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STUDIES ON THE EFFECT OF BLEOMYCIN ON *TRYPANOSOMA GAMBIENSE*

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SUMMARY The antibiotic bleomycin inhibits nuclear duplication and causes deformation of the nucleus without any effect on the kinetoplast of *Trypanosoma gambiense*. Consequently it induces the anucleate form, not the dyskinetoplastic form. Studies showed that in many respects the effect of bleomycin on trypanosomes was very similar to that of neocarzinostatin. Analysis of DNA by CsCl density gradient centrifugation indicated that bleomycin inhibits DNA synthesis in the nucleus but not that in the kinetoplast. Bleomycin also induced excess formation of pellicular microtubules and disorderly arrangement of the axonemal and pellicular microtubules.

INTRODUCTION

Ono and Nakabayashi (1978) reported that in *Trypanosoma gambiense* and *Trypanosoma evansi* the antibiotic neocarzinostatin caused deformation of both the nucleus and microtubules, and also induced the anucleate form by inhibiting nuclear division. Moreover, we found that in *T. gambiense*, treatment with the antitubulin drugs vinblastine and colchicine caused deformation of microtubules and induction of anucleate forms (Ono and Nakabayashi, 1979). In previous studies (Ono and Inoki, 1971; 1973; 1975), we never observed disorganization of microtubules or formation of anucleate parasites with chemicals inducing the dyskinetoplastic form.

Bleomycin, an antibiotic isolated from *Streptomyces verticullus* in 1966 (Umezawa et al., 1966), is an antitumor agent that inhibits growth and DNA synthesis of HeLa cells (See Umezawa, 1975). Krishan (1973) reported that bleomycin did not affect the formation of spindle microtubules, but there are several reports that the effect of bleomycin on DNA in mammalian cells both in vivo and in vitro is very similar to that of neocarzinostatin (Haidle et al., 1972; Sim and Lown, 1978; Kuo and Samy, 1978). Therefore, in this study we examined the effect of bleomycin on the nucleus and kinetoplast of *T. gambiense* by morphological and biochemical methods.

MATERIALS AND METHODS

1. *Strain of Trypanosoma gambiense*

The Wellcome strain of *T. gambiense* was used. This strain has been maintained in this laboratory by serial passages in mice for more than 20 years. Trypanosomes of this species have either one or two nuclei, and the percentage of the dyskinetoplastic

form in the whole population is always less than 1%.

2. *Light and electron microscopic observations*

Swiss albino mice were inoculated subcutaneously with 2.5×10^4 trypanosomes and 72 h later, they were injected intraperitoneally with 10 mg/kg of bleomycin. The following methods were used to examine the effect of the drug on trypanosomes. (1) Blood samples were taken at various times after the injection for light and electron microscopic observations. (2) Seven or 14 h after the injection with bleomycin, the mice were again injected with bleomycin and 2 or 5 h later, blood samples were examined by electron microscopy. The parasites were stained with Giemsa for light microscopic examination. Electron microscopy was carried out as described by Ono and Inoki (1976), except that materials were embedded in low viscosity epoxy resin by the method of Spurr (1969). Bleomycin (bleomycin hydrochloride) was purchased from Nippon Kayaku Co., Ltd.

3. *Labeling of cells with ^3H-thymidine*

When parasites had reached a level of 8×10^8 trypanosomes/ml in the blood stream in Swiss albino mice 3 days after intraperitoneal inoculation of about 1×10^5 trypanosomes, the blood was collected in 0.3% sodium citrate- 0.85% saline solution from the heart of animals under chloroform anesthesia. Trypanosomes were isolated from the blood on a DEAE—cellulose colum (Lanham, 1968).

A sample of 1 ml of sodium citrate—saline containing 8×10^9 trypanosomes was suspended in a mixture of 7.2 ml of Eagle's minimal essential medium, 1.0 ml of calf serum and 0.8 ml of bleomycin (final concentration 80 μg/ml). The parasites were cultivated for 2 h at 37°C, in vitro, then centrifuged at 3,000 rpm for 10 min to remove bleomycin and resuspended in a mixture of 9 ml of Eagle's minimal essential medium and 1 ml of calf serum. Thymidine-6-^3H was then added at a final concentration of 10 μCi/ml of total suspension and parasites were cultivated for 2.5 h at 37°C. As controls, trypanosomes that had not been treated with bleomycin were cultivated by the same method and labeled with ^3H-thymidine.

