* kills target cells on direct contact, while the target cell remains extracellular to the effector cell, and without phagocytosis.

We evaluated the potential role of secreted or cell-free cytotoxins in the extracellular killing of target cells by *T. vaginalis*. Filtrates of trichomonad cultures or of viable protozoa actively destroying CHO cells had no effect on CHO cell monolayers. In addition, filtrates did not increase the rate of monolayer disruption by low numbers of intact, viable trichomonads. Trichomonad sonic extracts also had no effect on target cells. Experiments were done in a serum-free system and with a serine protease inhibitor to decrease the potential for interference by serum or for proteolytic destruction of a cell-free toxin. These findings are in accord with the recent report by Alderete and Pearlman on the interaction of pathogenic *T. vaginalis* with a variety of tissue culture cell monolayers including human urogenital and vaginal (HeLa), human epithelial (HEp-2), normal baboon testicular, and monkey kidney (Vero) cells (1). There was no evidence of a role for toxic trichomonal products in disruption of these monolayers by the protozoa. Although it is theoretically possible that diffusible substances produced by the trichomonads are diluted away from sites of contact between effector and target cells or that such products are very short-lived, these studies provided no

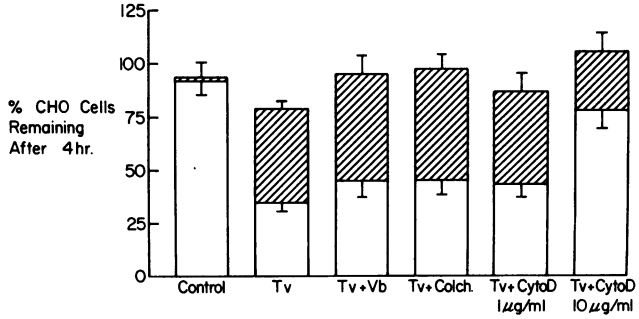

FIG. 3. Effect of microtubule and microfilament inhibitors on killing of CHO cells in pellets with *T. vaginalis* (Tv) after 4 h of incubation. The total of viable (unshaded portion) and nonviable (shaded portion) CHO cells in tubes with protozoa was less than 100% ($P < 0.05$). Trichomonad-mediated CHO cell killing was not inhibited by 10^{-6} M vinblastine (Vb), 10^{-6} M colchicine (Colch.), or 1 μg of cyto D (CytoD) per ml. However, at 10 μg/ml, cyto D significantly decreased the killing of CHO cells by *T. vaginalis* ($P < 0.0001$). Control experiments were done in serum-free medium without organisms, in medium containing dimethyl sulfoxide, and in medium containing each inhibitor at concentrations identical to those in experiments done with viable trichomonads. Each bar represents the mean ± SEM of 19 to 29 studies in four different experiments.

