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Taxol, a microtubule stabilizing agent, blocks the replication of *Trypanosoma cruzi*

(parasites/chemotherapy/electron microscopy)

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ABSTRACT **Taxol, an experimental antitumor agent and stabilizer of microtubules, inhibits *in vitro* replication of the human pathogenic hemoflagellate *Trypanosoma cruzi*. Micromolar concentrations of the drug prevent the completion of cell division in these organisms but allow the multiplication of cell organelles such as the nucleus, kinetoplast, and flagellum. The result is the formation of motile organisms that have extra organelles but cannot fully replicate. Division proceeds to a relatively fixed locus on the long axis of the organism, suggesting the presence of a specific affected structure or function at this site. It is postulated that taxol produces these effects by stabilizing a portion of the microtubular cytoskeleton of *T. cruzi*.**

Trypanosoma cruzi is the causative agent of South American trypanosomiasis or Chagas disease. Infection with this organism may lead to severe chronic disruption of the autonomic nervous system in 20–40% of those infected and is fatal in 5–10% of clinically ill patients (1, 2). Although many agents have been tested, there is currently no curative drug for this disease in man. *T. cruzi* is a eukaryote and presumably shares many metabolic pathways with human cells. Novel approaches will therefore be needed to identify drugs that have good therapeutic indices in this disease. In view of the prominent subsurface array of microtubules in *Trypanosoma*, we hypothesized that taxol, a drug affecting the stabilization of microtubules, might have a specific deleterious effect on these organisms.

Taxol is an experimental antitumor drug that was isolated from the plant *Taxus brevifolia* (3). Studies in our laboratory have shown that the drug has the unusual capacity to promote the assembly of microtubules in a cell-free system (4). Taxol also stabilizes microtubules, protecting them from the depolymerizing effects of low temperatures, both in cells and in a cell-free system (5). The drug has an unusual chemical structure and is a potent inhibitor of the replication of mammalian cells in culture (5). We have found that taxol in micromolar amounts produces unique morphologic changes in *T. cruzi* growing in culture and results in cessation of their replication.

MATERIALS AND METHODS

Trypanosomes. The Brazil strain of *T. cruzi* was originally obtained from William Hanson (University of Georgia, Athens, GA). Trypanosomes were cultured in LIT growth medium [2% liver infusion broth (wt/vol, Difco)/0.5% tryptose (Difco)/0.4% NaCl/0.046% KCl/0.8% Na_2HPO_4/0.2% glucose/0.4% hemin (Sigma)]. Just before use, the medium was supplemented with 5% (vol/vol) fetal calf serum (GIBCO). The organisms were suspended in 20 ml of LIT medium in a 125-ml Erlenmeyer flask and incubated at 27°C on a shaker platform. During its life cycle, *T. cruzi* assumes several forms having varying degrees of infectivity for the human host. The epimastigote or insect form is so named because its kinetoplast is situated anterior to the nucleus. This is the form that *T. cruzi* assumes when the organism is cultured in LIT medium. Replication of epimastigotes is asynchronous, with a doubling time of ≈24 hr. Under culture conditions, 85–90% of *T. cruzi* were in the epimastigote stage; the rest were trypomastigotes and other transitional forms.

Trypanosomes were counted in a hemocytometer by phase-contrast microscopy. For light microscopic examination, organisms were removed from the medium by centrifugation at 800 × *g*, washed twice in phosphate-buffered saline, pH 7.2, resuspended in the saline, and placed on slides. After air drying, the slides were fixed in absolute methanol for 5 min and stained with May–Gruenwald stain (Harelco) for 5 min and in Giemsa stain for 13 min. Slides were rinsed first in acetone, then in acetone/xylene (1:1), and finally in xylene. They were mounted in Permount and photographed by using a Zeiss photomicroscope.

Taxol, obtained from the National Cancer Institute, was dissolved in dimethyl sulfoxide (Me_2SO) at 10 mM and stored in the dark at −20°C.

