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Trypanosoma cruzi Proliferation and Differentiation Are Blocked by Topoisomerase II Inhibitors

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Bacterial topoisomerase II inhibitors (ofloxacin and its commercial derivative Tarivid, nalidixic acid, and novobiocin) were tested as blockers of *Trypanosoma cruzi* differentiation and proliferation. The transformation of either epimastigotes into metacyclic trypomastigotes or amastigotes into trypomastigotes was inhibited by the drugs in a dose-dependent manner. The inhibition of epimastigote differentiation was also dependent on the time of drug addition to the medium. Proliferation of *T. cruzi* was also blocked in a dose-dependent manner by the drugs, with the exception of novobiocin, which did not inhibit epimastigote replication and resulted in cell lysis when it was used at high concentrations. On the other hand, the transformation of amastigotes into epimastigotes in axenic culture was not inhibited; this process did not require either kinetoplast (mitochondrial) DNA replication or changes in the DNA network organization. Electron microscopy of cells treated with Tarivid (ofloxacin) showed damage to the kinetoplast, suggesting that this organelle might be the target of the drug. These results indicate that a bacterial-like topoisomerase II plays an important role in *T. cruzi* proliferation and differentiation.

Chagas' disease is a parasitic disease that affects more than 20 million individuals in Latin America (12). As yet, prophylaxis of the disease has been limited because of the lack of a vaccine or safe chemotherapy. *Trypanosoma cruzi*, the causative agent of this disease (4), displays a complex life cycle that involves two intermediary hosts (triatomines and mammals) and three differentiation stages: epimastigotes, which replicate in the insect vector; amastigotes, which replicate in mammalian cells; and the infective and nonreplicative trypomastigotes (11).

The transformation of replicative forms into infective forms involves important morphogenetic changes. One of the most striking modifications occurs at the level of kinetoplast ultrastructure. The kinetoplast is that portion of the unique mitochondrion from members of the order *Kinetoplastida* which contains the mitochondrial DNA (kinetoplast DNA [K-DNA]). During cell proliferation, the K-DNA must replicate, while on transformation of amastigotes or epimastigotes into trypomastigotes, the K-DNA changes from a compact (bar) to a more relaxed (basket) form (11). This suggests that topoisomerases may play an important role in both *T. cruzi* proliferation and differentiation.

Our recent work on *T. cruzi* metacyclogenesis (i.e., the transformation of epimastigotes into metacyclic trypomastigotes) resulted in the development of chemically defined in vitro differentiation conditions (1, 8). In addition to allowing the production of metacyclic trypomastigotes and the study of the metacyclogenesis process, these conditions are very useful for testing the effects of drugs on *T. cruzi* differentiation.

In this study we tested the effect of topoisomerase II inhibitors on *T. cruzi* metacyclogenesis. In addition, we also tested the effects of these inhibitors on *T. cruzi* proliferation and during the intracellular cycle of the parasite inside mammalian cells. The results indicated that both replication and differentiation of the parasite are blocked by topoisomerase II inhibitors.

MATERIALS AND METHODS

Chemicals. Ofloxacin and its commercial derivative Tarivid [9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid] were obtained from Hoechst (Frankfurt, Federal Republic of Germany) and were kindly provided by Uwe Sphor. All the other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Parasites. The *T. cruzi* Dm28c clone was obtained and was kept in the laboratory as described previously (6). Epimastigotes were grown in liver infusion tryptose (LIT) medium (2). In vitro metacyclogenesis was performed as described elsewhere (8). Briefly, epimastigotes were harvested from LIT medium and incubated for 2 h in artificial triatomine urine (TAU) (190 mM NaCl, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 8 mM phosphate buffer [pH 6.0]) and were then incubated in TAU supplemented with 10 mM L-proline (TAUP) for different times up to 96 h in the presence or absence of the drugs being tested. *T. cruzi* trypomastigotes of the Y strain (21) were obtained from the blood of albino mice at the peak of parasitemia and were isolated by differential centrifugation. Amastigote forms were obtained from the supernatant of a J774G-8 macrophage cell line, after 6 to 7 days after infection with bloodstream trypomastigotes (3). The proliferation of amastigotes in axenic culture (5×10^6 cells ml^{-1}) was performed in Warren medium at 29°C (10). In all cases, cells were counted in a Neubauer chamber.