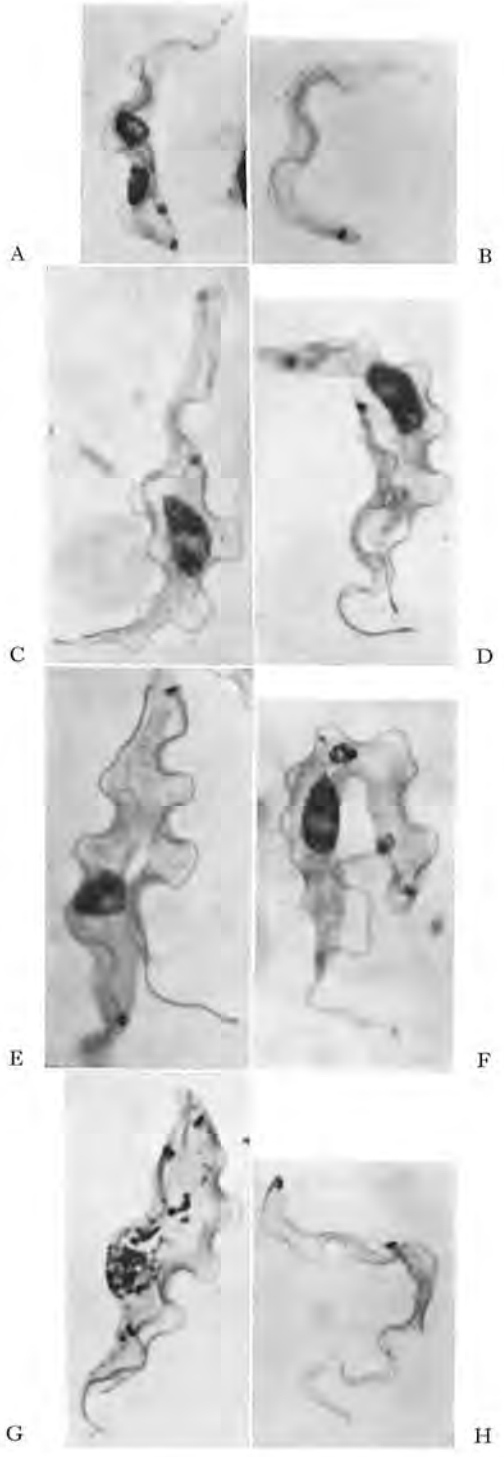
4. *Preparation of DNA and fractionation of DNA by CsCl density gradient centrifugation*

DNA of whole cells was extracted as described by Ono et al. (1971). CsCl density gradient centrifugation was carried out by the method of Meselson et al. (1957). ^{14}C-labeled DNA of *Micrococcus lysodeikticus* ($\rho=1.731$ g/ml) was used as a density marker. DNA was fractionated by ultracentrifugation at 40,000 rpm for 46 h at 5°C in a Beckman #65 angle rotor.

RESULTS

For analysis of the formation of the anucleate form, the rates of appearance of the various forms of trypanosomes were examined at intervals after injection of 10 mg/kg of bleomycin. The number of dividing forms with two nuclei and two kinetoplasts decreased greatly within 1 h and did not returned to the original level during the 12-h examination period. The number of dividing forms with one nucleus and two kinetoplasts increased 2 h after treatment with bleomycin. Anucleate forms without a stainable nucleus appeared 3 to 4 h after treatment with bleomycin and increased gradually in number to approximately 11% within 10 h. Treatment with bleomycin did not induce an increase in the number of the dyskinetoplastic form at any time. These results suggest that anucleate forms were formed as a result of selective inhibition of nuclear duplication by the drug without any disorder of kinetoplast division.

Figure 1A shows a light micrograph of an untreated trypanosome. Although the distance between the two kinetoplasts is moderately short, the two nuclei are already far apart. Figures 1B—H show the cytomorphological changes seen in trypanosomes treated with bleomycin. The parasites in Figs. 1B and H were obtained 12 h and 10 h after treatment, respectively. Figures 1C—G show trypanosomes obtained 7 h after treatment. Treated parasites were larger than untreated controls, except in the case of some anucleate forms. Figure 1B shows an anucleate form in which no nucleus is detectable. Although not shown in figures, anucleate forms varied in size. Figures 1C, D and E show formation of the anucleate form by inhibition of nuclear duplication. Most anucleate forms are produced