Growth Curves. Various concentrations of taxol in Me_2SO were added to trypanosome cultures either at the time of transfer to LIT medium or after logarithmic growth had been achieved. The final concentration of Me_2SO was 0.5%. Control cultures were exposed to the same concentration of Me_2SO. Previous studies in our laboratory have shown that this concentration of Me_2SO has no effect on growth as measured by cell count or by uptake of thymidine, uridine, or leucine (6).

Daily counts were performed during 4–7 days of continuous drug exposure. In addition, the percentage of abnormal morphologic forms was assayed by phase-contrast microscopy or by examination of stained and fixed slides. In general, examination by phase-contrast microscopy underestimated the percentage of abnormal forms. Abnormal organisms did not complete cytokinesis and had more than one flagellum and multiple nuclei. Drug-treated organisms also contained several copies of the kinetoplast, a giant mitochondrion.

Reversibility of the Taxol Effects. Trypanosomes were incubated with either 1 μM or 10 μM taxol for various periods of time in LIT media, washed three times in phosphate-buffered saline as described above, and suspended in media free of taxol. Daily counts were done, and the percentage of abnormal forms was determined.

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Abbreviation: Me_2SO, dimethyl sulfoxide; M/P_i, Millonig's phosphate buffer.

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Scanning Electron Microscopy. Samples to be examined were harvested as described above and fixed for 1 hr as a suspension in 2.5% (wt/vol) glutaraldehyde in 0.1 M Millonig's phosphate buffer, pH 7.4 (M/P_i), washed twice with M/P_i, and stored in this buffer at 4°C. For final processing, 1.5–3.0 × 10^3 cells from each sample were postfixed for 15 min with 1% osmium tetroxide in 0.05 M M/P_i, filtered onto a Diaflow filter, washed with M/P_i, and dehydrated in a graded series of alcohols. The filters were immersed in ethanol/Freon TF (Du Pont) (1:1) for 15 min, followed by two 15-min soakings in 100% Freon TF and critical point drying with CO_2 for 20 min. Filtration, dehydration, and critical point drying were done by using a Gelman Swinney-type syringe filter adapter modified to facilitate easy exchange of fluids. Immediately after critical point drying, the filters were sputter coated with gold and observed in a JEOL 25S scanning electron microscope.

Transmission Electron Microscopy. At various times after treatment with taxol, trypanosomes were counted, washed, and sedimented as described above. The resulting trypanosome pellet was fixed in 2.5% (wt/vol) glutaraldehyde in M/P_i for 2 hr at room temperature and postfixed with 1% osmium tetroxide for 1 hr at 4°C. The pellet was dehydrated with a graded series of alcohols, infiltrated with propylene oxide, and embedded in araldite resin. Untreated trypanosomes were prepared in the same way. Thin sections were obtained and stained first with 4% uranyl acetate in 40% (vol/vol) ethanol and then with 0.1% lead citrate. Sections were examined in a Siemens Elmiskop 1A electron microscope.

RESULTS

Effect of Taxol on Replication of *T. cruzi*. Addition of taxol at the time of transfer to LIT medium markedly inhibits the replication of the *T. cruzi* in a dose-dependent fashion (Fig. 1*A*). Taxol at 10 nM and 1 nM also inhibits replication (data not given). Fig. 1*B* shows that taxol is an effective growth inhibitor when added during the log phase of trypanosomal replication.

The inhibition of *T. cruzi* replication by taxol is accompanied by the formation of bizarre morphologic forms. Drug-treated organisms contain multiple nuclei and kinetoplasts and have multiple flagella that are motile. The data in Fig. 2*A* show the dose-related emergence of abnormal forms with time after treatment. Fig. 2*B* is a photomicrograph of stained *T. cruzi* treated with 10 μM taxol for 4 days.