Treatment of heart muscle cell culture. Cultures that were 3 to 5 days old were infected with bloodstream trypomastigotes by using a parasite:cell ratio of 10:1. After 24 h of interaction, the cells were washed and fresh medium (Dulbecco modified Eagle medium plus 5% fetal bovine serum and 10% horse serum) was added and changed every 2 days. Tarivid (molecular weight, 361.37) was added at concentra-

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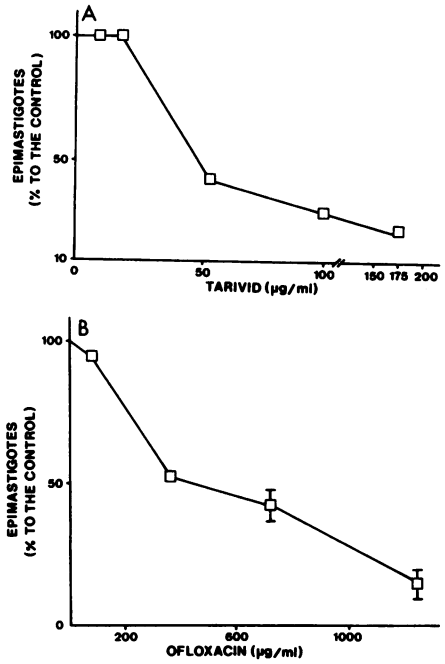

FIG. 1. Growth inhibition of *T. cruzi* epimastigotes by the topoisomerase II inhibitors Tarivid (A) and ofloxacin (B). Parasites were grown in LIT medium supplemented with the indicated amounts of inhibitors. The values are means ± standard deviations (which are included in the symbols or are indicated by vertical bars) of three independent experiments.

tions that ranged from 200 to 1,000 μM either during interaction or 1 to 3 days after infection and was maintained in the cultures throughout the experiment. The cells were fixed in Bouin fixative and stained with Giemsa stain for observation under a light microscope. Alternatively, the cells were processed for electron microscopy.

Transmission electron microscopy. The parasites or cultures were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h and rinsed in the same buffer. They were left at 4°C overnight and were then gently scraped off with a rubber policeman and collected by centrifugation. Following this they were postfixed with 1% OsO_4, dehydrated in acetone, and embedded in Epon resin. Thin sections were stained with uranyl acetate and lead citrate and observed in a microscope (EM10B; Zeiss).

Cell labeling and immunoprecipitation. Labeling of parasites with [^{35}S]methionine and polyacrylamide gel electrophoresis analysis were performed as described elsewhere (1). Immunoprecipitations were carried out with trypomastigote stage-specific antiserum and with antiserum that recognized antigens from both differentiation stages (8).

RESULTS

Effect of topoisomerase II inhibitors on epimastigote proliferation. We first tested the effect of Tarivid (whose active principle is ofloxacin) on the proliferation of *T. cruzi* Dm28c in LIT medium. The drug was added to LIT medium at different concentrations, and at several time points, portions of the cultures were removed for cell counting. The results are presented in Fig. 1A and show a dose-dependent inhibitory effect. A dose of 48 μg ml^{-1} resulted in 50% inhibition

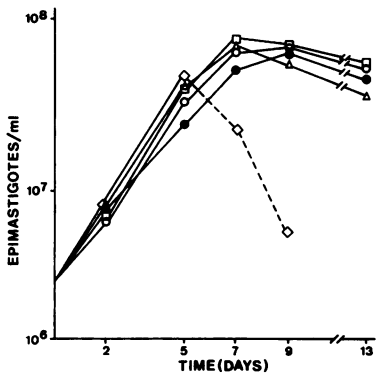

FIG. 2. Effect of novobiocin on epimastigote replication in LIT medium. The final drug concentrations were 0 (●), 122 (○), 200 (□), 245 (△), and 612 (◇) μg ml^{-1}. Values are means of three independent experiments, and standard deviations are included in the symbols.

of epimastigote proliferation. However, Tarivid exerted its maximum inhibitory effect only in the case of drug addition during the first 24 h of proliferation (data not shown). Surprisingly, Tarivid showed a stronger inhibitory effect than its active principle ofloxacin did. Indeed, the ofloxacin

