ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1990, p. 2097–2105
0066-4804/90/112097-09$02.00/0

Vol. 34, No. 11

Ultrastructural Alterations Induced by Two Ergosterol Biosynthesis Inhibitors, Ketoconazole and Terbinafine, on Epimastigotes and Amastigotes of *Trypanosoma (Schizotrypanum) cruzi*

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Received 10 May 1990/Accepted 9 August 1990

We report the ultrastructural alterations induced during the proliferative stages of *Trypanosoma (Schizotrypanum) cruzi*, the causative agent of Chagas' disease, by two ergosterol biosynthesis inhibitors, ketoconazole and terbinafine, which had previously been shown to be potent growth inhibitors whose effects are potentiated when used in combination (J. A. Urbina, K. Lazardi, T. Aguirre, M. M. Piras, and R. Piras, Antimicrob. Agents Chemother. 32:1237–1242, 1988). Epimastigotes treated with a low concentration of ketoconazole (1 μM), which blocks ergosterol biosynthesis at the level of C-14 demethylation of lanosterol and induces cell lysis coincident with total ergosterol depletion, showed gross alterations of the kinetoplast-mitochondrion complex, which swelled and lost the organization of its inner membrane and the electron-dense bodies of its matrix. Thus, coincident with the beginning of cell lysis, the kinetoplast-mitochondrion complex occupied >80% of the cell volume, while other subcellular structures such as the nucleus and subpellicular microtubules were not affected. Terbinafine, which blocks ergosterol synthesis in these cells at the level of squalene synthetase and thus leads to almost immediate arrest of growth at concentrations greater than 1 μM, produced proliferation of glycosomelike bodies, binucleated cells (arrest at cytokinesis), and eventually massive vacuolization. When the drugs were combined, the predominant effect was mitochondrial swelling, which was more drastic and took place earlier than that observed in cells treated with ketoconazole alone. In amastigotes proliferating in Vero cells, ketoconazole at the concentration required to eradicate the parasites (10 nM) produced mitochondrial swelling, the appearance of autophagic vacuoles containing partially degraded subcellular material, and finally a general breakdown of the subcellular structures. Terbinafine at 3 μM induced more limited ultrastructural damage to the amastigotes consistent with increased vacuolization of the cells and the appearance of occasional autophagic vacuoles. When the drugs were used in combination, just 1 nM was required for the total eradication of parasites, the ultrastructural effects were more extensive, and cell disintegration occurred earlier than when any of the drugs was used alone at a much higher concentration. No effect of the drugs on the ultrastructure of the host cells were observed at any of the concentrations tested.

Interest in ergosterol biosynthesis inhibitors (EBIs) has been increasing in the last decade as they have proven to be effective antiproliferative agents against lower eucaryotes such as fungi, yeasts, and protozoa, i.e., many of the important pathogens of human beings as well as of animals and plants of economic interest (36, 42, 48). The basis of this activity has been shown to be the fact that ergosterol and related C-24 methylated sterols, which are unique to this type of organism, have essential functions which cannot be fulfilled by cholesterol or phytosterol, the sterols produced by the host cells, and that the biosynthetic pathways of these compounds, although very similar, present crucial differences which can be exploited for the development of rational chemotherapeutic agents (9, 13, 26, 32). Imidazole and triazole derivatives are the best-known group of EBIs, blocking ergosterol biosynthesis at the level of the cytochrome P-450-dependent C-14 demethylation of lanosterol (9, 20, 39, 40, 42, 47–50); these compounds are potent broad-spectrum antimycotic agents (20, 42, 48) but are also very effective agents both in vitro and in vivo against pathogenic protozoa such as those from the genus *Leishmania* (3, 5–8, 18, 19, 46, 51) and *Trypanosoma (Schizotrypanum) cruzi*, the causative agent of Chagas' disease (2, 15, 17, 22–24, 44, 45). A second group of EBIs is the allylamines, which act at previous steps in the ergosterol biosynthetic pathway, squalene synthetase (44) or squalene epoxidase (31, 34–38), and also are potent antifungal and antiprotozoal agents (16, 28–31, 36). Recent studies from this and other research groups have shown that ketoconazole at a low concentration (1 μM) completely blocks ergosterol biosynthesis (2, 17, 22), but growth is only marginally affected (primary trypanostatic effect). However, after 120 h, cell lysis suddenly takes place coincident with the complete depletion of the preformed ergosterol pool (secondary trypanocidal effect) (22). At this point, gross changes in the physical and permeability properties of the plasma membrane of the treated cells are detected (45). On the other hand, the allylamine terbinafine has been shown to arrest growth long before the ergosterol pool is depleted, suggesting the accumulation of a toxic compound (44). Furthermore, we have also shown that when ketoconazole is used in combination with terbinafine, the antiproliferative effects of the drugs against epimastigotes and amastigotes are strongly potentiated (44). However, practically no information exists on the subcellular alterations induced by the drugs which underlie their lytic effects. In this report we present the results of a detailed analysis of the ultrastructural alterations

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produced by ketoconazole and terbinafine during the proliferative stages of *T. cruzi* as a function of time and concentration of the drugs and when used alone or in combination. We show that the primary subcellular alteration produced by the ketoconazole-induced ergosterol depletion in both epimastigotes and amastigotes of *T. cruzi* is a gross alteration of the unique kinetoplast-mitochondrion complex of the parasite which loses its osmoregulatory properties and finally causes cell lysis. These effects are strongly accentuated by terbinafine.