Ultrastructural Changes in *T. cruzi* Treated with Taxol. When the parasites were examined in the scanning electron microscope after 3 days of exposure to 10 μM taxol, the difference between treated and untreated organisms was evident. Fig. 3*A* is a photomicrograph of an untreated epimastigote with a single flagellum. Fig. 3 *B* and *C* are high- and low-power views of epimastigotes that have two or more flagella. When the distance from the tip of the flagella to the bifurcation point was measured in 30 treated organisms, it was found that the average ratio of this distance to the total length of the organism was quite constant (58 ± 6%).

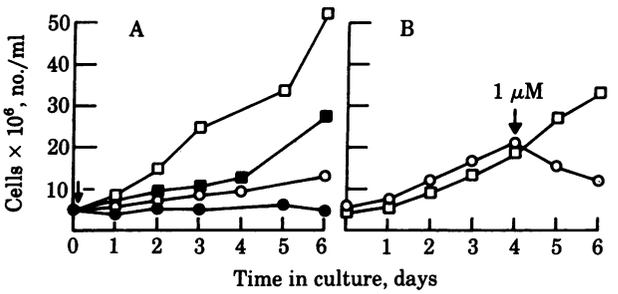


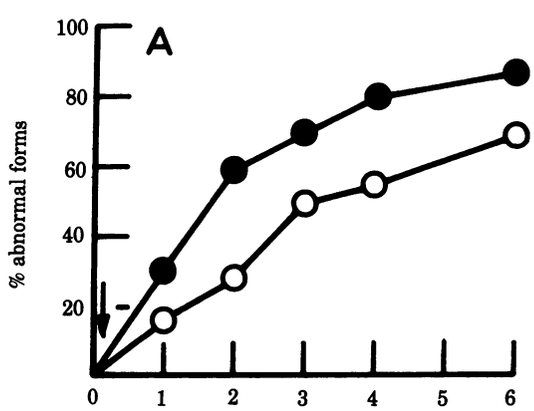
FIG. 1. Growth of *T. cruzi* in culture. (*A*) Various concentrations of taxol were added at the time organisms were transferred to growth medium. ●, 10 μM; ○, 1 μM; ■, 0.1 μM; □, no taxol. (*B*) With (○) and without (□) taxol added during exponential phase of growth. Arrow, time of addition.

T. cruzi examined by transmission electron microscopy (Fig. 4) were again seen to contain multiple copies of organelles in the treated organisms (Fig. 4*B*) as compared with untreated *T. cruzi* (Fig. 4*A*). At higher magnification (Fig. 5), the subpellicular microtubule network and multiple copies of organelles were easily appreciated. Subpellicular microtubules were 250 Å in diameter, and flagellar structures showed the typical axoneme pattern of nine pairs of microtubules arrayed in a circle around a central pair.

DISCUSSION

Trypanosomes and other protozoan hemoflagellates possess a microtubular skeletal network lying just below the surface membrane. After duplication of organelles, these organisms normally divide by longitudinal binary fission, which begins anteriorly and proceeds to the posterior end. The organisms can be grown *in vitro* and log phase division occurs about once in 24 hr.

T. cruzi, like many species of protozoa, fungi, and myxo-



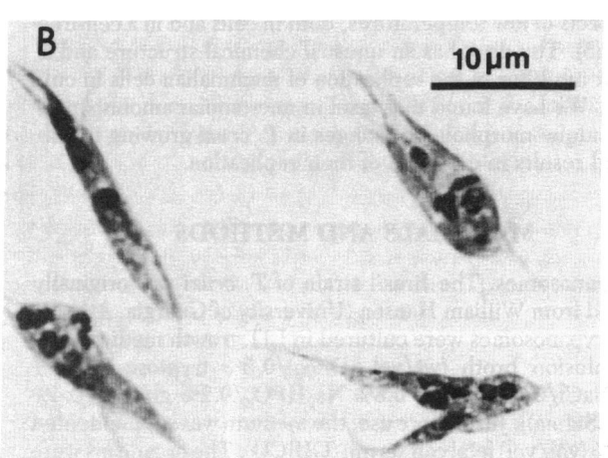


FIG. 2. (*A*) Percentage of abnormal forms of *T. cruzi* after addition of taxol at 10 μM (●) and 1 μM (○). Arrow, time of addition. (*B*) Photomicrograph of abnormal forms of *T. cruzi* grown in medium containing 10 μM taxol for 4 days. The organisms are partially divided, yielding two or more flagella and multiple copies of nuclei and kinetoplasts (dark bodies).