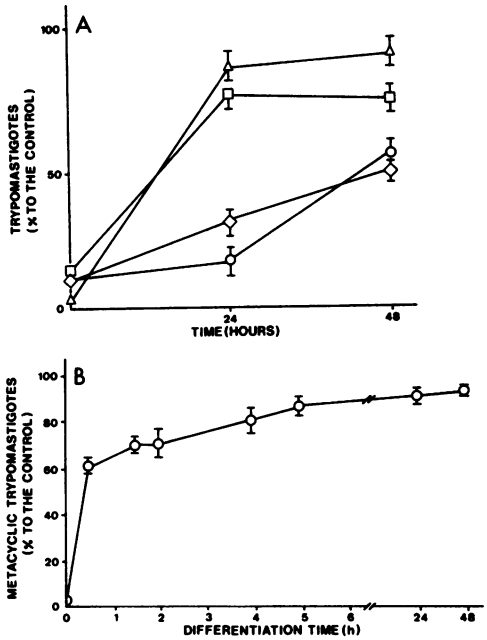

FIG. 3. (A) Effect of time of addition of topoisomerase II inhibitors on *T. cruzi* metacyclogenesis. The drugs were added at concentrations which resulted in full inhibition of the metacyclogenesis process when they were added at time zero and were added at the indicated times of the differentiation process. Parasites were counted after 96 h of differentiation. The values are means of triplicate values from two independent experiments, and the bars correspond to standard deviations. ○, Novobiocin, 245 μg ml^{-1}; ◇, nalidixic acid, 650 μg ml^{-1}; □, ofloxacin, 1,250 μg ml^{-1}; △, Tarivid, 100 μg ml^{-1}. (B) Time-dependent inhibition of *T. cruzi* metacyclogenesis by Tarivid. The drug (100 μg ml^{-1}) was added to the differentiation medium at the indicated times. After 96 h of incubation in the differentiation medium, cells were removed for differential counting. Each point represents the mean value of three independent experiments, and the bars represent standard deviations.