by division of abnormal dividing trypanosome, as shown in Fig. 1C. However, some anucleate forms are formed by division of dividing trypanosomes in which the anterior part of the body is elongated (Fig. 1D). Opposite polarity develops in a dividing trypanosome in which the kinetoplast is displaced anteriorly from its original position (Fig. 1E). Figure 1F shows a trypanosome with an unequally divided nucleus. One of the two flagella is growing away from the body. Another flagellum runs normally alongside the major axis of the parasite. Figure 1G shows a trypanosome with a degenerated nucleus. Fragmented nuclear elements lie scattered in the cytoplasm. Figure 1H shows an elongated anucleate form with two kinetoplasts far from each other. This type of parasite may divide at least once into two parasites.

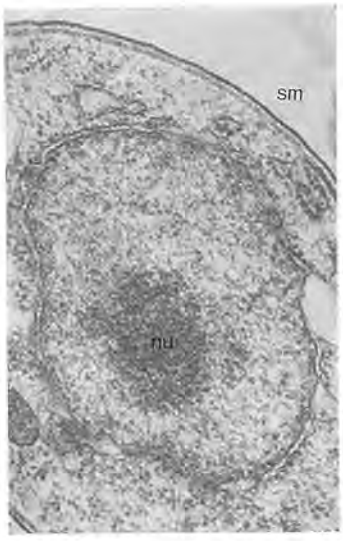
Figures 2A—C show electron micrographs of untreated *T. gambiense*. In the interphase,

◀

FIGURE 1A. Light micrograph showing a dividing form of *T. gambiense* not treated with bleomycin. ×1,900.

FIGURES 1B-H. Light micrographs showing cytomorphological changes in *T. gambiense* obtained 7 h (Fig. 1C-G), 12 h (Fig. 1B) or 10 h (Fig. 1H) after treatment with 10 mg/kg of bleomycin. ×1,900

The treated parasites are larger than the untreated control (Fig. 1A), except in the case of some anucleate forms (Fig. 1B, H). Although not indicated in the figures, anucleate forms varied in size. Anucleate form appears after treatment (Fig. 1B). Figures 1C, D and E show formation of anucleate forms. Most anucleate forms are produced by division of abnormal dividing trypanosomes, as shown in Fig. 1C. But, some anucleate forms are formed by division of dividing trypanosomes in which the anterior part of the body is elongated (Fig. 1D). In Fig. 1E, a dividing trypanosome shows opposite polarity. Figure 1F shows a trypanosome with unequally divided nuclei. One of the two flagella is growing away from the body. Figure 1G shows a trypanosome with a degenerated nucleus, whose elements are scattered about in the cytoplasm. Figure 1H shows an anucleate form in which the major axis of the parasite is elongated.



A

C

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FIGURE 2. Electron micrographs showing the nucleus (Fig. 2A, B) and the base of the flagellum (Fig. 2C) of untreated trypanosomes. In the interphase, the nucleolus is observed as a fine granular spherical structure (Fig. 2A). ×43,200 In Fig. 2B, chromatin material (arrow) is observed as slightly dense aggregates distributed over a wide zone beneath the nuclear membrane. ×50,600 The flagellum arises from a flagellar pocket (Fig. 2C). An extracellular flagellum and pellicular microtubules are seen. ×54,000

Abbreviation (Fig. 2A to 8B).

ax, axoneme; bb, basal body; fl, flagellum; fm, flagellar membrane; fp, flagellar pocket; kn, kinetonucleus; ke, kinetoplast envelope; n, nucleus ne, nuclear envelope; nu, nucleolus; pm, pellicular microtubules; pr, paraxial rod; rb, ribosome; sm, surface membrane.