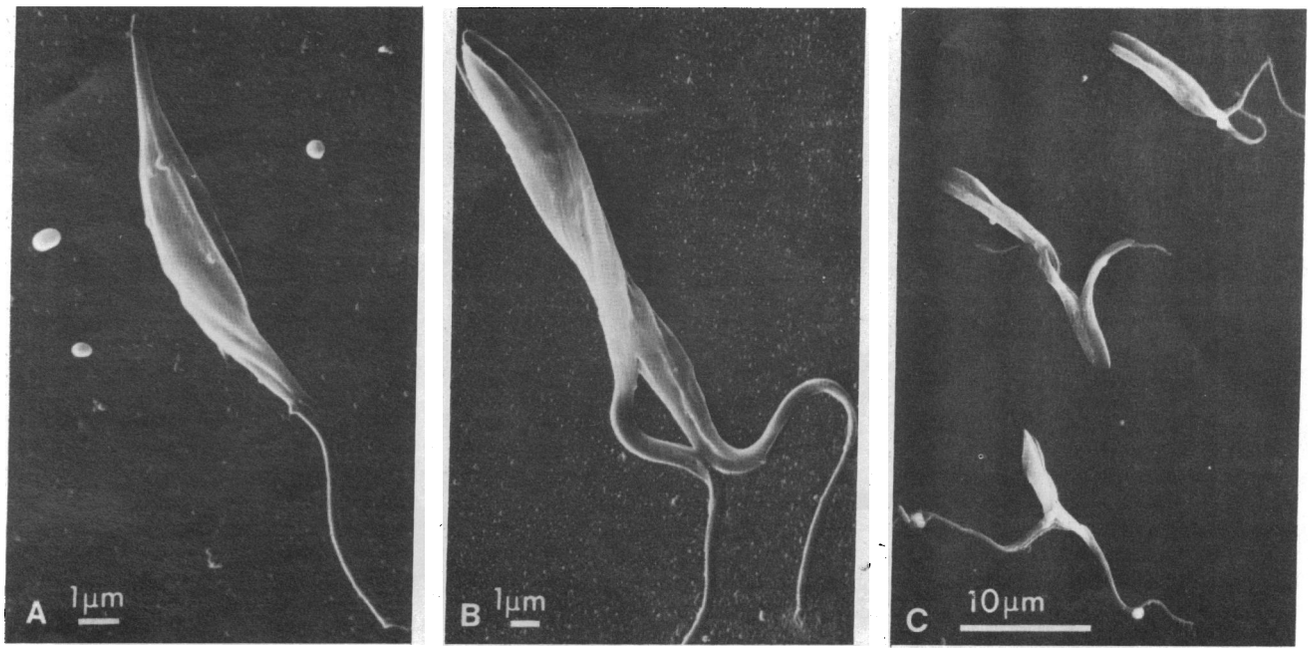


FIG. 3. Scanning electron micrographs of *T. cruzi*. (*A*) Untreated epimastigote form. (*B*) Epimastigote treated with 10 μM taxol for 72 hr, showing arrested division. (*C*) Taxol-treated *T. cruzi* at lower magnification, showing several organisms that have incompletely divided. Division begins at the split end and proceeds longitudinally for 58 ± 6% of the total length of the organism.

mycetes, undergoes mitosis with its nuclear membrane intact (7). The first sign of division in *T. cruzi* is replication of the basal body and flagellum. Microtubules subsequently form within the nucleus, and by the time the kinetoplast has divided, bundles of microtubules can be seen coursing from one end of the elongated nucleus to the other (7). After the daughter nuclei have pinched apart (interphase), intranuclear microtubules can no longer be seen and have evidently disassembled (7). No centriolar origin of these microtubules has been identified. As the cell membrane is duplicated to surround the daughter cells, new subpellicular microtubules must form. The means by which these subpellicular microtubules duplicate in dividing forms has not been determined. In the transitional spheromastigote form of the organism, there are two groups of four microtubules as-