MATERIALS AND METHODS

The Y stock of *T. cruzi* was used throughout this study. This stock displays the same susceptibility to the growth inhibitory effects of ketoconazole and terbinafine as the EP stock previously studied (22, 44, 45). The epimastigotes (equivalent to the form that develops in the reduviid vector) were cultivated in liver infusion-tryptose supplemented with 10% calf serum (22) at 28°C with strong agitation (120 rpm). The cultures were initiated at a cell density of 2×10^6 epimastigotes per ml, and the drugs were added at a cell density of 10^7 epimastigotes per ml. Cell densities were determined by direct counting with a hemacytometer. Amastigotes were cultivated in Vero cells maintained in minimal essential medium supplemented with 2% fetal calf serum in humidified 95% air-5% CO_2 at 37°C as previously described (44). The cells were infected by a 10:1 ratio of tissue culture-derived trypomastigotes to Vero cells for 24 h; the medium was then changed and incubation was continued for 96 h, with medium changes every 48 h. At this point, 80% of the cells were infected and the number of amastigotes per cell varied between 60 and 100. The drugs were then added to the medium, and the cells were incubated for various periods, with medium changes every 48 h.

For electron microscopy studies, the epimastigotes were collected at $1,000 \times g$ for 10 min and fixed in 0.1 M sodium phosphate buffer containing 3.5% saccharose, 2.5% glutaraldehyde, and 4% paraformaldehyde (pH 7.3) for 2 h. Amastigote-infected Vero cell monolayers were previously fixed under the same conditions described above and then scraped with a rubber spatula. Fixed cells were washed with 0.1 M sodium phosphate buffer containing 3.5% saccharose (pH 7.3) and postfixed in 1% OsO_4 with 1 mM $CaCl_2$ and 0.8% potassium ferricyanide for 2 h in the dark. Dehydration was carried out in ethanol, and embedding in Epon and ultrathin sections were obtained with an LKB Ultratome III (LKB Instruments Co., Rockville, Md.). The ultrathin sections were stained with uranyl acetate and lead citrate and observed in a JEOL 100 CX electron microscope. Terbinafine [SF-86327; (*E*)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphtalenemethanamine] (31) was provided by A. Lindenmann and H. Stähelin, Sandoz, Ltd., Switzerland, through Luis Rodriguez, Sandoz de Venezuela, South America. The drug was added as dimethyl sulfoxide solutions; the final dimethyl sulfoxide concentration never exceeded 1% (vol/vol) and had no effect by itself on the proliferation of parasites or Vero cells. Ketoconazole (*cis*-1-acetyl-4-[-4[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine) (20) was provided by J. Russe, Janssen Pharmaceutica, Caracas, Venezuela; it was added as an aqueous solution titrated to pH 2.4 with HCl and sterilized by filtration through membrane filters (mean pore size, 0.22 μm; Millipore Corp., Bedford, Mass.).

RESULTS

The ultrastructural alterations induced by the dioxolane-imidazole ketoconazole on *T. cruzi* epimastigotes are illustrated in Fig. 1. Control epimastigotes (Fig. 1A) showed the characteristic elongated shape of these cells, with a terminal flagellum, flagellar pocket, nucleus, and the single giant mitochondrion of branched structure characteristic of this group of organisms, which contains a disk-shaped aggregate of DNA called the kinetoplast. This single mitochondrion occupies 12% of the cell volume in normal epimastigotes (14). Cells treated with a low (1 μM) concentration of ketoconazole, which immediately blocks ergosterol biosynthesis but affects only marginally the growth rate of the organism (22, 44), retained essentially normal ultrastructures until 96 to 120 h of incubation with the drug. At that time the original endogenous ergosterol pool was exhausted by dilution, and cell growth stopped rather suddenly (22, 44). Concomitantly, a marked alteration in the kinetoplast-mitochondrion system rapidly (<24 h) developed which was consistent with swelling of the organelle, with loss of the inner membrane organization and disappearance of the electron-dense bodies of the matrix (Fig. 1B, D, and E). In many cells the organelle occupied >80% of the cell volume after 120 h of incubation with the drug (Fig. 1D), while other organelles such as the nucleus and subpellicular microtubules were not affected. After this point, cell lysis rapidly took place (Fig. 1C). When the cells were treated with 30 μM ketoconazole, which produces immediate growth arrest, the same ultrastructural effects were observed but they developed earlier; vacuolization and the appearance of autophagic vacuoles were also observed (results not shown).