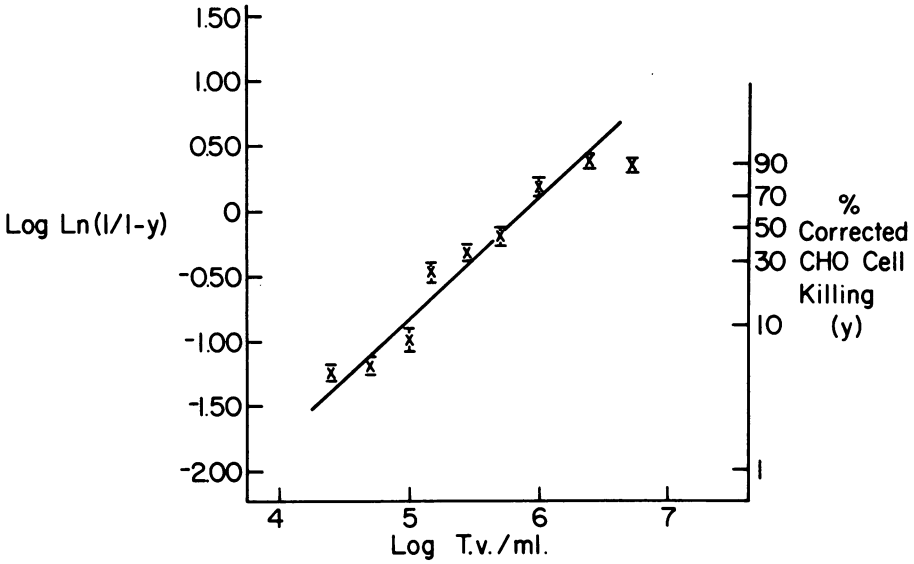

FIG. 4. Contact-dependent CHO cell killing by *T. vaginalis* (T.v.) in pellet studies. The corrected, specific CHO cell killing (y) was determined for each inoculum concentration of trichomonads. Corrected CHO cell killing (y) represents the reduction in target cell survival in pellets containing trichomonads compared with CHO cell survival in serum-free medium without trichomonads. Each point is the mean ± SEM for 5 to 9 experiments. The log ln(1/1 − y) was then plotted against the log number of protozoa. The slope of the linear regression line was 0.94, and the correlation coefficient was 0.97. Therefore, the probability of CHO cell death equals the probability of contact with a single trichomonad.

experimental evidence supporting a role for extracellular cytotoxins in the in vitro killing of cells in culture.

Electron microscopy investigations have concluded that *T. vaginalis* contains both microfilaments and microtubules (18, 24, 25). When trichomonads were in contact with epithelial cells, the parasite cytoskeleton was oriented such that most microfilaments were in the portion of the trichomonad in contact with the epithelium (24). *T. vaginalis* microtubules are primarily located in the flagella and axostyle (25). The potent micofilament inhibitor cyto D (37, 39) significantly inhibited trichomonad cytopathogenicity for tissue culture cell monolayers but was not toxic to trichomonads or CHO cells and did not abolish protozoan motility. In the pellet system, in which contact was ensured by centrifugation of protozoa and target cells together, eliminating the role for trichomonad motility, cyto D at a concentration of 10 μg/ml inhibited killing by 80%. These morphologic and physiologic findings suggest that intact trichomonad microfilament function was necessary for the killing of target cells.

Trichomonad microfilament function may be important in either the initial adherence of the parasites to target cells or the postadherence cytolytic events. In morphologic studies, *T. vaginalis* undergoes ameboid changes on contact with target cells or inert surfaces, and the areas of contact are rich in microfilaments (13). These cytoskeletal changes may be important for attachment to target cells. Cyto B, a less potent and less specific inhibitor of microfilament function than cyto D, reduced the adherence of *T. vaginalis* to glass surfaces (6). Studies demonstrating that surface adhesive proteins such as fibronectin align with internal microfilaments (19) suggest that alterations in microfilament function may directly influence the ability of cells to attach to one another. In other experimental systems, cytochalasins reduced lymphocyte- and macrophage-mediated cytolysis of tumor cells by interfering with adherence (2, 14, 33, 34), and cytochalasins also decreased target cell killing by a virulent

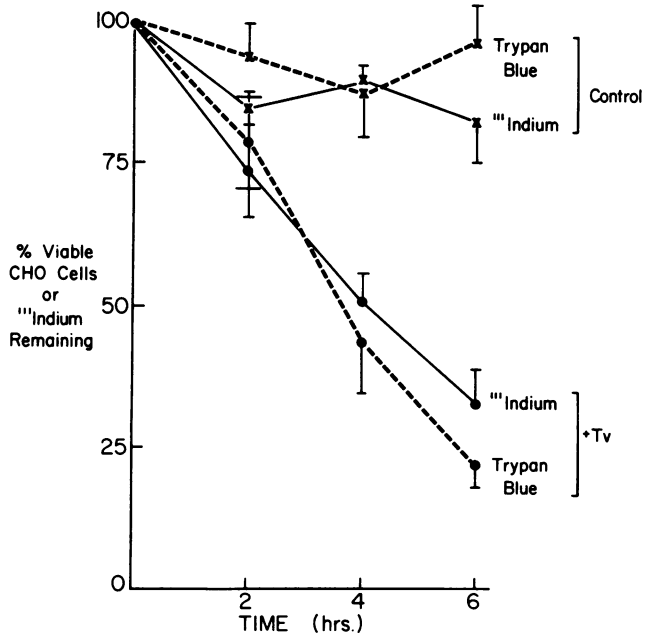

FIG. 5. CHO cell survival in trichomonad (Tv)-CHO cell pellets with both trypan blue (– – –) exclusion and ^{111}InOx counts (——). By trypan blue exclusion, there was significant killing of CHO cells in pellets with trichomonads ($P < 0.01$). Counts of ^{111}InOx in labeled CHO cells also demonstrated significant killing of CHO cells in pellets with trichomonads at 2, 4, and 6 h compared with that in control tubes ($P < 0.01$). The control experiments were done in serum-free medium without trichomonads. Each point represents the mean + SEM of six studies in three different experiments.