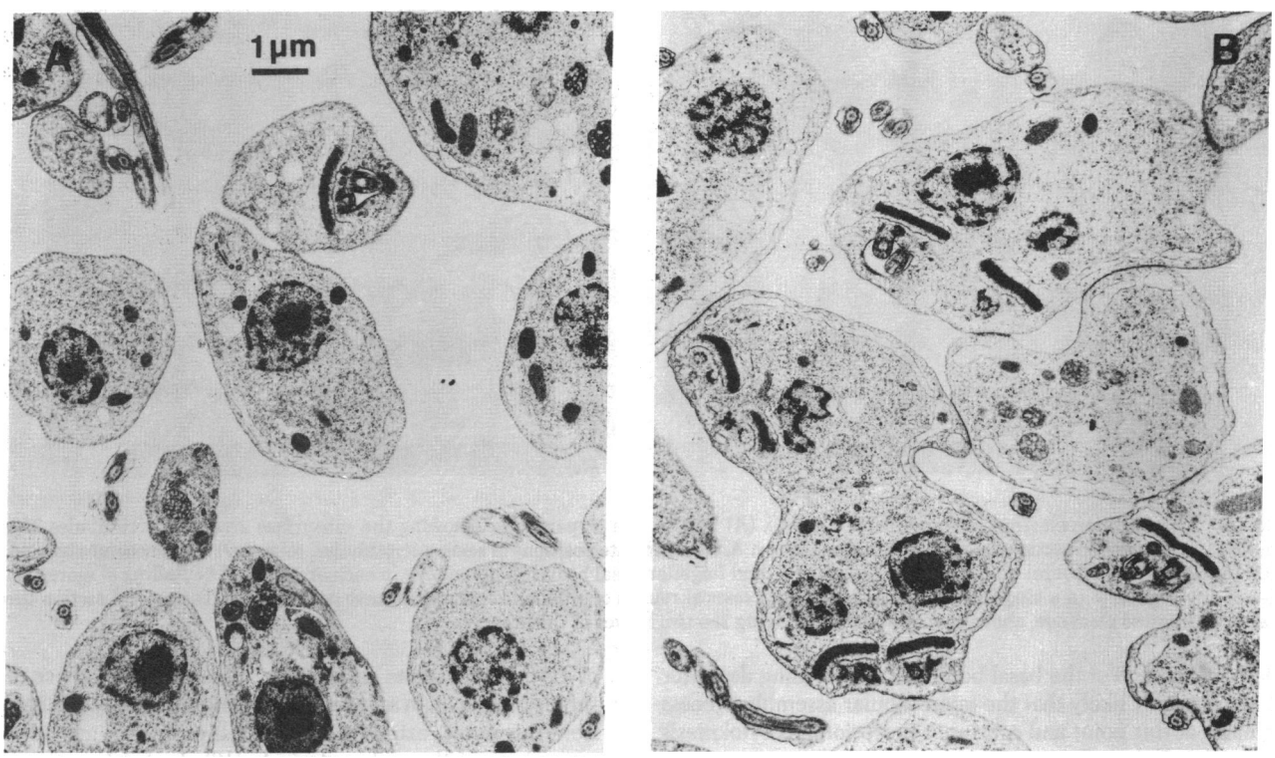


FIG. 4. Transmission electron micrographs of *T. cruzi*. (*A*) Control epimastigotes. (*B*) Epimastigotes treated with 10 μM taxol for 72 hr. Several organisms show multiple organelles including nuclei, kinetoplasts, and flagellar basal bodies.