B

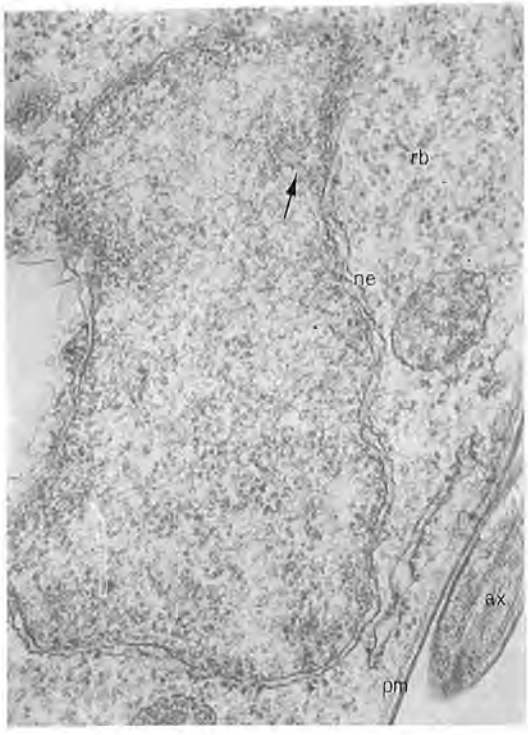

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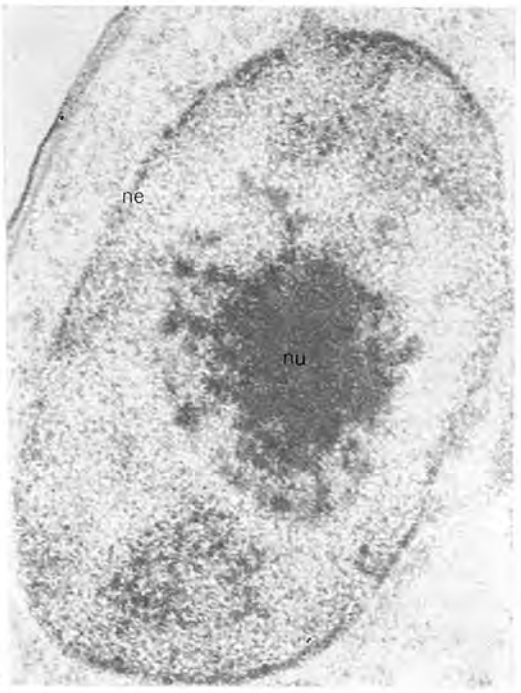
FIGURE 3. Electron micrographs showing the nuclei of trypanosomes obtained 16 h (Fig. 3A and B) and 12 h (Fig. 3C and D) after treatment with 10 mg/kg of bleomycin, respectively. In Fig. 3A, the nucleolus seems to be fragmented. ×50,600 Electron dense substances, instead of chromatin clumps, are seen in the area close to the nuclear membrane (Fig. 3B). ×37,950 In Fig. 3C, although the nuclear membrane has disappeared, a structure looking like a degenerated nucleus still remains. ×60,800 The nuclear substance is transformed into electron dense fragments (Fig. 3D). The nuclear membrane is seen as dense material. ×45,600



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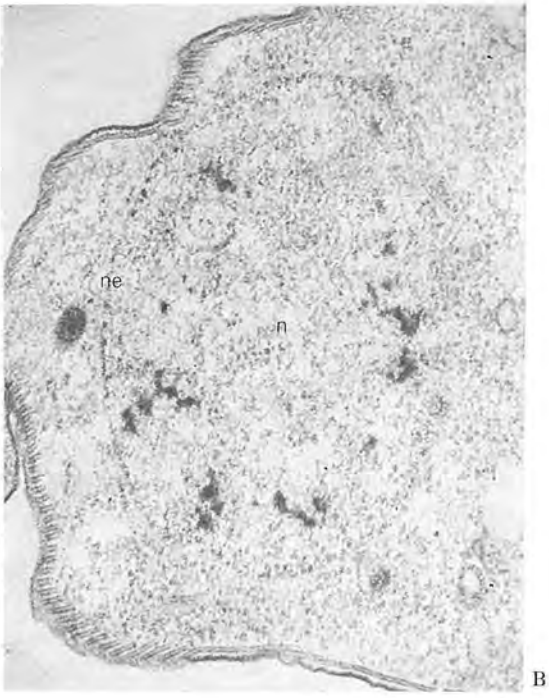

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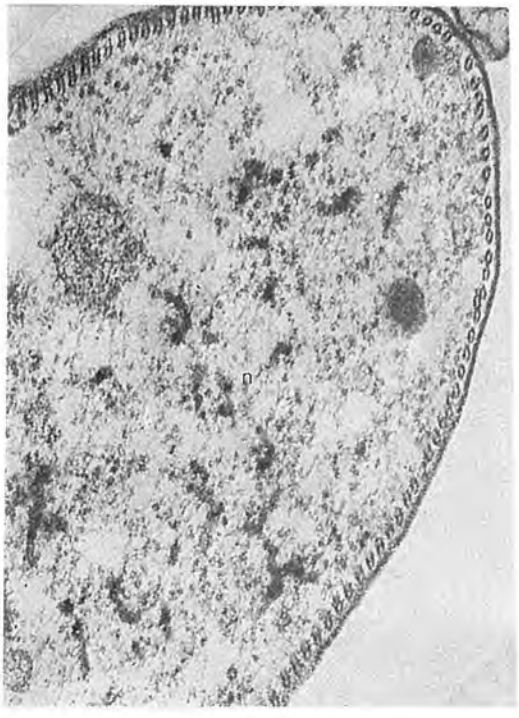