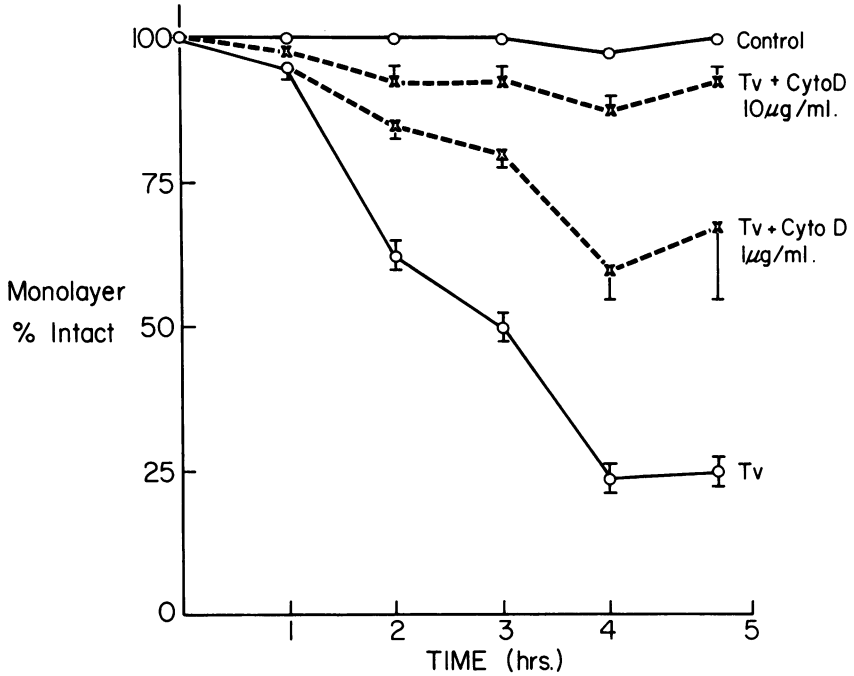

FIG. 6. Effect of microfilament inhibition on CHO cell monolayer destruction by trichomonads. Cyto D (– – –) at both 1 and 10 μg/ml significantly inhibited CHO cell monolayer destruction by *T. vaginalis* (Tv) ($P < 0.0001$). Control experiments were done in serum-free medium, in medium containing cyto D dissolved in dimethyl sulfoxide, and in medium containing dimethyl sulfoxide at concentrations identical to those in experiments done with viable trichomonads. Each point represents the mean ± SEM of 9 to 16 studies in six different experiments.