Figure 2 presents the ultrastructural effects induced in the epimastigotes by the allylamine terbinafine at a concentration which produces immediate arrest of growth (30 μM). The most remarkable feature of the epimastigotes treated for 48 h with this concentration of the drug (Fig. 2A through C) was the appearance of a large number of membrane-bound electron-dense bodies (Fig. 2B and C), whose morphological characteristics resembled those of the glycosomes, organelles present only in Kinetoplastida and which contain not only most of the enzymes required for glycolysis but also those required for lipid β-oxidation (10, 27, 41). The growth of many of these cells was found arrested at cytokinesis (Fig. 2C, after 48 h; Fig. 2D, after 144 h). After 144 h, the cells showed massive vacuolization and large multivesicular bodies appeared; the abundant glycosomelike bodies appear stacked (Fig. 2D and E). At this point, cell lysis begins.

The ultrastructural alterations of the epimastigotes produced by the incubation of the cells with ketoconazole and terbinafine simultaneously (at 1 μM each), which mutually potentiates their antiproliferative action, led to >80% inhibition of growth (44) (Fig. 3). Characteristically, the changes in the kinetoplast-mitochondrion complex appeared much earlier when the cells were treated with the drug combination than when they were incubated with ketoconazole alone (marked mitochondrial swelling after 48 h for the combination [Fig. 3A] versus 96 to 120 h required to produce the same effects for the azole alone [Fig. 1B, D, and E]). The alterations observed for the drug combination were more drastic, and many cells were lysed after 120 h of incubation (Fig. 3B).

We also characterized the effect of the EBIs on the ultrastructure of *T. cruzi* amastigotes proliferating inside Vero cells. Figure 4A shows a typical amastigote inside the