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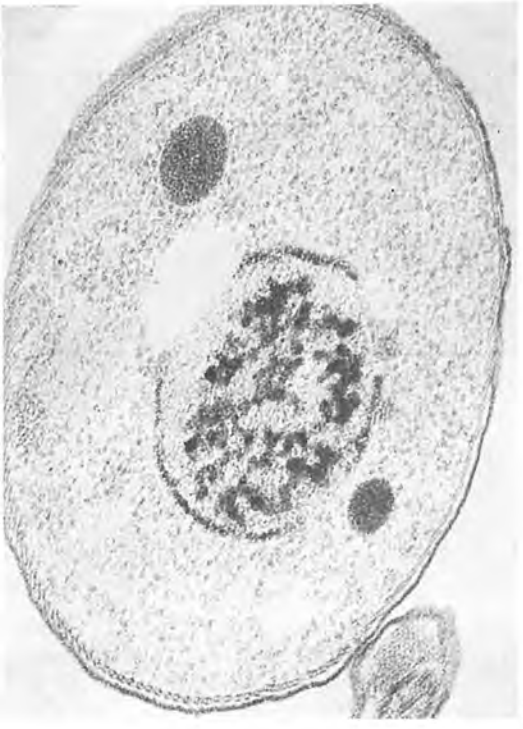


D

the nucleus is spherical and the nucleolus is situated centrally or slightly eccentrically as a large electron-dense finely granular spherical structure (Fig. 2A). The nucleolus becomes elliptical and disappears during division. The nuclear membrane remains unchanged throughout division. In Fig. 2B, chromatin material (arrow) is observed as slightly dense aggregations extending over a wide zone beneath the nuclear membrane. The single flagellum arises from a flagellar pocket, which is a bulbous reservoir and its membrane is connected to the flagellar membrane and surface membrane (Fig. 2C). The extracellular flagellum includes the axoneme complex and, running alongside it, the paraxial rod. Pellicular microtubules are seen in a single layer below the surface membrane.

Figures 3A and B, and 3C and D show the nuclei of trypanosomes obtained 16 h and 12 h after treatment with 10 mg/kg of bleomycin, respectively. In Fig. 3A, the nucleolus seems to has been transformed into electron dense fragments or coarse granular material especially at the periphery. The fine structure of the nuclear membrane is not visible. In Fig. 3B, the nuclear membrane is indistinct in some regions. Instead of chromatin clumps, small amounts of electron dense material are seen in the intranuclear area close to the nuclear membrane. In Fig. 3C, although the nuclear membrane has disappeared, a structure looking like a degenerated nucleus still remains. A large number of electron dense fragments derived from the nuclear substance are observed surrounded by the nuclear membrane, seen as a line of electron dense material (Fig. 3D).