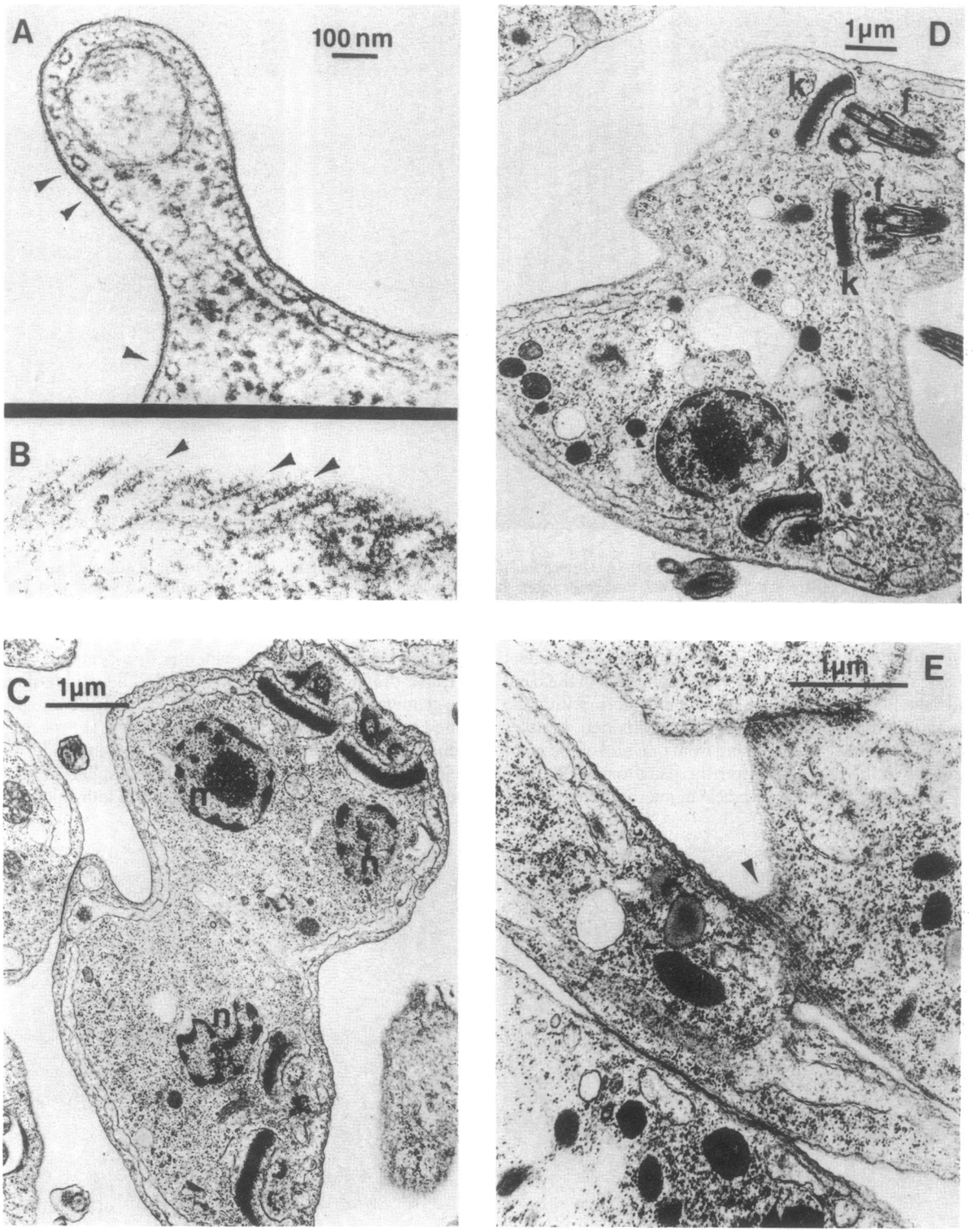


FIG. 5. Transmission electron micrographs of *T. cruzi*. (*A*) Portion of a trypanosome, showing the subsurface array of microtubules 250 Å in diameter. (*B*) Tangential section through microtubule array. Arrows indicate positions of some microtubules. (*C* and *D*) Trypanosomes treated with 10 μM taxol for 72 hr. Several nuclei (n), kinetoplasts (k), and flagellar basal bodies (f) are visible in each organism. The finding of more than two copies of any organelle in a single organism indicates that several rounds of organelle replication have occurred. (*E*) Tangential section through the area of arrested cleavage, showing microtubules bridging the two halves of the organism.