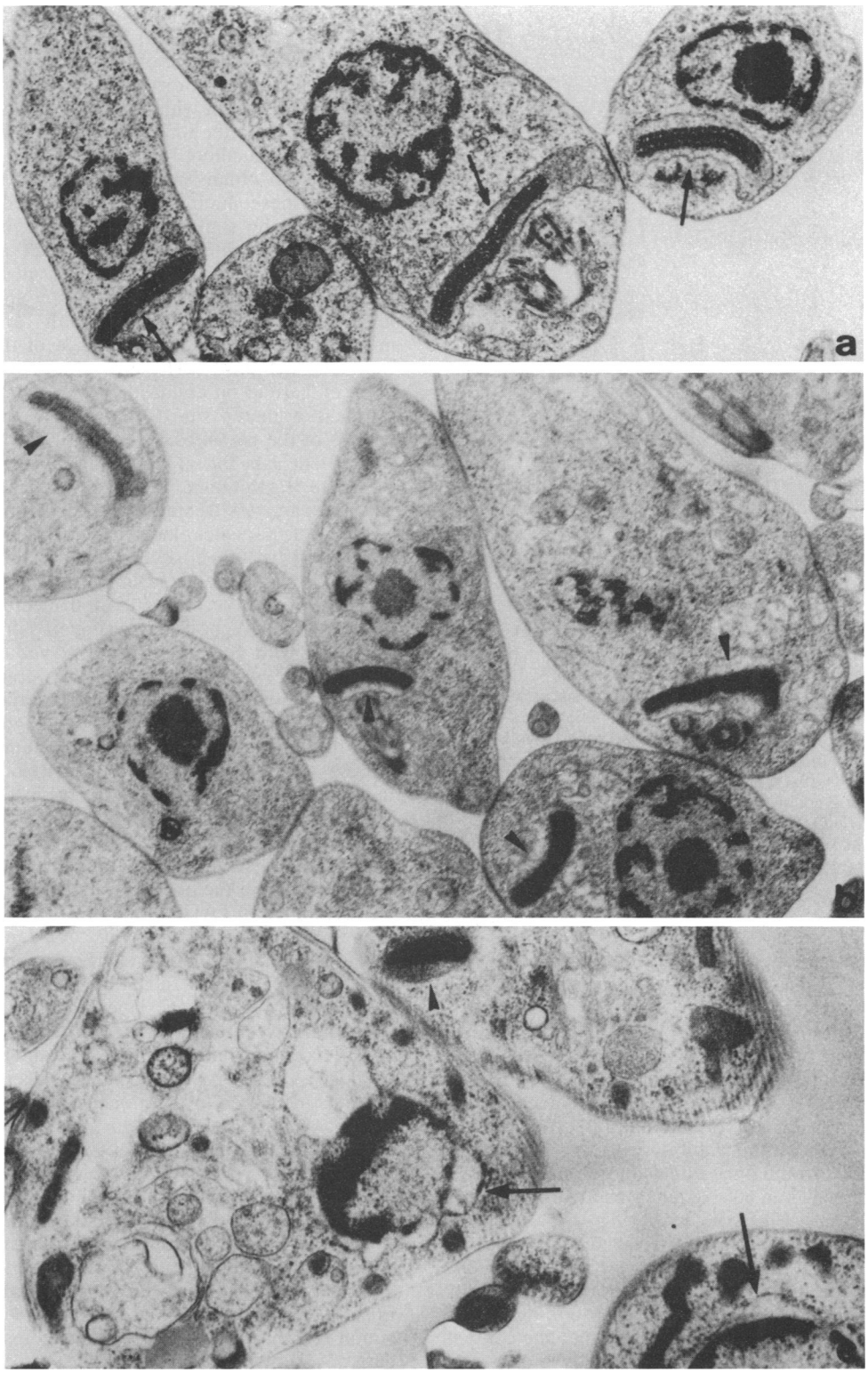

FIG. 4. Effect of Tarivid on epimastigote morphology. The parasites were treated with 100 μg of the drug ml^{-1} for three days in LIT medium, in which they displayed a swollen kinetoplast with loss of matrix and cristae (arrowhead; magnification, ×15,000) (b), and in TAU medium, in which they presented an intense vesiculation and damage in the kinetoplast and nucleus (arrowhead; magnification, ×25,000) (c). (a) Nontreated control epimastigotes.

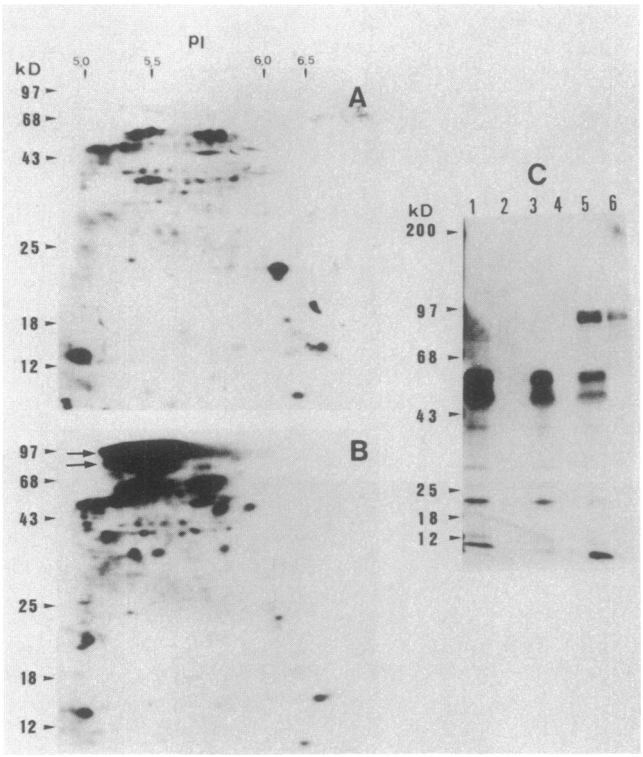

FIG. 5. (A and B) Two-dimensional polyacrylamide gel electrophoresis analysis of the metabolic labeling products of parasites incubated in the presence (A) or absence (B) of Tarivid (100 μg ml^{-1}) during metacyclogenesis in TAUP. The first dimension was an isoelectric focusing gel, and the second dimension was a 13% polyacrylamide gel. (C) Polyacrylamide gel electrophoresis analysis of the products immunoprecipitated with epimastigote antiserum (lanes 1, 3, and 5) and with trypomastigote antiserum (lanes 2, 4, and 6) from differentiating epimastigotes incubated for 96 h in TAUP in the presence of Tarivid (100 μg ml^{-1}; lanes 1 and 2), ofloxacin (1,250 μg ml^{-1}; lanes 3 and 4), and novobiocin (245 μg ml^{-1}; lanes 5 and 6). kD, Kilodaltons.

concentration that resulted in 50% inhibition of *T. cruzi* proliferation after 7 days in culture was approximately 480 μg ml^{-1} (Fig. 1B).

A similar inhibitory effect was observed with nalidixic acid, which is a structural analog of ofloxacin and, hence, is also inhibitory for topoisomerase II subunit A. In this case, 50% inhibition after 7 days in culture was attained with a drug concentration of 173 μg ml^{-1} (data not shown).