Figure 4 shows a trypanosome 12 h after treatment with 10 mg/kg of bleomycin. An

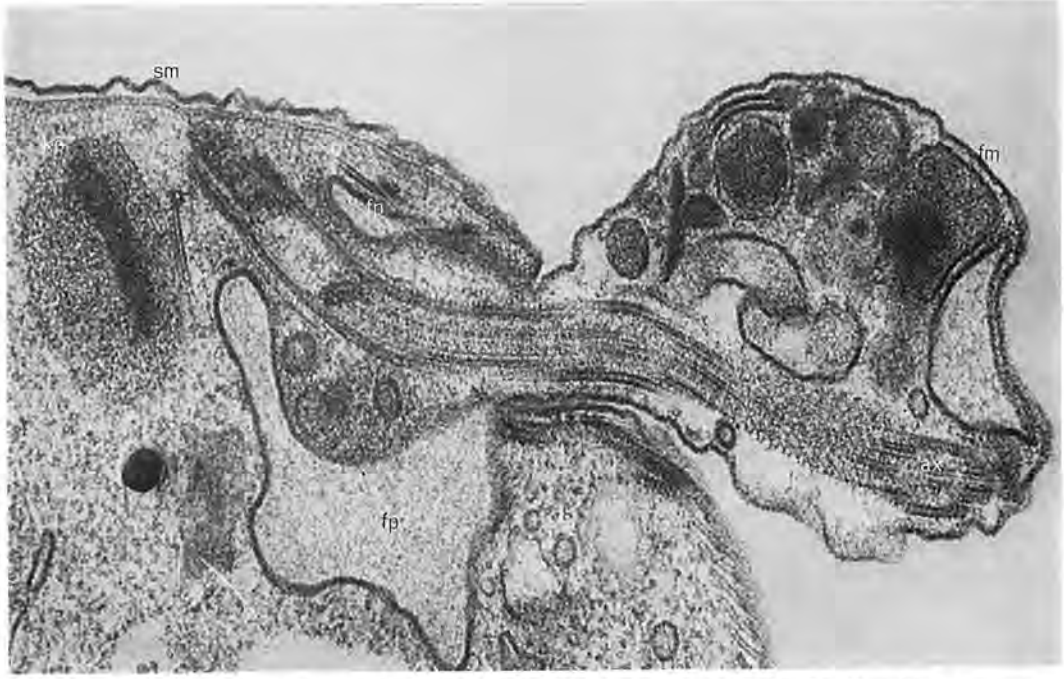
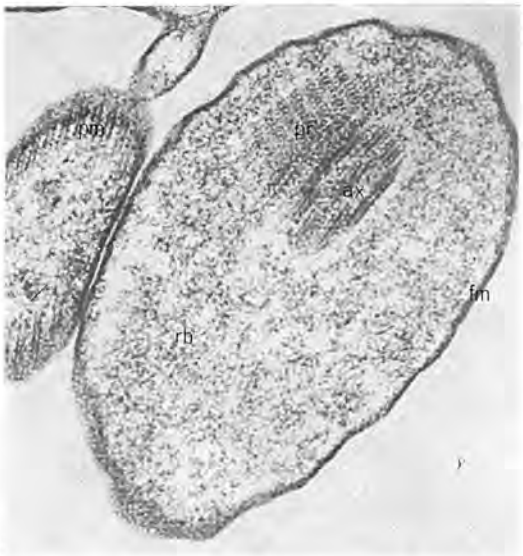


FIGURE 4. Electron micrograph showing a mushroom-like enlargement of the flagellum in a trypanosome obtained 12 h after treatment with 10 mg/kg of bleomycin. Some vesicles in this flagellum seem to be derived from the cytoplasm. No modification of the kinetonucleus or kinetoplast envelope can be detected. ×58,000



A

electron micrograph indicates a mushroom-like enlargement of the basal area of the flagellum; this contains a section of the axoneme, small vesicles and undetermined membrane-bounded structures. Some vesicles seem to be derived from the cytoplasm, because small vesicles can be seen in the vicinity of the transitional zone between the cytoplasm and flagellum. No modification of either the kinetonucleus or kinetoplast envelope can be detected.

Figures 5A and B show trypanosomes obtained 5 h after a second treatment with 10 mg/kg of bleomycin, 7 h after the first treatment (10 mg/kg of bleomycin). In Fig.