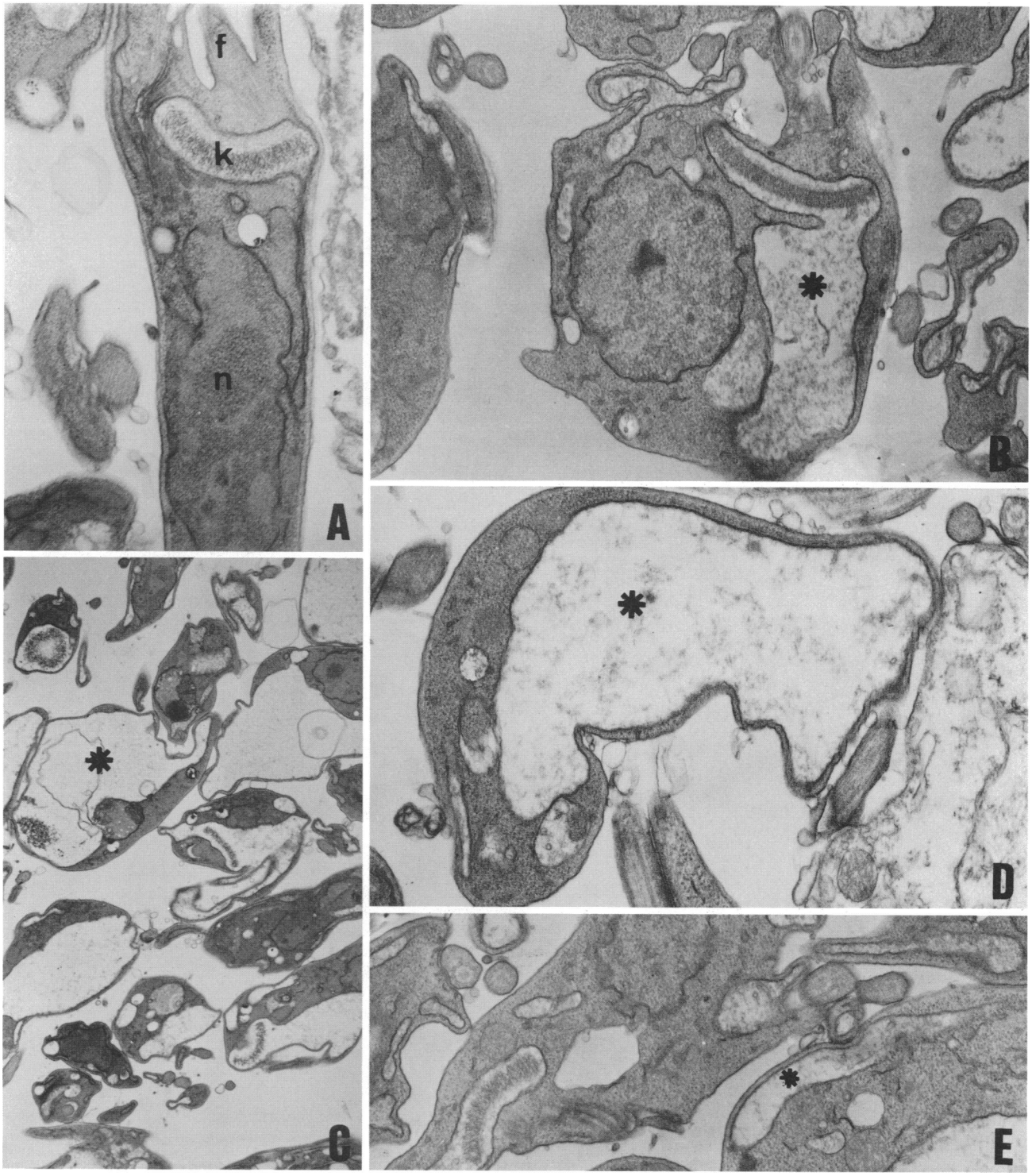

FIG. 1. (A) General view of an untreated *T. cruzi* epimastigote form showing the kinetoplast (k), nucleus (n), and flagellum (f). Magnification, ×22,000. (B through E) Epimastigotes incubated for 120 h in the presence of 1 μM ketoconazole. Intense swelling of the mitochondrion (asterisks) is visible. Magnifications: B, D, and E, ×22,000; C, ×6,500.

vertebrate cell in the absence of drugs. The short nonprotruding flagellum, the kinetoplast, and the branched mitochondrion are clearly visible. In the presence of ketoconazole at 10 nM, which is the minimal concentration of this drug capable of complete eradication of the parasites (44), alteration of the mitochondrion was readily apparent (Fig. 4B), the presence of autophagic vacuoles was very prominent, and finally a general breakdown of the subcellular structures was observed (Fig. 4C and D). No parasites were discernible inside the cells after 120 h of incubation with the drug, after starting with 80% of infected cells and 60 to 100 amastigotes per cell.