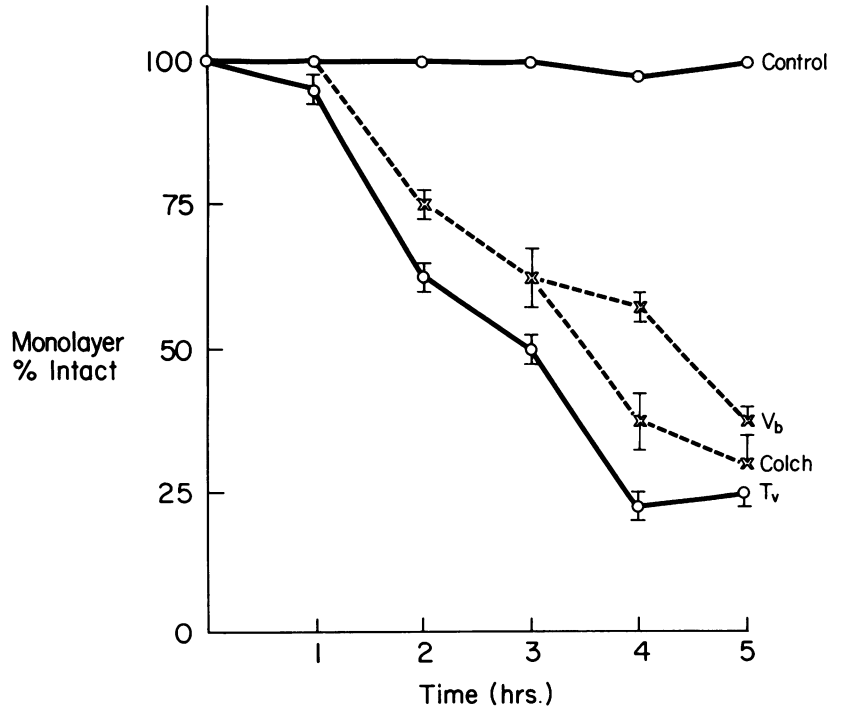

FIG. 7. Effect of microtubule inhibition on CHO cell monolayer destruction by trichomonads (Tv). Colchicine (Colch) at 10^{-6} M did not significantly reduce trichomonad-mediated CHO cell monolayer destruction. Vinblastine (V_b) at 10^{-6}M caused statistically significant inhibition of monolayer destruction at both 4 and 5 h ($P < 0.020$). Control studies were done in serum-free medium and in serum-free medium containing each inhibitor at concentrations identical to those in experiments with trichomonads. Each point represents the mean ± SEM of 9 to 18 studies in six different experiments.

axenic strain of *Entamoeba histolytica* by inhibiting both adherence and postadherence events (28, 29).

The microtubule inhibitors colchicine and vinblastine, at 10^{-6} M, are specific for different sites on mammalian microtubules and have been shown to reduce macrophage-mediated cytolysis (5, 26, 35, 40). Colchicine had no effect on protozoal killing of CHO cells in monolayers or in pellets. Although vinblastine slightly reduced CHO cell monolayer disruption, it had no effect in pellet studies. This result implies that the relatively small reduction in CHO cell monolayer destruction was a consequence of decreased trichomonad motility. It is also possible that colchicine and vinblastine merely have no activity for the microtubules of *T. vaginalis*. It is of interest, in this respect, that colchicine and vinblastine, even in concentrations 25- to 30-fold higher than those used in our study, had no effect on trichomonad adherence to glass (6). The inhibition seen with cyto D and the lack of effect of colchicine and vinblastine may indicate a vital role for an adherence event in trichomonad cytopathogenicity.

Using an in vitro model, we examined the pathogenic mechanisms of *T. vaginalis*. Trichomonads appear to kill target cells exclusively by direct contact, not by a cell-free or secreted cytotoxin. Contact-dependent killing does not involve phagocytosis of living CHO cells by *T. vaginalis*. Target cell killing is dependent on intact trichomonad microfilament function.

ACKNOWLEDGMENTS

This work was partially supported by research grants no. NIH SO7 RR05432-22, NIH NIAID AI 12192, and NIH L R23 A121422-01A1.

We thank Richard L. Guerrant of the Division of Geographic Medicine, University of Virginia, for his encouragement and Cathy Dickens for her expert technical assistance.

