# CYTOPATHOGENIC MECHANISMS OF *ENTAMOEBA HISTOLYTICA* *

By JONATHAN I. RAVDIN, BARBARA Y. CROFT, AND RICHARD L GUERRANT

*From the Divisions of Geographic Medicine and Infectious Diseases, Department of Medicine; and the Division of Nuclear Medicine, Department of Radiology, University of Virginia Medical Center Charlottesville, Virginia 22908*

*Entamoeba histolytica* causes disease by invading tissue. How the organism exerts this cytolytic effect is not understood.

We have examined three proposed mechanisms of *E. histolytica* virulence: cytophagocytosis, contact-dependent cell killing, and effects of a cell-free cytotoxin. Chinese hamster ovary (CHO)[1] cells in tissue culture served as target cells for the amebae. Studies were done with CHO cells in pellets or in monolayers, similar to the system of Mattern et al. (1).

Several investigators have found evidence of *E. histolytica* contact-dependent cell killing. Eaton et al. (2) proposed a surface-active lysosome, the existence of which has been disputed (3–9). Electron microscopy reveals *E. histolytica* contact cytolysis of target cells (4, 5, 7–10). In vitro examples of *E. histolytica* contact-dependent cell killing with leukocytes (11)[2] and with various tissue culture cell lines (12–15) has been described. Lushbaugh et al. (16) recently described a cytopathic effect of *E. histolytica* sonicate that was inhibited by serum; other workers, with serum-containing systems, have failed to observe any evidence of *E. histolytica* cell-free cytopathogenicity (7, 8, 13).

Our present studies show that *E. histolytica* exerts a cytolethal effect only on direct contact with a target cell. The ameba cytolethal effect occurs extracellularly and usually precedes ameba phagocytosis. *E. histolytica* sonicate has a nonlethal, reversible, cytopathic effect, different from that of whole, viable amebae. Microfilament inhibitors—cytochalasins A, B, and D—decreased *E. histolytica* cytopathogenicity, whereas microtubule inhibitors, colchicine and vinblastine, did not alter ameba target cell destruction.

## Materials and Methods

*Cultivation and Harvesting of E. histolytica.* Axenic *E. histolytica* (*E.h.*) strain HM1 (formerly ABRM [17]) was obtained from L. Diamond at the National Institutes of Health (Bethesda, Md). The method of obtaining bacteria-free cultures of *E.h.* has been described (18). The amebae are grown in TYI-S-33 medium (trypticase, yeast extract, iron, and serum), as developed by Diamond et al. (19), that contained penicillin (Pfizerpen G; Pfizer Inc, New York) (100 U/ml), and streptomycin sulfate (Pfizer Inc.) (100 μg/ml). The amebae are subcultured at 38-

* Supported by The Rockefeller Foundation.

[1] *Abbreviations used in this paper.* CHO, Chinese hamster ovary; Cyto, cytochalasin(s), *E.h.*, *Entamoeba histolytica*, FCS, fetal calf serum, $^{111}$InOx, $^{111}$indium oxine, TYI, trypticase, yeast extract, and iron

[2] Guerrant, R L., J Brush, J I. Ravdin, J A Sullivan, and G L. Mandell The interaction between *Entamoeba histolytica* and human polymorphonuclear leukocytes Manuscript submitted for publication

to 96-h intervals, depending upon amebae inoculum size and growth rate. The cultures were monitored periodically for bacterial contamination, with standard aerobic and anaerobic techniques.

The amebae used in experiments are in the logarithmic phase of growth, within 96 h after subculture (18, 20). *E.h.* are harvested by chilling culture tubes in an ice bath for 5–15 min and centrifuging at 50 *g* for 5 min in 16- × 125-mm plastic tubes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) After removal of the supernate, the amebae pellet is resuspended in 1 ml trypticase, yeast extract, and iron (TYI) broth, counted in a hemocytometer, then further diluted with TYI broth to the desired amebae concentration.

*Cultivation and Harvesting of CHO Cells.* CHO cells were grown in F-12 medium (Grand Island Biological Co., Grand Island, N. Y.), with 10% fetal calf serum (FCS) (Grand Island Biological Co.), penicillin (100 U/ml), and streptomycin (100 μg/ml) (21). For monolayer experiments, the CHO cells are grown to confluency in 24-well, flat-bottomed tissue culture plates (3.5 ml capacity/well; Linbro Chemical Co., Hamden, Conn.) CHO cells were then washed once with TYI before adding *E.h.* suspension, or were washed twice with TYI before adding amebae sonicate or filtrates.

For pellet experiments, trypsinized CHO cells were suspended in TYI broth, washed once in TYI in 12- × 75-mm polystyrene tubes (Falcon Labware, Div. of Becton, Dickinson & Co.), and adjusted to $2.0 \times 10^5$ cells/tube.

*Studies of Contact-dependent Cell Killing by Whole, Viable E.h.*

CINEMICROGRAPHY. *E.h.* were harvested as described. CHO cells were grown to confluency at 37°C on 22-$mm^2$ cover slips (Number 1½, Corning Glass Works, Science Products Div., Corning N. Y.) in a tissue culture dish (3002, Falcon Labware, Div. of Becton, Dickinson & Co.). Amebae, $10^4$/ml, were placed on a slide, the cover slip with CHO cells was inverted on the amebae suspension, and the interaction was examined on a prewarmed (35°C) stage with both phase- and interference-contrast optics and photographed with a 6-mm Bolex (Bolex International S. A., Yverdon, Switzerland) camera at 120 frames/min.

MONOLAYER EXPERIMENTS. CHO cell monolayers were prepared as described; 1 ml of the amebae suspension in TYI broth (with $10^4$ amebae total) was added to each well and incubated at 37°C at 6% $CO_2$. At desired times the percentage of intact CHO cell monolayer was scored as 0–4 for 0–100% of the cells remaining Gradations of 0.5 scoring (12 5%) were recorded. Each reading represented three random observations from each well at each point in time. Results were tabulated as the percentage of intact CHO monolayer remaining.

PELLET EXPERIMENTS. Pellets of CHO cells and amebae were prepared by adding $1.0 \times 10^4$ amebae strain HM1 to $2.0 \times 10^5$ CHO cells (20:1 CHO cell:amebae ratio), suspended in TYI, and centrifuged at 150 *g* for 5 min. After incubation at 37°C and 6% $CO_2$ for the desired time intervals, the pellets were dispersed with 0.1 ml of 4% aqueous trypan blue (22), placed in a hemocytometer, and the total number of live and dead CHO cells remaining were counted. These experiments were performed in serum-free systems or with 1% FCS.

EVALUATION OF TARGET CELL KILLING BY *E.H* WITH THE ONE-HIT HYPOTHESIS. With the same number of CHO cells, $2.0 \times 10^5$, the number of amebae added was varied from $10^2$ to $10^4$ *E.h.*/ml in a serum-free system. The complete derivation of the one-hit hypothesis as applied to T lymphocyte contact-dependent cytopathogenicity, has been described (23, 24). Briefly, let t = initial number of target cells (CHO cells), x = number of amebae added, n = fraction of amebae which are effector cells, y = fraction of target cells killed or corrected specific killing.