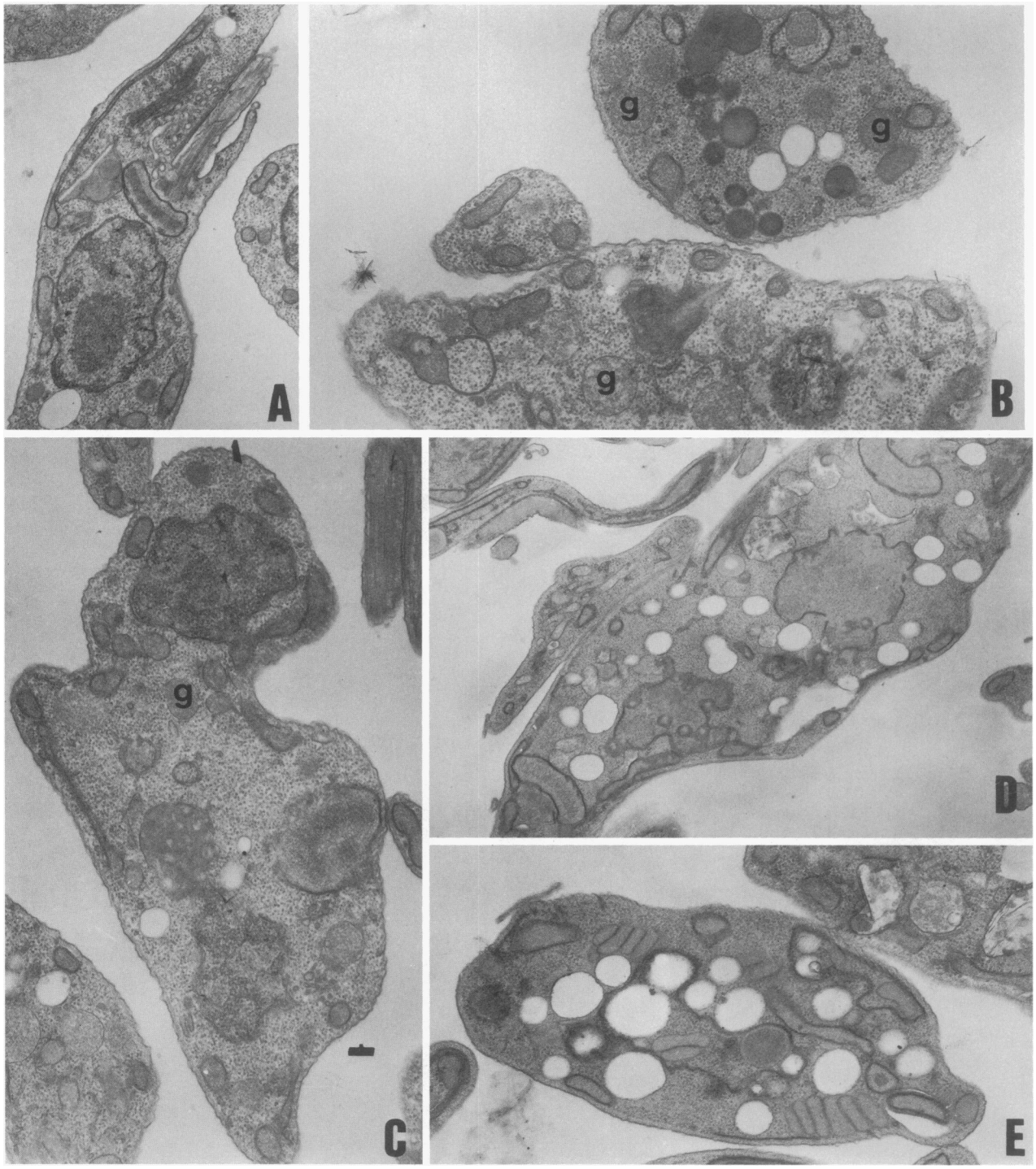

FIG. 2. *T. cruzi* epimastigotes incubated for 48 h (A through C) or 144 h (D and E) in the presence of 30 μM terbinafine. At 48 h the general structure appeared normal; however, there was an increase in the number of profiles of structures which may correspond to glycosomes (g). At 144 h intense vacuolization is visible and the glycosomelike bodies are arranged in stacks (E). Magnifications: A, ×18,000; B and C, ×22,000; D, ×9,000; E, ×12,000.

When the infected monolayers were treated with up to 3 μM terbinafine, the parasites showed only moderate damage after 72 h of treatment, consisting of increased vacuolization of the cells and the appearance of occasional autophagic vacuoles (Fig. 5A); large numbers of damaged but still identifiable parasitic cells were present after 120 h. However, when the treatment was carried out with the combination of ketoconazole plus terbinafine (both at a concentration as low as 1 nM), a combination previously shown to produce complete growth inhibition and clearance of the parasites

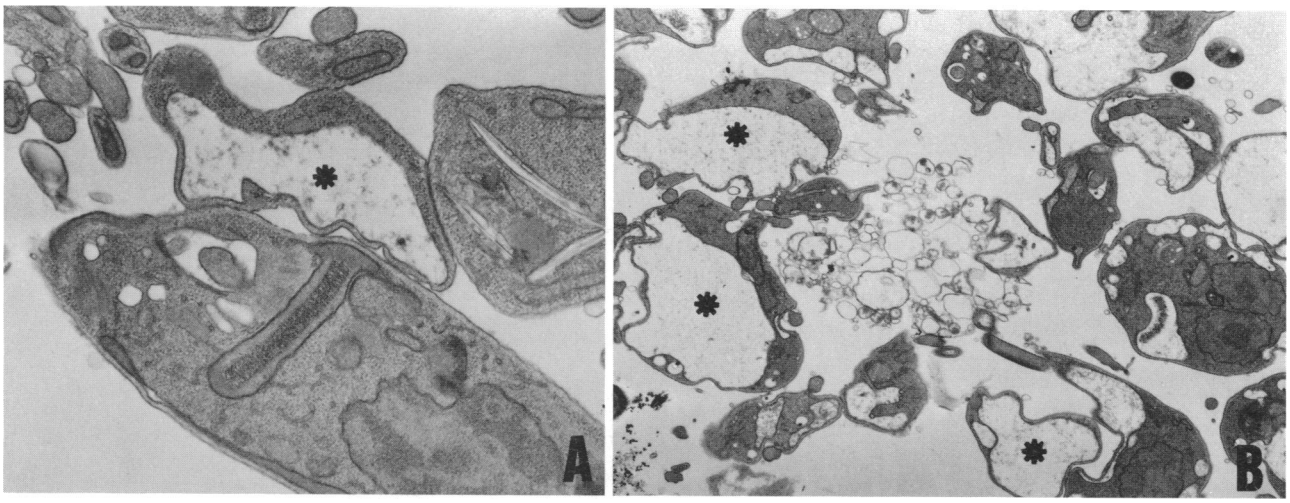

FIG. 3. *T. cruzi* epimastigotes incubated with 1 μM ketoconazole plus 1 μM terbinafine for 48 (A) and 120 (B) h. Most of the cells show mitochondrial swelling (asterisks). Magnifications: A, ×17,000; B, ×7,000.

from the infected monolayers (44), the ultrastructural analysis revealed that the parasites were rapidly destroyed inside the cells and the few remaining cells showed large autophagic vacuoles (Fig. 5B), loss of plasma membrane, and finally complete breakdown of the subcellular organization (Fig. 5C and D). No parasites were visible with either a light or electron microscope after 96 h of treatment. On the other hand, no ultrastructural alterations were observed in the host Vero cells with any of the drug treatments.