However, novobiocin (an inhibitor of the B subunit) displayed a slightly stimulatory effect on cell proliferation, although doses higher than 600 μg ml^{-1} resulted in cell lysis after 4 days in culture (Fig. 2).

Topoisomerase II inhibitors and *T. cruzi* metacyclogenesis. During the transformation of epimastigotes into metacyclic trypomastigotes, the kinetoplast, in addition to altering its position relative to the flagellum, changes from a bar (more compact) to a basket (more relaxed) form. Since the content of K-DNA is the same irrespective of the developmental stage (14), the fact that the K-DNA occupies a larger volume in trypomastigotes might be due to a topoisomerase II decatenating activity.

The results presented in Fig. 3A show that different topoisomerase II inhibitors block the metacyclogenesis process. In this case, novobiocin, in contrast to its effect on *T. cruzi* proliferation, also inhibited transformation. However, it is interesting that the inhibitory effect was less pronounced (for novobiocin and nalidixic acid) or was almost nonexistent (for Tarivid and ofloxacin) if the drugs were added some hours after the beginning of the differentiation process. This time-dependent inhibitory effect was very pronounced for Tarivid (Fig. 3B), in which inhibition was diminished if the drug was added after 1 h of incubation of the parasites in the differentiation medium.

In order to obtain information about the ultrastructural effects of these inhibitors, we examined parasites treated with Tarivid by transmission electron microscopy. In the case of epimastigotes incubated in LIT medium, the parasites showed a swelling of the kinetoplast together with a loss of mitochondrial matrix and cristae (Fig. 4b). In the case of parasites incubated in TAUP with Tarivid, we observed an intense vesiculation and damage of the kinetoplast and the nucleus (Fig. 4c). These results indicate that the major effect of Tarivid on epimastigote proliferation and differentiation is primarily due to damage of the kinetoplast and nucleus of the parasites.

Metabolic labeling of epimastigotes incubated with topoisomerase II inhibitors. In order to characterize biochemically the parasites treated with topoisomerase II inhibitors, proteins of parasites labeled with [^{35}S]methionine were analyzed by two-dimensional polyacrylamide gel electrophoresis. The results depicted in Fig. 5 are those for parasites incubated in TAUP differentiation medium for 72 h in the presence (Fig. 5A) or absence (Fig. 5B) of Tarivid. Parasites incubated with the inhibitor displayed a protein pattern characteristic of nondifferentiating epimastigotes, while those incubated in the absence of the drug showed a profile characteristic of metacyclic trypomastigotes (1). This indicates that in the presence of Tarivid, epimastigotes do not express the characteristic trypomastigote antigens in the range of 85 to 90 kilodaltons (arrows in Fig. 5B).

However, although the parasites incubated with novobiocin maintained the epimastigote morphology, they expressed trypomastigote stage-specific antigens. This is shown in Fig. 5C, in which the metabolically labeled proteins from parasites incubated in TAUP in the presence of Tarivid (Fig. 5C, lanes 1 and 2), ofloxacin (Fig. 5C, lanes 3 and 4), and novobiocin (Fig. 5C, lanes 5 and 6) were immunoprecipitated with antiserum against the *T. cruzi* epimastigote and trypomastigote forms (Fig. 5C, lanes 1, 3, and 5) or with trypomastigote stage-specific antiserum (Fig. 5C, lanes 2, 4, and 6). The trypomastigote stage-specific antigens in the range of 78 to 86 kilodaltons were immunoprecipitated only from parasites incubated in the presence of novobiocin.

Effect of Tarivid on amastigote proliferation and differentiation. Ultrastructural analysis of amastigotes (Fig. 6a) and tissue culture trypomastigotes (Fig. 6b) treated with Tarivid (288 μg ml^{-1}) for 24 h showed that the drug provoked damage (swelling) in the kinetoplast. Similar results were observed for blood trypomastigotes, with additional severe alterations in the nucleus and the absence of cytoplasmic material (Fig. 6c). Amastigote proliferation in axenic cultures (10) was also inhibited by Tarivid, while the transformation of amastigotes into epimastigotes was not affected (data not shown).