Y = corrected specific CHO cell killing

$$= \frac{\left(\begin{array}{c}[\text{percent dead CHO cells with amebae added}] \\ -[\text{percent dead CHO cells in control}]\end{array}\right)}{100 - \text{percent dead CHO cells in control}} \times 100.$$

The fraction of CHO cells which survive (1 − y) is equal to the probability of a target cell not being contacted by an effector cell, by using the Poisson distribution.

$$\begin{aligned} 1 - y &= \exp(-nx/t), \\ \ln(1 - y) &= -nx/t, \\ \ln\{1/(1 - y)\} &= nx/t, \text{ and} \\ \log \ln\{1/(1 - y)\} &= \log(nx/t) \end{aligned}$$

$^{111}$INDIUM OXINE ($^{111}$INOX)-LABEL STUDIES. $^{111}$InOx was obtained from Diagnostics Isotopes, Inc. (Bloomfield, N. J.), in its complete chelated form, that contained 1 mCi with 0.05 ng oxine in 0.05 ml ethanol. Approximately $3 \times 10^6$ CHO cells were labeled in flasks by adding 120 $\mu$Ci of $^{111}$InOx in F-12 medium with 10% FCS for 15 min at 37°C. Approximately 33% of the $^{111}$InOx label was taken up by CHO cells. CHO cells were then trypsinized, suspended in TYI in 5 ml polystyrene tubes, washed once, and adjusted to $2 \times 10^5$ CHO cells/tube in 1 ml of TYI. Less than 1 % of $^{111}$InOx label was lost during passage and washing. Although $^{111}$InOx also labels dead cells via passive diffusion (25, 26), >98% of CHO cells were viable before labeling. Viability of CHO cells immediately after $^{111}$InOx labeling was no different than controls (>97% excluded trypan blue). The gamma activity of the adjusted CHO cell suspension was ascertained in a Beckman Gamma counter (Beckman Instrument, Inc., Fullerton, Calif.). Amebae were then added and centrifuged with the CHO cells, pellets were maintained at 37°C until desired times, when supernates were removed, and pellet and supernatant activities were counted separately. Counts were corrected for background and decay to time zero (with $e^{-\lambda t}$, where $\lambda = (\ln 2/t_{1/2})$, $t_{1/2} = 67.2$ h).

After indium activity was counted, pellets were resuspended in 1 ml of fresh TYI, and hemocytometer counts were made. 70% of the $^{111}$InOx label was released by 500 $\mu$g/ml amphotericin B (E. R. Squibb & Sons, Princeton, N. J.). This approximates the 80% of $^{111}$InOx released by freeze-thaw experiments reported for other tissue culture cell lines (26).

*Cytopathogenicity of E.h. Filtrates and Sonicates*

AMEBAE FILTRATE STUDIES. *E.h.* suspensions ($10^4$/ml in TYI broth without serum) were incubated for 1 h at 37°C (*E.h.* viability >90% by trypan blue exclusion), and spun at 100 *g* for 5 min. The supernate was removed and passed through a sterile 0.45-$\mu$m filter (Acrodisc; Gelman Instrument Co., Ann Arbor, Mich.), and used as *E.h.* filtrate. Filtrates from *E.h.* plus CHO cells were prepared by using supernates of $10^4$ amebae/ml added to CHO monolayers or pellets. Filtrates of *E.h.* or *E.h.* plus CHO cells were then added to confluent CHO monolayers and evaluated in pellet experiments as well.

AMEBAE SONICATE STUDIES Suspensions of *E.h.* were washed three times with phosphate-buffered saline (to remove serum-containing medium), counted, then diluted in TYI broth to desired concentrations, and sonicated on ice (Sonifer Cell Disrupter, model W140D; Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) at microtip-limit-setting 6 for 3 min. The sonicate was inspected microscopically to insure that no whole amebae remained. The sonicate was centrifuged at 150 g for 5 min and filtered through a 0.45-$\mu$m filter (Acrodisc).

Sonicates of *E.h.* were added to CHO cell monolayers at concentrations of $1 \times 10^4$ to $1 \times 10^6$ *E.h* /ml. Cytopathogenicity was scored as described; CHO cells rounded and released from the tissue culture plate were not considered part of the intact CHO monolayer. With a $5 \times 10^4$ *E.h.*/ml sonicate, the effects of bovine serum (Biofluids, Inc., Rockville, Md.) and a protease inhibitor, equine alpha II globulin (Fraction IV; ICN K&K Laboratories Inc., Plainview, N. Y.), were evaluated.

*Studies with Microfilament and Microtubule Inhibitors*

MICROFILAMENT INHIBITORS, CYTOCHALASINS A, B, AND D. Cytochalasins A, B, and D (Sigma Chemical Co., St. Louis, Mo.) were diluted in dimethyl sulfoxide (Sigma Chemical Co.) to a concentration of 500 $\mu$g/ml and stored at 0°C until used. Upon use, the solution was further diluted in TYI broth to a desired concentration: 5 $\mu$g/ml, 0.5 $\mu$g/ml, or 0.158 $\mu$g/ml. Harvested *E.h.* were then suspended in the cytochalasin (Cyto) solution to a concentration of $10^4$ amebae/ml and added to CHO cell monolayers or used in pellet studies.

STUDIES OF FILTRATES OF AMEBAE PLUS CYTO B. To examine whether cyto B induced secretion of toxic products by the amebae, *E.h.* were suspended in Cyto B-TYI solution (*E.h.* viability >90% by trypan blue exclusion) and used to prepare *E.h.* filtrates as described.

MICROTUBULE INHIBITORS, COLCHICINE, AND VINBLASTINE. Colchicine (Sigma Chemical Co ) was diluted in Puck's saline F to a concentration of $1 \times 10^{-4}$ M. Colchicine was then further diluted to $1 \times 10^{-6}$ M in TYI broth and used to suspend *E.h.* to $10^4$ amebae/ml. After a 2-h incubation, the suspension was added to confluent CHO cell monolayers or pellets to evaluate *E.h.* CHO cell destruction.