sociated with each of the basal bodies in the dividing daughter cell (8). It seems likely that the microtubular assembly process originates at this point and continues posteriorly as cytokinesis proceeds. In other Trypanosomatidae, a similar arrangement and origin of the subpellicular microtubules has been described (9). Cell disruption studies by Angelopoulos in seven species of Trypanosomatidae support the hypothesis that these microtubules originate in association with the basal body and elongate from this point, extending to the cell periphery and then spiraling around the cell posteriorly (10). In *Leishmania*, Lewis reported that the subpellicular microtubules double just before the formation of the division furrow (11). It seems evident that,

for daughter cells to separate during cytokinesis, the subpellicular microtubules must depolymerize and later reassemble.

Recognition of the subpellicular microtubule network described by others led us to hypothesize that a drug that stabilized microtubules might inhibit the replication of trypanosomes by preventing disassociation of the microtubules. Taxol in micromolar concentrations was indeed found to markedly inhibit the replication of the Brazilian strain of *T. cruzi*. In the presence of taxol, replication ceased when the drug was added in the lag or in the log phase of growth. Cessation of replication was accompanied by the formation of bizarre organisms in which multiplication of all identifiable organelles took place but division into daughter cells was prevented. The fact that cytokinesis proceeded to a relatively fixed region along the length of the organism suggests that some crucial structure or event occurs at that locus. The nature of this structure or event remains to be elucidated but would appear to be related to the microtubular skeletal network. It is interesting that the basal body, the site from which the subpellicular microtubules are thought to arise, is at about this region in the epimastigote form of *T. cruzi*.

Inhibition by 1 μM taxol appears to be reversible because when the drug is removed division proceeds. It is not clear at present whether this apparent reversibility represents the recovery and division of affected organisms or whether the increase in the number of organisms on withdrawal of taxol is solely due to replication of unaffected forms. Inhibition by 10 μM taxol, which led to the production of 90% bizarre forms, was not reversible. Despite the inhibition of replication, the organisms remained viable in taxol and continued to exhibit the specialized function of motility.

Our studies suggest that there are at least two classes of microtubules in *T. cruzi* as defined by their sensitivity to taxol. Those comprising the flagellar apparatus are apparently not susceptible to the effects of taxol. In contrast, a portion of those microtubules that make up the subpellicular cytoskeleton are stabilized and cannot disassemble to allow efficient duplication and cytokinesis. It is of course possible that the effects of taxol on *T. cruzi* are not the result of the microtubule-stabilizing activity of this drug. However, the inability of the trypanosomes to completely divide is a predictable result of such activity.

Colchicine and vinblastine, drugs that also affect microtubules, were assayed for their actions on *T. cruzi*. In preliminary experiments, vinblastine inhibited replication and led to the induction of shortened forms of *T. cruzi*. At micromolar concentrations, colchicine had no effect on replication or morphology. Other workers have recently reported a damaging effect of colchicine on *Trypanosoma* (12), but concentrations used in those experiments were 1000-fold those that we used.

Studies with drugs such as taxol may prove useful in designing chemotherapeutic agents for *T. cruzi* infection and also may allow us to determine the functions of different classes of microtubules within the cell. Although taxol itself may prove too toxic to mammalian cells (5) to be therapeutically useful, the specific effect on a class of protozoal microtubules gives promise that derivatives of this drug may be found that have good therapeutic indices.

Finally, our data suggest that microtubular arrays in *Trypanosoma* and other hemoflagellates may prove a useful substrate for determining the exact action of drugs that have a primary activity on microtubules.

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