▼B

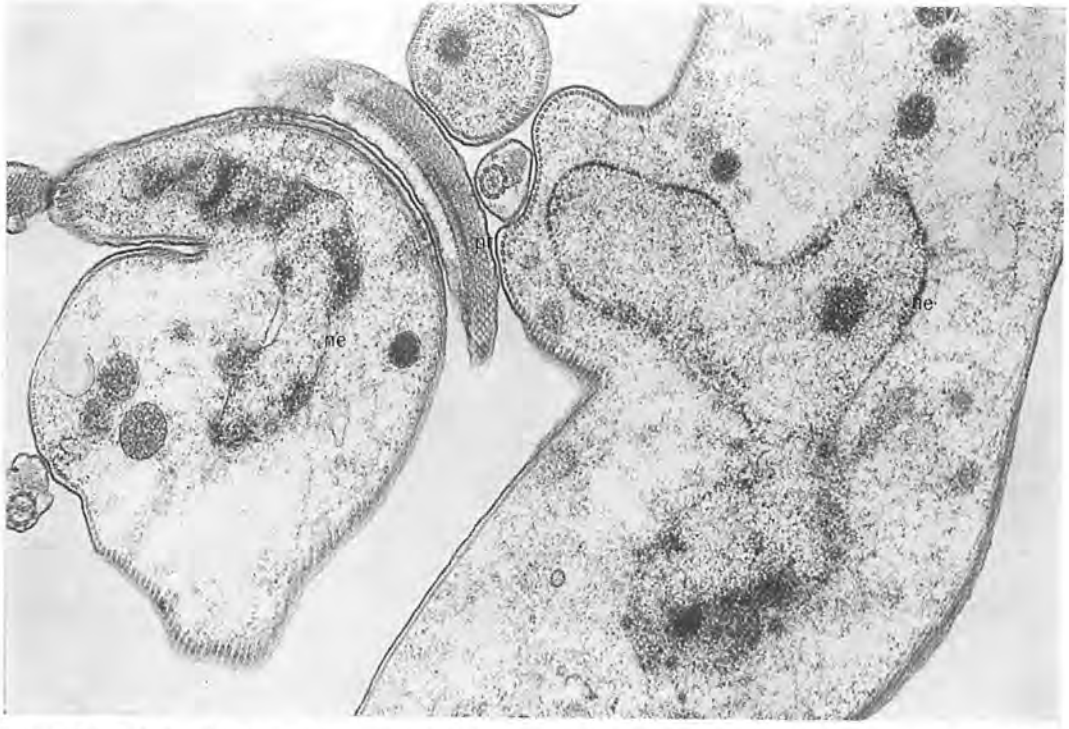
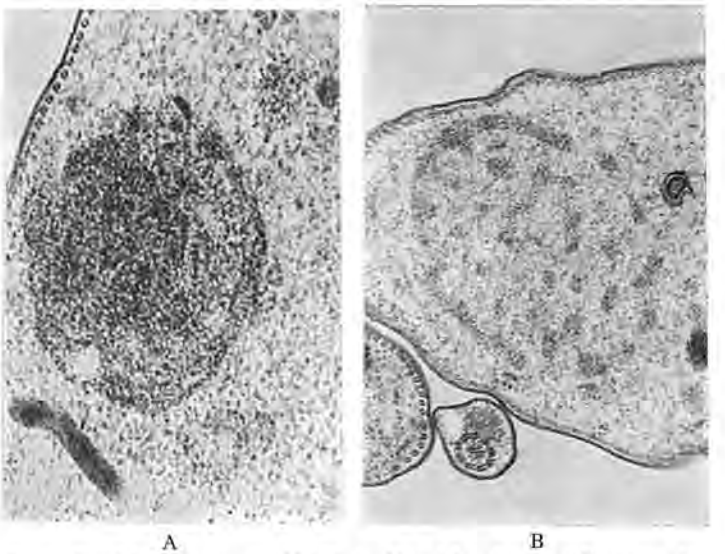


FIGURE 5A and B. Electron micrograph showing trypanosomes 5 h after a second treatment with 10 mg/kg of bleomycin, 7 h after the first treatment (10 mg/kg of bleomycin). In Fig. 5A, many ribosomes, an axoneme, and a paraxial rod are seen within an enlarged extracellular flagellum. ×60,800 In Fig. 5B, the nucleus in the upper parasite has a similar outline to the cytoplasm. In the lower parasite, although division of the cytoplasm is proceeding by incision, the nucleus is still incompletely divided. ×28,000

5A, a large number of ribosomes, an axoneme and a paraxial rod are seen within an enlarged extracellular flagellum. In Fig. 5B, the nucleus in the upper parasite which is incompletely divided, has a similar outline to the cytoplasm. In the lower parasite, division of the cytoplasm is proceeding by incision, but the nucleus is still incompletely divided.

Figures 6A—D show unusual structures in trypanosomes 5 h after a second treatment with 10 mg/kg of bleomycin, 7 h after the first treatment (10 mg/kg of bleomycin), unless otherwise noted. Electron dense structures are seen in Figs. 6A, B and C. In Fig. 6A, the structure is distinctly outlined against the peripheral cytoplasm. Figure 6B shows a trypanosome 2 h after a second treatment with 10 mg/kg of bleomycin, 14 h after the first treatment (10 mg/kg of bleomycin). In Figure, electron dense fragments are dispersed forming a nearly concentric circle. Some of these fragments are similar to those in the electron dense structure shown in Fig. 6C. In Fig. 6C, the structure is seen to be associated with the kinetoplast envelope (arrow). Small particles are seen in an electron dense structure (double arrow) and in the vicinity of the flagellar pocket. Figure 6D shows many round particles of less than 60 nm in diameter scattered in the cytoplasm. These particles have an electron dense part, appearing as a central core. They are similar to those in Fig. 6C.