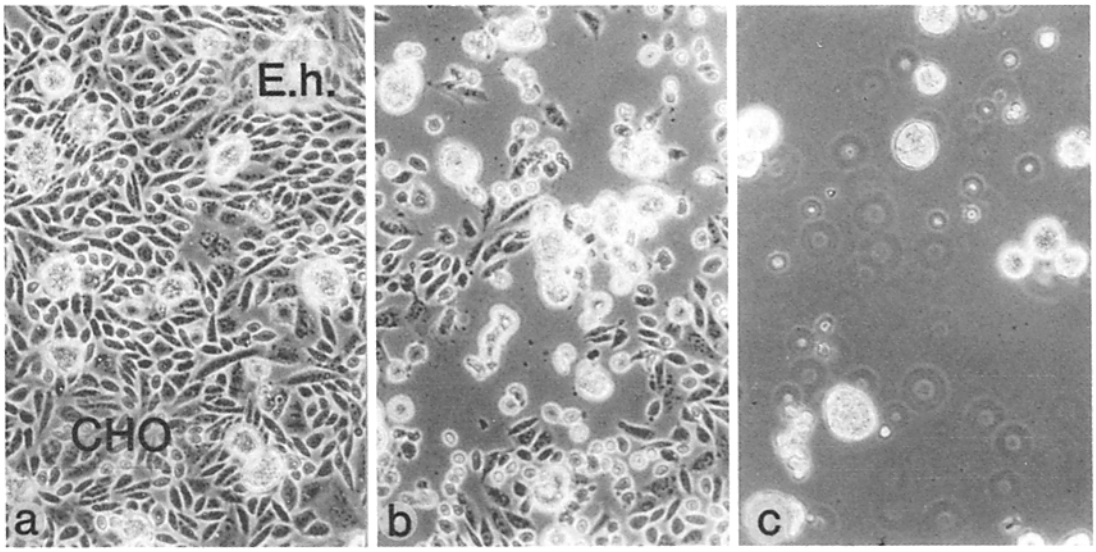


FIG 1 CHO cell monolayer destruction by *E h* strain HM1, added at $10^4$ amebae/ml in TYI At time zero (a), there is an intact CHO cell monolayer (CHO) with amebae (*E.h*) added By 4 h (b), only 25% of the CHO cell monolayer remains, and, at 8 h (c), there is complete destruction of the CHO cell monolayer by the amebae

Vinblastine sulfate (Eli Lilly and Co., Indianapolis, Ind.) was diluted with normal saline to $1 \times 10^{-3}$ M and then further diluted in TYI broth to $1 \times 10^{-6}$ M, which was used for *E.h.* suspension

## Results

### *Cytopathogenicity of Whole, Viable E.h.*

MONOLAYER EXPERIMENTS. The destruction of 75 and 100% of CHO cells in monolayers by whole, viable *E.h.* at $10^4$ amebae/ml at 4 and 8 h, respectively, is shown in Fig. 1.

With cinemicrography, separate components of *E.h.* cytopathogenicity are noted (Fig. 2a–e). Blebbing and death of CHO cells are seen upon contact with amebae (CHO cells that are not in direct contact with amebae remain viable). Amebae are seen phagocytizing CHO cells that had undergone contact-associated blebbing. One ameba can establish contact with numerous CHO cells and amebae phagocytosis does not always immediately follow contact-mediated CHO cell damage.

Approximately 50% of the CHO monolayer is destroyed by the amebae at 2 h, and, by 5 h, <25% of the monolayer remains intact ($P < 0.01$ vs. control, 30 studies).

CHO CELL AMEBAE PELLETS, SERUM FREE. By using a serum-free system, we evaluated the CHO cell killing by amebae in a pellet form, varying the concentration of amebae over a two-log range. By using the one-hit hypothesis, we found a linear relationship of a log ln plot of CHO cell killing vs. log of ameba concentration ($r = 0.99840$, $P < 0.001$) (Fig. 3), which supported cinemicrographic observations that *E.h.* kills CHO cells only on direct contact.

$^{111}$INOX-LABELED CHO CELL AMEBAE PELLET EXPERIMENTS. The comparison of manual quantitative counts, with trypan blue, vs. simultaneous $^{111}$InOx label reveals

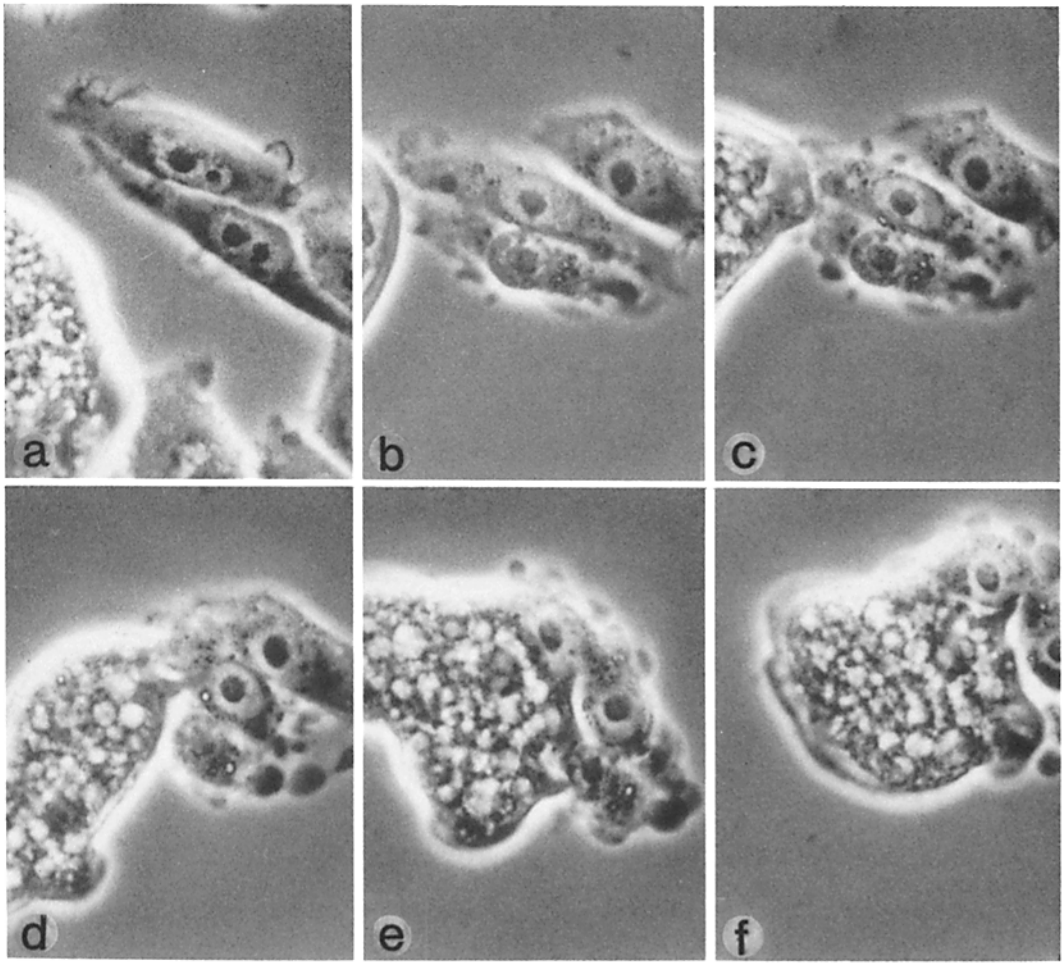


FIG 2 Cinemicrography of the interaction of *E h* and CHO cells. CHO cells not in direct contact with amebae remain intact (a), upon ameba contact, CHO cell membrane blebbing and release from the glass surface occurs (b-d). After contact-mediated CHO cell blebbing occurs, ameba phagocytosis begins (e-f)

several components of *E.h.* cytopathogenicity. At 3 and 6 h, there is significant killing of CHO cells by *E.h.* with both methods ($P < 0.01$, Fig. 4). At 3 h, there are significantly more blue CHO cells present when amebae are added vs. control (35.7 vs. 6.4%, $P < 0.01$). Further extensive studies using only trypan blue exclusion confirmed these observations (Fig. 8, $P < 0.01$), which indicated that amebae kill numerous cells without phagocytosis. At 6 h, only 1.6% of the CHO cells remained alive, the pellet (containing the live CHO cells, blue CHO cells, and amebae with phagocytized CHO cells) had 15.1% of its original $^{111}$InOx label. The amebae in the pellet have phagocytized numerous CHO cells by 6 h (visual count >6 and up to 12 cells/ameba, a minimum of 30% of the original number of CHO cells). These findings indicate that the CHO cells are killed by amebae before phagocytosis (extracellular).