Effect of Tarivid on *T. cruzi*-heart muscle cell interaction. The experimental system of primary heart and skeletal muscle cell cultures (15) is suitable for studies of the interaction of *T. cruzi* with mammalian host cells, since muscle cells are targets for *T. cruzi* infections. The study of the interiorization and fate of *T. cruzi* on infection of muscle

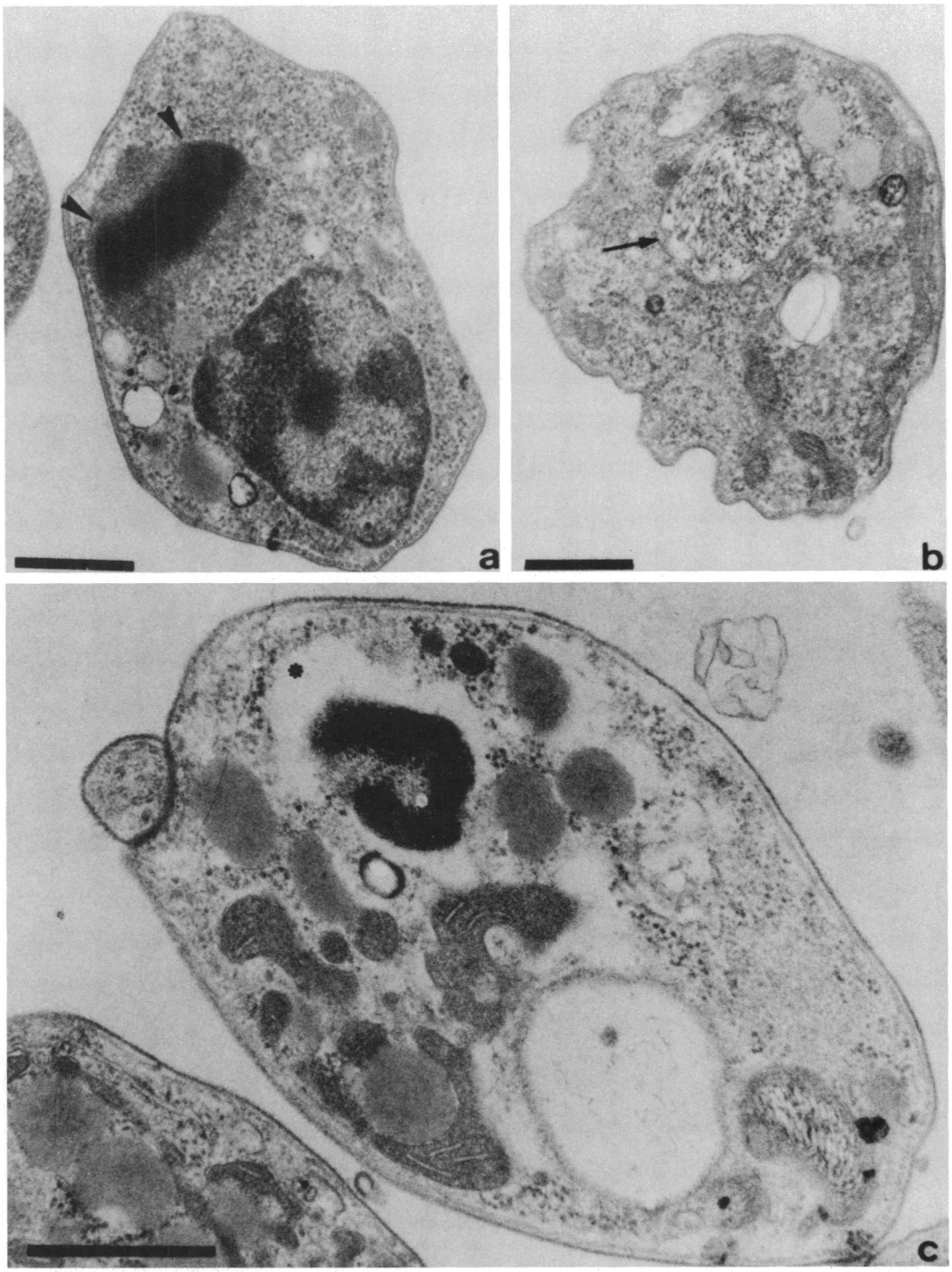

FIG. 6. Effect of Tarivid on *T. cruzi* amastigote and trypomastigote forms. The parasites were treated with the drug (288 μg ml^{-1}) for 24 h. (a) Amastigote form with a swollen kinetoplast (arrowhead; magnification, ×33,000); (b) culture trypomastigote with intense damage of the kinetoplast (arrow; magnification, ×30,000); (c) bloodstream trypomastigote with severe alterations on the nucleus (*) and loss of cytoplasmic material (magnification, ×52,000). Bars, 2.5 μm.