LITERATURE CITED

1. **Alderete, J. F., and E. Pearlman.** 1984. Pathogenic *Trichomonas vaginalis* cytotoxicity to cell culture monolayers. Br. J. Vener. Dis. **60**:99–105.
2. **Allison, A. C., and J. Ferluger.** 1976. How lymphocytes kill tumor cells. N. Engl. J. Med. **295**:165–167.
3. **Asherson, G. L., and J. Ferluga.** 1973. Contact sensitivity in the mouse, a non-specific cytotoxicity of T blasts in the draining lymph nodes. Immunology **25**:471–483.
4. **Asherson, G. L., J. Ferluga, and G. Janossy.** 1973. Non-specific cytotoxicity for T cells activated with plant mitogens *in vitro* and the requirement for plant agents during the killing reaction. Clin. Exp. Immunol. **15**:573–589.
5. **Bryan, J.** 1972. Definition of three classes of binding sites in isolated microtubule crystins. Biochemistry **11**:2511–2516.
6. **Cappuccinelli, P., and L. Varesio.** 1975. The effect of cytochalasin B, colchicine and vinblastine on the adhesion of *Trichomonas vaginalis* to glass surfaces. Int. J. Parasitol. **5**:57–61.
7. **Catterall, R. D.** 1972. Trichomonal infections of the genital tract. Med. Clin. North Am. **56**:1203–1209.
8. **Christian, R. T., N. F. Miller, P. O. Ludovici, and G. M. Riley.** 1963. A study of *Trichomonas vaginalis* in human cell culture. Am. J. Obstet. Gynecol. **85**:947–954.
9. **Farris, V. K., and B. M. Honigberg.** 1970. 1970. Behavior and pathogenicity of *Trichomonas vaginalis* Donne in chick liver cell cultures. J. Parasitol. **56**:849–882.
10. **Fouts, A. C., and S. J. Kraus.** 1980. *Trichomonas vaginalis*: reevaluation of its clinical presentation and laboratory diagnosis. J. Infect. Dis. **141**:137–143.
11. **Garcia-Tamoyo, J., J. T. Nunez-Montiel, and H. P. deGarcia.** 1978. An electron microscopic investigation of the pathogenesis of human vaginal trichomoniasis. Acta Cytol. **22**:447–454.
12. **Goodman, G. D., and A. F. Miranda.** 1978. Cellular contractility and the visible effects of cytochalasin, p. 279–429. *In* S. W. Tanenbaum (ed.), Cytochalasins: biochemical and cell biological aspects. Elsevier/North-Holland Publishing Co., New York.
13. **Heath, J. P.** 1981. Behavior and pathogenicity of *Trichomonas vaginalis* in epithelial cell cultures: a study of light and scanning electron microscopy. Br. J. Vener. Dis. **57**:106–117.
14. **Henney, C. S.** 1975. T-cell mediated cytolysis: consideration of the role of a soluble mediator. J. Reticuloendothel. Soc. **17**:231–235.
15. **Hogue, M. J.** 1943. The effect of *Trichomonas vaginalis* on tissue-culture cells. Am. J. Hyg. **37**:142–152.
16. **Honigberg, B. M.** 1978. Trichomonads of importance in human medicine, p. 275–454. *In* J. P. Kreier (ed.), Parasitic protozoa, vol. 2. Academic Press, Inc., New York.
17. **Honigberg, B. M., and A. Ewalt.** 1961. Preliminary observations on pathogenicity of *Trichomonas vaginalis* for cell cultures. Proc. Int. Congr. Protozool. **1**:568–569.
18. **Honigberg, B. M., D. Volkmann, R. Entzeroth, and E. Scholltysek.** 1984. A freeze-fraction electron microscope study of *Trichomonas vaginalis* Donne and *Tritrichomonas foetus* (Riedmuller). J. Protozool. **31**:116–131.
19. **Hynes, R. O., A. T. Destree, and D. D. Wagner.** 1982. Relationships between microfilaments, cell-substratum, adhesion and fibronectin. Cold Spring Harbor Symp. Quant. Biol. **46**:659–670.
20. **Jirovec, O., and M. Petru.** 1968. *Trichomonas vaginalis* and trichomoniasis. Adv. Parasitol. **6**:117–188.
21. **Kotcher, E., and A. C. Hoogasian.** 1957. Cultivation of *Trichomonas vaginalis* Donne 1837, in association with tissue cultures. J. Parasitol. **43**(Suppl.):39.
22. **Krieger, J. N.** 1981. Urologic aspects of trichomoniasis. Invest. Urol. **16**:411–417.
23. **Kulda, J.** 1967. The effect of different species of trichomonads on monkey kidney cell cultures. Folia Parasitol. **14**:295–310.
24. **Nielsen, M. H., and R. Nielsen.** 1975. Electron microscopy of *Trichomonas vaginalis* Donne: interaction with vaginal epithelium in human trichomoniasis. Acta Pathol. Microbiol. Scand. Sect. B **83**:305–320.
25. **Ovcinikov, N. M., V. V. Delektorskij, E. N. Turanova, and G. N. Yashkova.** 1975. Further studies of *Trichomonas vaginalis* with transmission and scanning electron microscopy. Br. J. Vener. Dis. **51**:357–375.
26. **Pesanti, E. L., and S. G. Axline.** 1975. Phagolysosome formation in normal and colchicine-treated macrophages. J. Exp. Med. **142**:903–913.
27. **Phillips, H. J.** 1973. Dye exclusion tests for cell viability, p. 406–408. *In* P. F. Krause and M. K. Patterson (ed.), Culture methods and applications. Academic Press, Inc., New York.
28. **Ravdin, J. I., B. Y. Croft, and R. L. Guerrant.** 1980. Cytopathogenic mechanisms of *Entamoeba histolytica*. J. Exp. Med. **152**:377–390.
29. **Ravdin, J. I., and R. L. Guerant.** 1981. Role of adherence of cytopathogenic mechanisms of *Entamoeba histolytica*: study with mammalian tissue culture cells and human erythrocytes. J. Clin. Invest. **68**:1305–1313.
30. **Rein, M. F., and K. K. Holmes.** 1983. "Nonspecific vaginitis," vulvovaginal candidiasis, and trichomoniasis: clinical features, diagnosis and management, p. 281–315. *In* J. S. Remington and M. N. Swartz (ed.), Current clinical topics in infectious diseases, vol. 4. McGraw-Hill Book Co., New York.
31. **Rein, M. F., J. A. Sullivan, and G. L. Mandell.** 1980. Trichomacidal activity of human polymorphonuclear neutrophils: killing by disruption and fragmentation. J. Infect. Dis. **142**:575–585.
32. **Remington, R. D., and M. A. Schork.** 1970. Statistics with applications to the biological and health sciences, p. 192–225. Prentice-Hall, Inc., Englewood Cliffs, N.J.
33. **Sanderson, C. J.** 1976. The mechanism of T cell-mediated cytotoxicity. The release of different cell components. Proc. R Soc. Lond. (Biol.) **192**:221–229.
34. **Sanderson, C. J.** 1976. The mechanisms of T-cell mediated cytotoxicity. II. Morphological studies in cell death by time-lapse microcinematography. Proc. R. Soc. Lond. (Biol.) **192**:241–255.