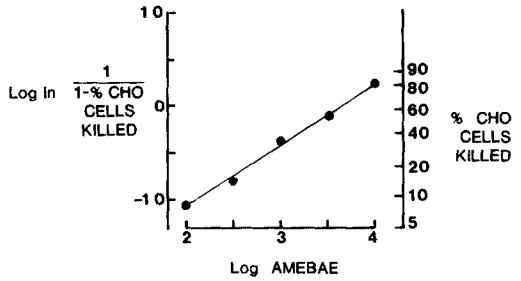


FIG 3 Contact-dependent CHO cell killing by *E.h.*, as demonstrated by the one-hit hypothesis. The percentage of CHO cells killed was ascertained (the mean of 8-10 pairs of amebae: control studies) at each ameba quantity; the log ln(1/[1 − mean percentage of CHO cells killed]) was then calculated. The log of the number of amebae plotted against the log ln (1 / [1 − percentage of CHO cells killed]) was linear ($r = 0.9984$, $P < 0.001$)

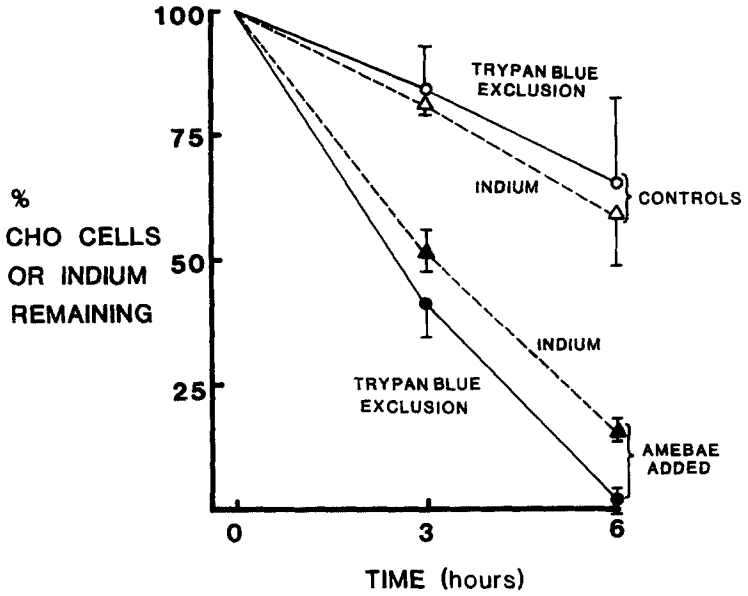


FIG. 4 CHO cell survival in ameba-CHO cell pellets with both trypan blue exclusion and $^{111}$InOx counts By trypan blue exclusion, significant killing of CHO cells by *E h* occurred at 3 and 6 h (each point represents the mean of nine studies ± SE, $P < 0.01$). Retained indium in the pellet was also less than in controls at 3 and 6 h (each point represents the mean of nine studies ± SE, $P <$ 0 01)

*Studies of Cell-free E.h. Cytopathogenicity*

AMEBA FILTRATE STUDIES. At the same concentration of *E.h.* ($10^4$/ml) as the whole ameba contact studies, neither filtrates of *E.h.* alone nor of *E.h.* plus CHO cells had any effect on the CHO cell monolayer when compared with control for up to 24 h. CHO pellet studies confirmed that filtrates of *E.h.* plus CHO cells had no cytolethal effect.

AMEBA SONICATE STUDIES. *E.h.* sonicate at $10^4$ amebae/ml had no effect on CHO monolayers. Lushbaugh et al. (16) have demonstrated a cytopathic effect of higher concentrations of amebae sonicate. We found that CHO cells exposed for 6 h to 5 × $10^4$ amebae/ml sonicate are rounded and released from the tissue culture plate, but

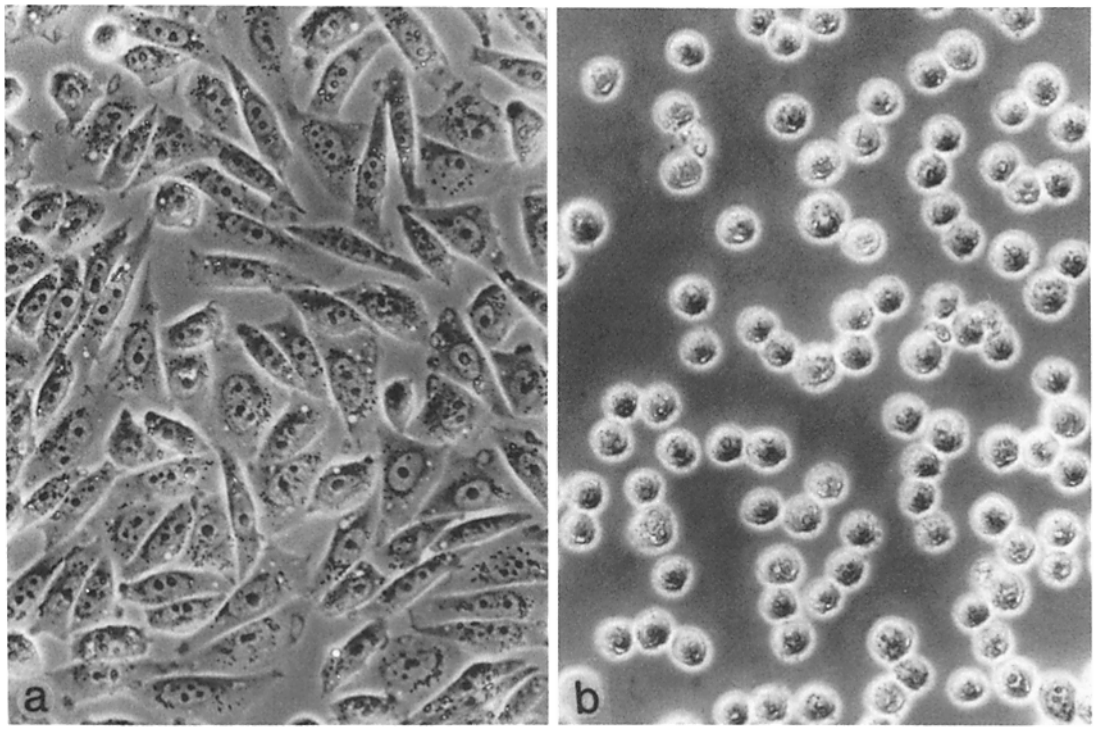


FIG 5. Effect of *E.h.* sonicate on CHO cell monolayer. Photomicrograph of a control CHO cell monolayer (a) contrasted with a monolayer exposed to $\geq 5 \times 10^4$ *E.h.*/ml sonicate (b). The sonicate-exposed CHO cells are rounded and released from the tissue culture plate.

otherwise appear intact (Fig. 5). Filtered supernate of sonicated amebae ($5 \times 10^4$ amebae/ml) had the same effect as the unfiltered suspension. At higher sonicate concentrations ($10^6$ amebae/ml), CHO cells were released within 10 min. 15% heat-inactivated bovine serum and 0.2% equine alpha II macroglobulin (a protease inhibitor) completely inhibited the amebae sonicate effect. In contrast, serum factors were not inhibitory to whole *E.h.* CHO cell monolayer destruction.