cells in the presence of Tarivid showed that the drug does not block either the entry of bloodstream trypomastigotes or their transformation into amastigotes. However, the proliferation of amastigotes as well as their transformation into trypomastigotes were inhibited (Fig. 7). When the drug was added following 48 h of infection, the differentiation of amastigotes to trypomastigotes was inhibited by a drug concentration of 288 μg ml^{-1}, but no effect was observed with a drug concentration of 144 μg ml^{-1} (data not shown), indicating that the inhibition is dose dependent. Similar results were obtained for metacyclic trypomastigotes (data not shown). It is important that the Tarivid concentrations used in these experiments were not harmful to the control noninfected heart muscle cells (data not shown).

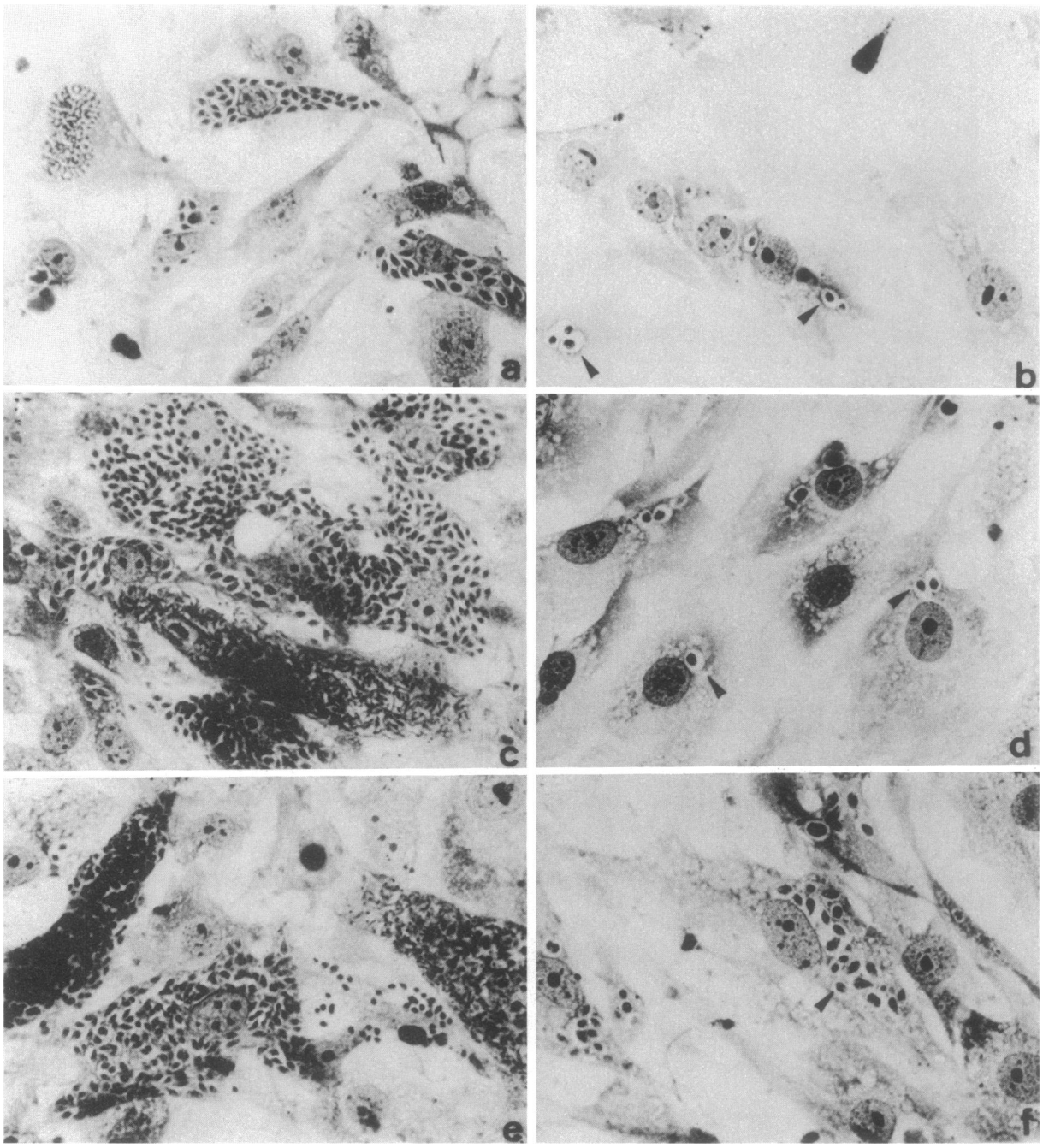

FIG. 7. Effect of Tarivid on *T. cruzi*-heart muscle cell interaction. The drug (360 μg ml^{-1}) was added at the beginning of the infection of the cultures with bloodstream trypomastigotes. Panels a, c, and e show control cultures after 2, 3, and 5 days after infection, respectively; panels b, d, and f show drug-treated cultures after 2, 3, and 5 days of infection, respectively. The drug inhibited both amastigote proliferation and differentiation. Magnifications, ×900.

DISCUSSION

The results presented above demonstrate that bacterial topoisomerase II inhibitors block both the proliferation and differentiation of *T. cruzi* epimastigotes in a dose- and time-dependent manner. The doses required for inhibition of these processes varied according to the drug that was used and could be due to differences in the permeabilities of the cells to the drugs. Accordingly, Tarivid was 10 times more active than its active principle ofloxacin (Fig. 1A and B, respectively). The proliferation of amastigotes (Fig. 7) as well as the transformation of trypomastigotes into amastigotes in heart muscle cells were inhibited in the presence of Tarivid. All these processes involved either replication or changes in the conformation of K-DNA. On the other hand, the transformation of epimastigotes into amastigotes (a process that does not require either DNA replication or any conformational change of the kinetoplast) was not inhibited by the drug. This suggests that processes involved in DNA

replication or in changing the DNA conformation would be the targets of the drug. Interestingly, transmission electron microscopy showed that the major ultrastructural changes in parasites treated with Tarivid occurred in the kinetoplast and the nucleus (Fig. 6).