DISCUSSION

There have been very few studies dedicated to investigate the ultrastructural alterations induced by EBIs in susceptible cells in general and parasitic protozoa in particular. Cannon and Kerridge (11), by using scanning electron microscopy, reported the appearance in *Candida albicans* of stunted mycelia rounded at the tip as a result of incubating the fungus with ketoconazole or terbinafine. They attributed this effect to an alteration in the biosynthetic apparatus of the cell wall produced as a result of the altered sterol composition and concluded that the ergosterol content of the membrane is "of greater importance than the increase of sterol precursors in determining cell shape." Previous studies with the allylamines naftifine and terbinafine on the filamentous fungus *Trichophyton mentagrophytes* (25) also found gross alterations at the hyphal tips (growth zones), but the most prominent alteration at the ultrastructural level was the appearance of spherical or drop-shaped osmiophyllic deposits of different sizes within the cell. The lipidic nature of these deposits was ascertained by various methods, and it was concluded that they are formed by the accumulation of the hydrocarbon precursors of ergosterol induced by these drugs. This interpretation received support at the biochemical level when it was shown that the allylamines act in this group of organisms by blocking the squalene epoxidase, leading to the accumulation of squalene, a polyunsaturated hydrocarbon precursor of ergosterol (31, 34–38). An early report by Docampo et al. (15) on Kinetoplastida indicated that the main effects of two imidazole antifungal compounds, miconazole and econazole, which are potent growth inhibitors against *T. cruzi* epimastigotes in vitro, are a marked reduction in the 5,7-diene sterol content and, at the ultrastructural level, a strong reduction in the nuclear chromatin, the occurrence of vacuolization, and a decrease in the electron density of the cytoplasm. A modification of the agglutination response of the epimastigotes to several lectins was also observed by Docampo et al., an observation we made as well in a previous study with ketoconazole (22). On the other hand, in a study of ketoconazole-treated *Leishmania tropica* amastigotes growing inside human macrophages, Langreth et al. (21) found condensed parasite cytoplasm and alterations in the distribution of cytoplasmic ribosomes.

The marked alteration of the single giant mitochondrion produced by the ketoconazole-induced depletion of ergosterol in both epimastigotes and amastigotes of *T. cruzi* has never been reported before as a cellular effect of azole EBIs. The notion that ergosterol is involved in the mitochondrial function of *T. cruzi* as suggested by the results of the present study is also supported by two lines of evidence. (i) The effects of the drug on cell growth, morphology, and ultrastructure can be reversed by the presence of exogenous ergosterol but not cholesterol (22; J. A. Urbina and K. Lazardi, unpublished data). (ii) Turrens et al. (43) found, by using several subcellular fractionation schemes for *T. cruzi* epimastigotes, that ergosterol and related 5,7-diene sterols (identified by gas-liquid chromatography and UV spectroscopy) are concentrated in typical plasma membrane fractions but are also preferentially associated with those subcellular fractions containing succinate dehydrogenase, a marker enzyme for the inner mitochondrial membrane. This contrasts with the lipid composition of the inner membranes of mitochondria from vertebrates and plants, which are typically devoid of sterols (33).

The observed alterations in the structure and function of the single mitochondrion, the main energy-transforming organelle of the parasite, which include the loss of the inner membrane and its associated functions such as osmotic regulation of the organelle, are most probably among the main factors underlying the loss of *T. cruzi* viability observed after total ergosterol depletion induced by ketoconazole (22, 44). Another important factor is undoubtedly the alteration of the lipid-packing and permeability properties of the plasma membrane reported by us previously (45). Direct proof of the involvement of ergosterol in the function of the *T. cruzi* mitochondrion is currently being sought in our group