CHO cells released by, and remaining in, ameba sonicate ($5 \times 10^4$ amebae/ml) for 24 h had a viability of 97.8% by trypan blue exclusion. $^{111}$InOx-labeled CHO cells exposed to *E.h.* sonicate ($1 \times 10^6$ amebae/ml) for 3 h released only 4.27% of the indium label (indium release in control 4.22%, mean of six studies), and 99.8% of those cells excluded trypan blue.

To evaluate for reversibility of *E.h.* sonicate effect, CHO cells were exposed for 10 min to a high concentration *E.h.* sonicate ($1 \times 10^6$ *E.h.*/ml), incubated until 100% of CHO cells were rounded and released from the well surfaces (30 min), and then F-12 medium (CHO cell medium) was added (Fig. 6). After 3 h, 50% of the CHO monolayer was reestablished, and, at 24 h, there was a confluent CHO monolayer, no different than control (Fig. 6). Trypsin, at 0.025%, exhibits a similar reversible effect (Fig. 6).

*Studies with Microfilament and Microtubule Inhibitors.* We evaluated the effects of Cyto A, B, and D (microfilament inhibitors) on the CHO cell monolayer destruction by whole, viable *E.h.*.

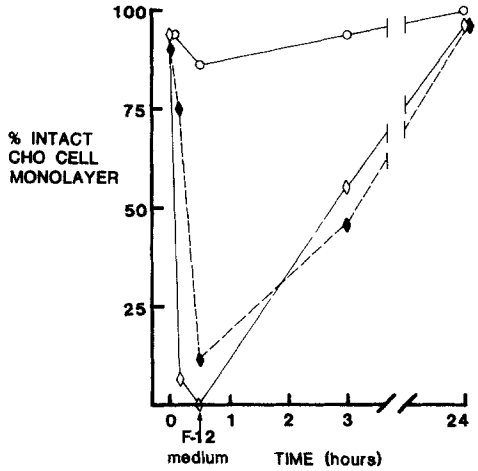


FIG. 6. Reversibility of *E.h.* sonicate-effected CHO cell release. CHO cell monolayers were exposed to control trypticase medium (○), $10^6$ *E.h* /ml sonicate (◇), or 0 025% trypsin (◆) for 10 min. Each point represents the mean of six studies. Solution were removed, and monolayers were incubated for an additional 20 min, at which time F-12 medium was added. The *E.h.* sonicate-exposed CHO cells had complete rounding and release at 30 min, but, with F-12 medium added, were able to reattach and form confluent monolayers by 24 h, 0 025% trypsin had an analogous effect.

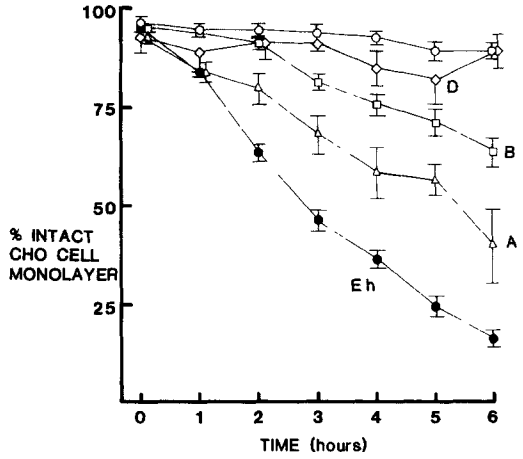


FIG 7 Inhibition by Cyto of CHO cell monolayer destruction by *E h*. The percentage of remaining CHO cell monolayer was observed for control trypticase medium (○, mean of 24 studies ± SE), $10^4$ *E.h* /ml (●, mean of 30 studies ± SE), $10^4$ *E h.*/ml with Cyto A (5 μg/ml) (△, mean of 6 studies ± SE), Cyto B (5 μg/ml) (□, mean of 24 studies ± SE), and Cyto D (5 μg/ml) (◇, mean of 6 studies ± SE) added. There was significant inhibition by all Cyto of CHO monolayer destruction by *E h* (from 2 to 6 h, $P < 0.01$) The inhibitory effect varied with Cyto D > Cyto B > Cyto A (at 5 and 6 h, $P < 0$ 027)

Cyto B (5 μg/ml) significantly inhibited CHO monolayer destruction by $10^4$ *E.h.*/ml ($P < 0.01$ at 2–6 h) (Fig. 7). Cyto B alone was nontoxic to CHO cells, and *E.h.* viability was >90% in Cyto B (5 μg/ml) at 6 h, no different than control.

Cyto B causes macrophage release of acid hydrolases with or without a phagocytic stimulus (27, 28). However, filtrates of amebae in Cyto B (5 μg/ml) or amebae plus CHO cells in Cyto B (5 μg/ml) had no effect on CHO cell monolayers.

Cinemicrography of *E.h.* with Cyto B added showed decreased ameba motion, spike-like cytoplasmic projections, and decreased ameba phagocytosis. Cyto D, a more potent and specific microfilament inhibitor (27, 29), without the metabolic effects of Cyto B, at 5 μg/ml also significantly inhibited CHO monolayer destruction by *E.h.* ($P < 0.01$ at 2–6 h, Fig. 7). Cyto A, which reacts directly with free sulfhydryl groups (27, 30), at 5 μg/ml as well, significantly inhibited CHO cell monolayer destruction by *E.h.* ($P < 0.01$ at 4–6 h, Fig. 7). Preincubation of *E.h.* with Cyto A (5 μg/ml) for 2 h followed by washing amebae resulted in no inhibition of CHO cell monolayer destruction.

Colchicine at $10^{-6}$ M is a specific inhibitor of microtubule function without the metabolic effects seen at higher drug concentrations (31, 32). Colchicine ($10^{-6}$ M) was nontoxic to CHO cells and, when added to *E.h.* ($10^{-4}$/ml), had no inhibitory effect on *E.h.*-mediated CHO cell monolayer destruction (comparison of mean of six studies at 0–6 h).

Vinblastine, which binds at a different site on the microtubule than colchicine (32), inhibits microtubule function at $10^{-6}$ M (31, 33). Vinblastine ($10^{-6}$ M) was nontoxic to CHO cells and, when added to *E.h.* at $10^4$ amebae/ml was well, had no inhibitory effect on *E.h.*-mediated CHO cell monolayer destruction (comparison of means of six studies at 0–6 h).