Our results are not in full agreement with those obtained by Pate et al. (17). Those investigators found that nalidixic acid and novobiocin (at concentrations of 200 and 500 μg ml^{-1}, respectively) did not inhibit epimastigote proliferation. When we used those two drugs at those concentrations, use of nalidixic acid resulted in 60% inhibition of cell proliferation (data not shown) and use of novobiocin resulted in cell lysis (Fig. 2). Furthermore, in contrast to our findings and those of others (17), in one study (9) 4-quinolones (e.g., ofloxacin and nalidixic acid) were found to be inactive against *T. cruzi* amastigotes. These discrepancies might result from such differences as the culture media and *T. cruzi* strains that were used in the experimental protocols.

However, all the drugs tested in this study inhibited the metacyclogenesis process. In this case, the drugs had to be present from the beginning of the differentiation process (Fig. 3). This feature was particularly striking for Tarivid, for which addition of the drug after 30 min of metacyclogenesis induction resulted in only 40% inhibition of differentiation (Fig. 3B). These results suggest that topoisomerase type II might participate in a very early event during *T. cruzi* metacyclogenesis. Indeed, recent results indicate that on metacyclogenesis there is a more than threefold increase in the volume density of the K-DNA network (22) without a corresponding increase in the amounts of DNA (14).

Metabolic labeling of parasites incubated in the differentiation medium with topoisomerase II inhibitors showed that they displayed a protein profile characteristic of nondifferentiating epimastigotes (Fig. 5A) (1). However, in the presence of novobiocin, although the cells displayed an epimastigote morphology, they synthesized the 78- to 86-kilodalton (1, 8) trypomastigote stage-specific surface antigens (Fig. 5C). We cannot yet interpret this result, although it has been described previously (7) that under some circumstances stage-specific gene expression precedes the morphological changes that occur during *T. cruzi* metacyclogenesis.

It remains to be determined whether type II topoisomerase is the target for the bacterial topoisomerase II inhibitors that we tested. It is worth mentioning that type II topoisomerases have been described in several trypanosomatids (5, 11, 13, 16, 18–20), including *T. cruzi*, which expresses an ATP-independent enzyme (13). However, only in *Crithidia fasciculata* (16) and *Trypanosoma equiperdum* (18) has evidence been presented which supports the localization of the enzyme in the kinetoplast.

Taken together, the data indicate that *T. cruzi* type II topoisomerase might be a good target for the chemotherapy of Chagas' disease. In this case, the experimental systems of in vitro metacyclogenesis under chemically defined conditions (1, 8) and primary muscle cells (15) would be important tools for the testing of new drugs.

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