35. **Sharmar, S. D., and W. F. Piessens.** 1978. Tumor cell killing by macrophages activated *in vitro* with lymphocyte mediators. III. Inhibition by cytochalasins, colchicine and vinblastine. Cell Immunol. **38**:176–285.
36. **Spence, M. R., D. H. Hollander, J. Smith, L. McCaig, D. Sewell, and M. Brockman.** 1980. The clinical and laboratory diagnosis of *Trichomonas vaginalis* infection. Sex. Transm. Dis. **7**:168–171.
37. **Tamm, C. H.** 1978. Chemistry and biosynthesis of cytochalasins, p. 15–51. *In* S. W. Tanebaum (ed.), Cytochalasins: biochemical and cell biological aspects. Elsevier/North-Holland Publishing Co., New York.
38. **Weber, K., and M. Osborn.** 1975. Proteins and sodium dodecyl sulfate: molecular weight determination on polyacrylamide gels and related procedures, p. 179–223. *In* R. L. Hill (ed.), Proteins. Academic Press, Inc., New York.
39. **Weihing, R. R.** 1978. *In vitro* interactions of cytochalasins with contractile proteins, p. 431–444. *In* S. W. Tanenbaum (ed.), Cytochalasins: biochemical and cell biological aspects. Elsevier/North-Holland Publishing Co., New York.
40. **Wilson, L.** 1978. Minireview: action of drugs on microtubules. Life Sci. **17**:303–310.
41. **Wiltrout, R. H., P. Frost, and G. P. Cummings.** 1978. Isotope release cytotoxicity assay with the use of indium: advantage over chromium-51 in long-term assays. J. Natl. Cancer Inst. **61**:83–188.
42. **Wisdom, A. R., and E. M. C. Dunlop.** 1965. Trichomoniasis: study of the disease and its treatment. I. The disease and its treatment in women. Br. J. Vener. Dis. **41**:90–96.