The CHO cell amebae pellets decrease the contribution of ameba motility to cytopathogenicity. With quantitative CHO cell counts with trypan blue, we still found significant inhibition ($P < 0.01$) of *E.h.* cytopathogenicity when Cyto D (0.5 μg/ml and 0.158 μg/ml) was added (Fig. 8). There was evidence that some CHO cells were killed extracellularly by amebae, as the number of dead CHO cells present were significantly greater with *E.h.* plus Cyto D than with Cyto D alone ($P < 0.05$).

Colchicine ($10^{-6}$ M) or vinblastine ($10^{-6}$ M) had no effect on *E.h.* contact-dependent extracellular killing in ameba CHO cell pellet studies (Fig. 8). With a submaximal inhibitory dose of Cyto D (0.158 μg/ml), we found no additive inhibition of CHO killing when colchicine ($10^{-6}$ M) or vinblastine ($10^{-6}$ M) was added to Cyto D (Fig. 8).

## Discussion

Whole, viable *E.h.*, strain HM1, kill and phagocytize CHO cells. This CHO cell killing occurs exclusively on direct contact. These observations support previous work that shows *E.h.* contact-dependent cytolysis (5, 7–13).[2]

By using cinemicrography, components of *E.h.* cytopathogenicity can be dissected: (*a*) target cell cytolysis occurs only on contact with whole, viable *E.h.*, neighboring cells not in contact with amebae remain intact; (*b*) *E.h.* phagocytize cells after contact-dependent damage; and (*c*) target cell release from a surface occurs on exposure to sufficient concentrations of *E.h.* sonicate. Interference with *E.h.* motility, establishment of effective ameba:cell contact, or phagocytosis would decrease *E.h.* cytopathogenicity.

Our observations, applying the one-hit hypothesis with different concentrations of amebae in a serum-free system, support the cinemicrographic observations that amebae kill cells solely on direct contact. If the amebae elaborated a cell-free cytotoxin, the target cell killing would be independent of the probability of amebae CHO cell contact and the log ln(1[1 − percent CHO cells killed]) vs. log amebae would not be a linear relationship (23, 34).

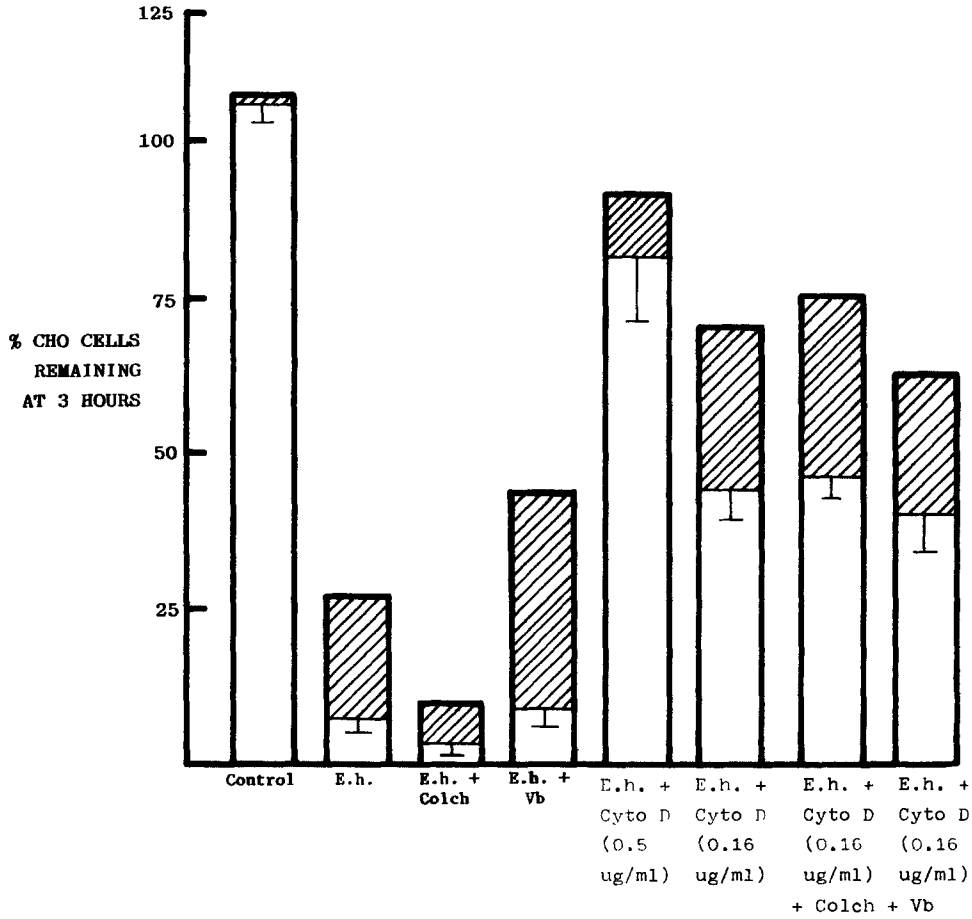


FIG 8 Effect of Cyto D, colchicine (Colch), and vinblastine (Vb) on *E h* killing of CHO cells in ameba CHO cell pellets at 3 h The clear area of the bar represents viable CHO cells that exclude trypan blue, the cross-hatched area represents CHO cells that take up trypan blue The percentage of remaining CHO cells at 3 h was obtained for control trypticase medium with 1% FCS (mean of 29 studies ± SE), $10^4$ *E.h* (mean of 24 studies ± SE) and $10^4$ *E.h.* with colchicine ($10^{-6}$ M) (mean of 6 studies ± SE), vinblastine ($10^{-6}$ M) (mean of 6 studies ± SE), Cyto D (0.5 and 0 158 μg/ml) (mean of 6 and 17 studies ± SE, respectively), and Cyto D (0 158 μg/ml plus colchicine [$10^{-6}$ M] (mean of 12 studies ± SE) or vinblastine ($10^{-6}$ M) (mean of 6 studies ± SE) added Colchicine or vinblastine alone had no effect on *E.h* CHO cell killing Cyto D (0 5 and 0 158 μg/ml) significantly decreased *E h* CHO cell killing ($P < 0.01$ vs. *E h* alone) Adding colchicine or vinblastine to Cyto D (0 158 μg/ml) did not increase the inhibitory effect seen with Cyto D alone

The $^{111}$InOx studies combined with trypan blue exclusion confirm the cinemicrographic observation that *E.h.* kills extracellularly and before phagocytosis. At 6 h, 15.1% of the $^{111}$InOx label was retained in the pellet. This pellet label represents the sum of the activity of live CHO cells (1.6%), retained $^{111}$InOx of killed cells (37.3% × 0.2 [retained $^{111}$InOx in dead cells] = 7.6%), and the retained activity of phagocytized CHO cells within amebae. $^{111}$InOx, once released from target cells, is altered sufficiently to prevent reutilization (25) by the amebae. Therefore, only ~6% of remaining activity in the pellet can be accounted for by phagocytized CHO cells. This indicates that ≥94% of all CHO cells were killed extracellularly before phago-

cytosis. As amebae phagocytized ≥30% ($6 \times 10^4$) of the total CHO cell population ($2 \times 10^5$), the 6% residual activity can be accounted for by the retained $^{111}$InOx of CHO cells first killed extracellularly before phagocytosis.