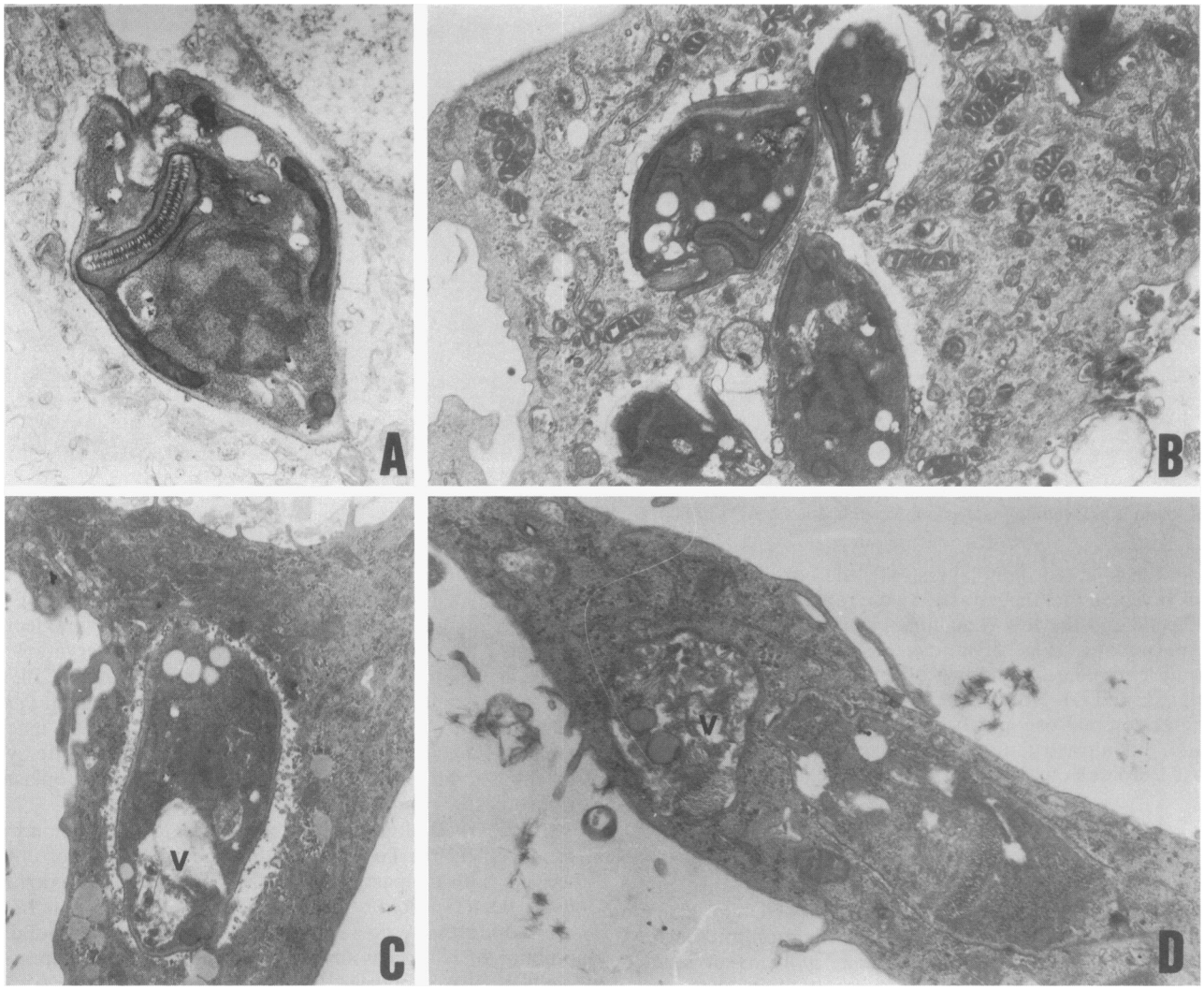

FIG. 4. (A) Untreated *T. cruzi* amastigote inside a Vero cell. Magnification, ×10,000. (B through D) Cell cultures previously infected with *T. cruzi* trypomastigotes and then incubated with 10 nM ketoconazole for 72 h. The amastigotes are damaged, showing mitochondrial swelling and autophagic vacuoles (v). Magnifications: B, ×6,500; C, ×10,000; D, ×12,000.

by using isolated, coupled mitochondrial vesicles (1, 4). However, a recent study by Clarkson et al. (12) reports that in a related organism, *Trypanosoma brucei*, digitonin, a nonionic detergent which interacts specifically with sterols, is capable of producing complete inhibition of the respiration of the parasite over a narrow range of detergent concentration. This was taken as evidence that digitonin was acting by disrupting the inner mitochondrial membrane, which in turn would indicate that this membrane is rich in sterols. Thus, the unusual role of sterols in the structure and function of the mitochondrial apparatus could be a general characteristic among trypanosomatids and is a novel biochemical aspect of these parasites which deserves further study because of its potential chemotherapeutic applications.

The appearance of glycosomelike organelles is the most characteristic feature of the epimastigotes whose growth has been arrested by terbinafine. These membrane-bound electron-dense bodies have a very narrow size distribution and increase their number as a function of time of incubation with the drug (Fig. 2). They are very different from the lipid droplets which accumulate in *T. mentagrophytes* mycelia treated with allylamines (25) which are much more osmiophyllic, have a very broad size distribution, and are devoid of a limiting membrane. The cause of the increase in the number of glycosomes or glycosomelike structures in *T. cruzi* epimastigotes treated with terbinafine is not clear at the moment. However, one possible explanation is that they proliferate as a response to the accumulation of the hydrocarbonated precursor ergosterol observed in these cells (44), since glycosomes contain, together with the enzymes that catalyze most of the glycolytic sequence, those enzymes responsible for lipid β-oxidation (10, 27, 41). The epimastigotes treated with the synergistic combination of EBIs are afflicted by a more drastic alteration of the kinetoplast-mitochondrion complex, in which the structural and functional alterations produced by the reduced ergosterol content are probably compounded by the accumulation of surface-active phosphorylated hydrocarbons produced by the allylamine (44).

The intracellular amastigotes are clearly much more susceptible to the EBIs used in this study than the epimastigotes (44; present work), a fact that suggests a different mecha-