The CHO cell destruction with whole, viable *E.h.* does not appear to be mediated by a cell-free cytotoxin. *E.h.* sonicate causes reversible rounding and release of CHO cells from a tissue culture plate, an effect that can be mimicked by 0.025% trypsin. This sonicate cell-releasing effect is inhibited by serum and, more specifically, a protease inhibitor, alpha II macroglobulin. These observations are consistent with those of Lushbaugh et al. (16) regarding serum inhibition of *E.h.* sonicate cytopathic effect. It is possible that a component of *E.h.* sonicate on the ameba surface could be delivered in high concentration on contact, thus avoiding inhibition by a serum factor, and, therefore, contribute to the contact-mediated effect of whole amebae. However, *E.h.* sonicate at $5 \times 10^4$ amebae/ml did not exert a cell-killing effect with a 24-h incubation, and, as the effects of still higher concentrations are reversible, it is unlikely that this substance is responsible for the amebae contact cytolysis we and others observe.

We found no evidence that *E.h.* secrete a cytolethal substance in either filtrate or pellet kinetic studies. This is consistent with previous observations in serum-containing systems (7, 13).

Cyto A, B, and D, microfilament inhibitors (35, 36), all significantly decreased CHO cell monolayer destruction by *E.h.*. These Cyto are secondary metabolites of various fungi; Cyto A and B are very similar in structure and activity, whereas Cyto D has a different configuration and ~10 times the activity of Cyto A or B (37, 38). The Cyto used have been reported not to be toxic to protozoa or bacteria (38). Although Cyto B also inhibits metabolic pathways in some cell lines (39), this would not explain the inhibition of ameba cytopathogenicity seen with Cyto D present. On electron microscopy, *E.h.* has been observed to have microtubule- and microfilament-like structures associated with digestive vacuoles and the cell membrane (39–41).

We have observed, on cinemicrography, decreased *E.h.* phagocytosis and disordered amebae motility in the presence of Cyto B (5 μg/ml). In CHO cell amebae pellet studies, Cyto D (0.5 and 0.158 μg/ml) significantly inhibited *E.h.* cytopathogenicity without completely abolishing ameba extracellular killing. Cyto D inhibition of motility should not be a major factor in a pellet experiment with 20 target cells/ameba. As we have shown that ameba extracellular target cell killing normally precedes phagocytosis, Cyto D inhibition of ameba phagocytosis, as well, should not account for the decreased CHO cell killing observed. Therefore, it appears that microfilaments are required for contact-dependent extracellular killing by amebae. Events that may require microfilament function include cell adherence or an event occurring at the ameba membrane after adherence is established.

Analogous effects of Cyto on other contact-dependent killer cells have been reported. Activated human macrophages and T lymphocytes have been reported to cause contact cytolysis of tumor cells (34, 42–45). The lymphocyte- and macrophage-mediated cytolysis is inhibited by Cyto B by decreasing the ability of the killer cells to establish intimate contact with the target cell (34, 42, 45).

Colchicine and vinblastine, used at concentrations ($10^{-6}$ M) at which they are specific microtubule inhibitors (31, 32) and at which they reduce macrophage-mediated cytolysis (45), did not inhibit CHO cell destruction by *E.h.*. When added to

submaximal Cyto D concentrations (0.158 μg/ml), colchicine and vinblastine ($10^{-6}$ M) exhibited no additive inhibition of *E.h.* cell killing. Therefore, even with partial impariment of microfilament function, an intact microtubule system does not appear to be required for ameba contact-dependent cell killing.

## Summary

Cinemicrography of *Entamoeba histolytica* destruction of Chinese hamster ovary (CHO) cells shows that ameba cytopathogenicity consists of separate components: a contact-dependent cytolethal effect, and phagocytosis. Cells not in contact with amebae remain intact. Quantitation of ameba destruction of CHO cells by applying the one-hit hypothesis confirms that the cytolethal effect of amebae is contact dependent. Studies with $^{111}$Indium oxine-labeled cells provide further evidence of extracellular killing by *E. histolytica* and indicate that >94% of the target cells are killed before phagocytosis.

When we examined for a cytotoxin release by *E. histolytica*, we found no effect on CHO cells with filtrates of amebae, and a nonspecific effect of cell rounding and release with sonicates of amebae. The ameba sonicate effect was time-dose dependent, was not cytolethal, was reversible, and was inhibited by alpha II macroglobulin.

Cytochalasin B altered ameba motility and morphology, and monolayer experiments confirmed that cytochalasins A, B, or D inhibited CHO cell destruction by *E. histolytica*. Cytochalasin D also inhibited extracellular killing of CHO cells by amebae in pellets, apparently independent of effects on ameba motility or phagocytosis. Colchicine and vinblastine, alone or in combination with cytochalasin D, did not inhibit *E. histolytica* cytopathogenicity, which indicates that microtubule function is not required for target cell killing by amebae.

The authors wish to express their thanks to James A. Sullivan and Jean Wakefield for their technical assistance, and to Leigh A. Patrick for secretarial and graphics assistance.

*Received for publication 13 March 1980 and in revised form 29 April 1980*

## References

1. Mattern, C. F T , D. B Keistar, and P A. Casper. 1978. Experimental amebiasis. a rapid *in vitro* assay for virulence of *Entamoeba histolytica. Am. J. Trop. Med. Hyg.* **27:**882
2. Eaton, R. D. P., E. Meerovitch, and J. W. Casterton 1976. Correspondence a surface active lysosome in *Entamoeba histolytica. Trans. R. Soc Trop. Med Hyg.* **63:**678.
3. Rondanelli, E. G., G. Caron, G R. Filice, and I. Carver. 1974. Observations of surface activity lysosomes and other organelles in monoaxenically cultivated trophozoites of *Entamoeba histolytica* and *Entamoeba coli. Int. J. Parasitol.* **4:**433
4 Pactor, E. M., and M. A. Gregory. 1972. The observation of a surface active lysosome in the trophozoites of *Entamoeba histolytica* from the lower colon. *Ann. Trop. Med Parasitol.* **66:** 339.
5 Deas, J. E., and J H. Miller. 1977 Plasmalemmal modifications of *Entamoeba histolytica in vivo. J. Parasitol.* **63:**25
6 El-Hashimi, W., and F. Pittman. 1970. Ultrastructure of *Entamoeba histolytica* trophozoites obtained from the colon and from *in vitro* cultures. *Am. J. Trop. Med Hyg.* **19:**215.
7 Knight, R., R. G. Bud, and T. F McCaul 1975. Fine structural changes at *Entamoeba histolytica* rabbit kidney cell (RK 13) interface. *Ann. Trop. Med. Parasitol.* **69:**197

8. Eaton, R. D., E. Meerovitch, and J. W. Casterton. 1970. The functional morphology of pathogenicity of *Entamoeba histolytica. Ann. Trop. Med. Parasitol.* **64:**229.
9. Griffin, J. E. 1972. Human amebic dysentery, electron microscopy of *Entamoeba histolytica* contacting, ingesting and digesting inflammatory cells *Am. J. Trop. Med. Hyg.* **21:**895.
10. McCaul, T. F., and R. G. Bird. 1977. Surface features of *Entamoeba histolytica* and rabbit kidney (RK 13) cell surface changes after trophozoite contact: observations by scanning EM. *Int. J. Parasitol.* **7:**383.
11. Jarumilinta, R., and F. Kradolfer. 1964. The toxic effect of *Entamoeba histolytica* on leukocytes. *Ann. Trop. Med. Parasitol.* **58:**375.
12. Knight, R. 1977. An *in vitro* model for measuring the cytopathic effect of *Entamoeba histolytica* J. Parasitol. **63:**388.
13 McCaul, T. F., R N Paston, and R. G. Bud. 1977. *Entamoeba histolytica* and *Entamoeba* envaders: chromium release from labeled human liver cells in culture. *Exp. Parasitol.* **43:** 342
14. Ravdin, J. I., and R. L. Guerrant. Studies in cytopathogenicity of virulent *Entamoeba histolytica.* Eighth Seminar on Amebiasis B. Sepulveda, editor. Instituto Mexicano del Seguro Social. Mexico City, Mexico. In press.
15. Ravdin, J. I., J. A. Sullivan, G. L. Mandell, and R L. Guerrant. 1979. Effect of *Entamoeba histolytica* on mammalian cells. *Clin. Res.* **27:**788A
16. Lushbaugh, W. B., A. B. Kairalla, J. R. Cantey, A. F. Hofbauer, and F. E. Pittman. 1979. Isolation of a cytotoxin-enterotoxin from *Entamoeba histolytica. J. Infect Dis.* **139:**9.
17 Phillips, B. P., L. S. Diamond, I. L. Bartgis, and S. A. Stuppler. 1972 Results of intracellular inoculation of germ-free and conventional rats with axenically cultivated *Entamoeba histolytica. J. Protozool.* **19:**498.
18. Diamond, L 1968. Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn 1903 and *E. histolytica*-like amebae. *J. Parasitol.* **54:**1047.
19 Diamond, L. S., D. R. Harlow, and C. C. Cunnick. 1978 A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba. Trans R. Soc. Trop Med. Hyg.* **72:**431.
20. Wittner, M. 1968. Growth characteristics of axenic strains of *Entamoeba histolytica* Schaudinn, 1903. *J. Protozool.* **15:**403.
21 Guerrant, R. L., L. L Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G Gilman 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive, *in vitro* assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli. Infect. Immun.* **10:**320.
22. Phillips, H A. 1973 Dye exclusion tests for cell viability. *In* Tissue Culture Methods and Applications. P. F. Krause and M. K. Patterson, editors. Academic Press, Inc., New York 406.
23. 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.
24. Asherson, G. L , and J. Ferluga 1978. Contact sensitivity in the mouse, a non-specific cytotoxicity of T blasts in the draining lymph nodes. *Immunology.* **25:**471.
25 Frost, P., J. Smith, and H. Frost. 1978. The radio-labeling of lymphocytes and tumor cells with $^{111}$Indium (39991). *Proc. Soc. Exp. Bio. Med.* **157:**61.
26. Wiltrout, R. H., P Frost, and G D. Cummings. 1978 Isotope-release cytotoxicity assay with the use of Indium$^{111}$: advantage over chromium-51 in long-term assays. *J Natl. Cancer Inst.* **61:**183.
27. Davies, P , and A. C. Allison. 1978 Effects of cytochalasin B on endocytosis and exocytosis *In* Cytochalasins—Biochemical and Cell Biological Aspects. S. W. Tannenbaum, editor. Elsevier North-Holland, Inc., New York. 143.

28. Hanson, P M., and Z. G. J. Oades. 1973 Enhancement of immunologically induced granule exocytosis from neutrophils by cytochalasin B. *Jour. Immunol.* **110:**290.
29. Czech, M. P., D. G. Lynn, and W S. Lynn. 1973. Cytochalasin B-sensitive 2-deoxyglucose transport in adipose cell ghosts. *J. Biol. Chem.* **248:**3636.
30. Goodman, G. C., and A. F. Miranda. 1978. Cellular contractility and the visible effects of cytochalasin. *In* Cytochalasins—Biochemical and Cell Biological Aspects. S. W. Tannenbaum, editor. Elsevier North-Holland, Inc., New York. 279.
31 Pesanti, E. L., and S. G Axline 1975. Phagolysosome formation in normal and colchicine-treated macrophages *J Exp Med* **142:**903.
32 Wilson, L. 1978. Minireview: action of drugs on microtubules. *Life Sci.* **17:**303.
33. Bryan, J. 1972 Definition of three classes of binding sites in isolated microtubule crystals. *Biochemistry.* **11:**2611.
34. Henney, C. S 1975 T cell-mediated cytolysis: consideration of the role of a soluble mediator *J. Reticuloendothel. Soc.* **17:**231.
35. Weihing, R. R. 1978. *In vitro* interactions of cytochalasins with contractile proteins *In* Cytochalasins—Biochemical and Cell Biological Aspects. S. W. Tannenbaum, editor Elsevier North-Holland, Inc., New York. 431
36 Weilring, R. R. 1976. Cytochalasin B inhibits actin related gelation of HeLa cell extracts. *J. Cell Biol.* **71:**303
37 Tamm, C. H 1978. Chemistry and biosynthesis of cytochalasins. *In* Cytochalasins—Biochemical and Cell Biological Aspects. S. W. Tannenbaum, editor. Elsevier North-Holland, Inc., New York. 15.
38. Thilly, W. G., H. L. Liber, and G N. Wagner. 1978. Toxicity and structure-activity relationships of cytochalasins. *In* Cytochalasins—Biochemical and Cell Biological Aspects. S W Tannenbaum, editor Elsevier North-Holland, Inc., New York. 53.
39. Rosenbaum, R. M , and M. Wittner. 1970 Ultrastructure of bacterized and axenic trophozoites of *Entamoeba histolytica* with particular reference to helical bodies. *J Cell Biol.* **45:**367.
40. Martínez-Palomo, A , A. González-Robles, and B. Chevez de Ramírez 1976. Ultrastructural studies of various *Entamoeba* strains *In* Memorias de la Conferencia Internacional Sobre Amibiasis. B Sepulveda and L. S. Diamond, editors. Instituto Mexicano del Seguro Social, Mexico City, Mexico. 226.
41 Michel, R., and E. Schupp. 1976. Cytoplasmic fibrils and their relation to ameboid movement of *Entamoeba histolytica In* Memorias de la Conferencia Internacional Sobre Amibiasis. B Sepulveda and L S Diamond, editor Instituto Mexicano del Seguro Social, Mexico City, Mexico. 300.
42 Allison, A. C., and J. Ferluger. 1976. Editorial· how lymphocytes kill tumor cells. *N. Engl J Med.* **295:**165
43 Sanderson, C. J. 1976. The mechanism of T cell-mediated cytotoxicity. I. The release of different cell components. *Proc. R. Soc. Lond. B Biol. Sci.* **192:**221.
44 Sanderson, C J. 1976 The mechanism of T cell-mediated cytotoxicity. II. Morphological studies of cell death by time-lapse microcinematography *Proc R Soc. Lond B Biol Sci* **192:** 241.
45 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:**276.