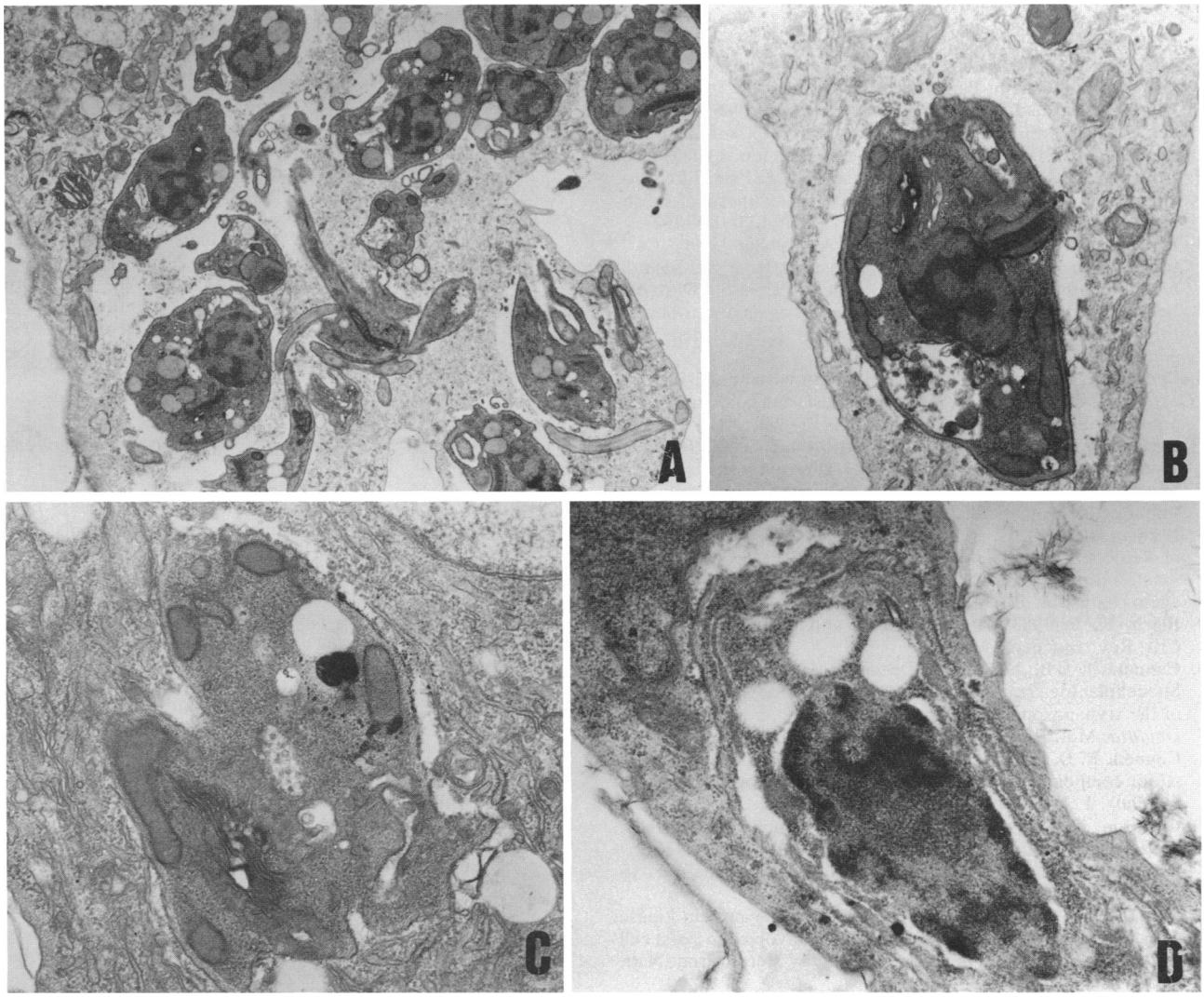

FIG. 5. Cell cultures previously infected with *T. cruzi* trypomastigotes and then incubated with 3 μM terbinafine for 72 h (A) or with a combination of 1 nM terbinafine plus 1 nM ketoconazole for 72 (B and C) or 96 (D) h. Amastigote lesions are evident such as autophagic vacuoles (B), disorganization of the plasma membrane (C), and swelling of the nuclear envelope (D). Magnifications: A, ×6,500; B, ×10,000; C and D, ×20,000.

nism of action or a different susceptibility of this form of the parasite to reduced ergosterol production. Although no studies have reported on the sterol composition of *T. cruzi* amastigotes treated with EBIs, they have been carried out in *Leishmania mexicana mexicana* amastigotes proliferating inside macrophages and treated with ketoconazole (7, 19). It was shown that the reduction of the ergosterol content and the type of precursors accumulated are similar to those observed in the promastigote (culture) form, suggesting that the mechanism of action is the same in both forms of the parasite. Thus, there are grounds to assume that in *T. cruzi* the mechanism of action of the drugs is similar in both forms and that the increased susceptibility of the amastigote could be a result of a more stringent requirement of ergosterol in the parasite functions. Support for this interpretation comes from the fact that although mitochondrial alterations are clearly observed in ketoconazole-treated amastigotes (Fig. 4B), the predominant pathological feature of these cells is the appearance of autophagic vacuoles, which indicates a generalized alteration of the membranous subcellular structures leading to complete cell disintegration in a much shorter time than that required for the epimastigotes. The allylamine effects are moderate, but again the simultaneous presence of the azole and the allylamine produces more rapid and drastic ultrastructural effects than any of the drugs used alone, as was found previously in a study of proliferation of the parasites (44).

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Marlene Cazuza and Sebatião Cruz.

This work was supported by a Scientific Exchange grant by the Consejo de Desarrollo Cientifico y Humanistico, Universidad Central de Venezuela (to J.U.), Financiadora de Estudos e Projetos, Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, and Conselho de Pesquisa da Universidade Federal do Rio de Janeiro (to W.S.).

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