Figure 7 shows a trypanosome obtained 12 h after treatment with 10 mg/kg of bleomycin. The electron micrograph indicates increase in the number of microtubules in the inner part of the cytoplasm and below the surface membrane. Some microtubules in the cytoplasm appear to intersect one another (arrow). Pellicular microtubules are seen to be closely associated with each other. Similar findings are also seen in Fig. 3C and D. Figures 8A and B show disorder in the arrangement of axonemal microtubules in a trypanosome 5 h after a second treatment with 10 mg/kg of bleomycin, 7 h after the first treat-



A B

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FIGURE 6. Electron micrographs showing unusual structures in trypanosomes 5 h after a second treatment with 10 mg/kg of bleomycin, 7 h after the first treatment (10 mg/kg of bleomycin), unless otherwise noted. Electron dense structures are seen in Fig. 6A, B and C. Magnification ×45,600 in Fig. 6A. Figure 6B shows dispersion of fragments in a traypanosome 2 h a second treatment with 10 mg/kg of bleomycin, 14 h after the first treatment (10 mg/kg of bleomycin). ×32,400 In Fig. 6C, a structure is seen to associate with the kinetoplast envelope (arrow). Small particles are seen in an electron dense structure (double arrow) and in the vicinity of the flagellar pocket. ×45,600 Figure 6D shows many round particles similar to those in Fig. 6C. Some of them contain an electron dense part, looking like a central core. ×50,600

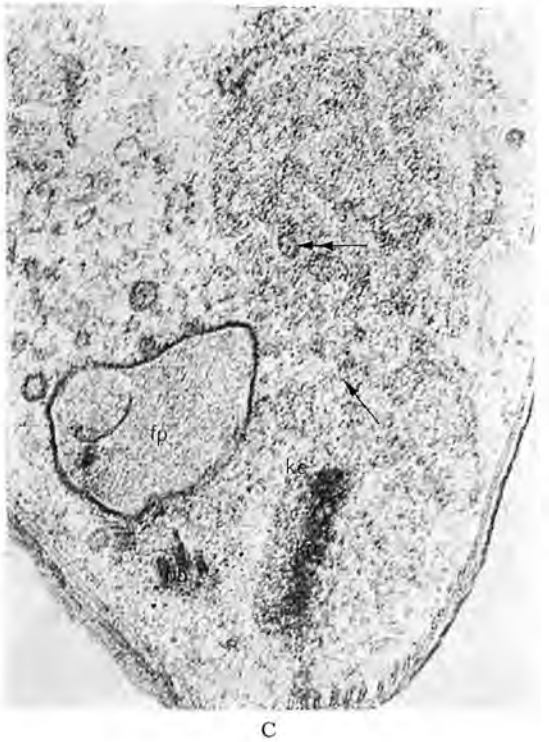
ment (10 mg/kg of bleomycin).

By CsCl density gradient centrifugation of whole cell DNA extracted from bleomycin-treated and untreated trypanosomes, a main band and a satellite band were obtained (Fig. 9A, B). The main band had a buoyant density of $\rho=1.703$ g/ml, and the satellite band had one of $\rho=1.688$ g/ml. These bands seemed to be nuclear and kinetoplast DNA, respectively, judging from results in a previous report (Ono et al., 1971). The buoyant densities of the main and satellite bands were unchanged by treatment with bleomycin. However, incorporation of ^{3}H-thymidine into nuclear DNA was significantly less in bleomycin-treated trypanosomes (Fig. 9B), than in untreated trypanosomes (Fig. 9A). Incorporation into kinetoplast DNA was unchanged by bleomycin treatment. In Figs. 9A and B, the kinetoplast DNA amounts to 22% and 7% of the total cell DNA in bleomycin-treated and untreated trypanosomes, respectively. These results indicate that treatment with bleomycin inhibits DNA synthesis in the nucleus, but not in the kinetoplast.

DISCUSSION

Bleomycin is known to decrease the mitotic index and inhibits the growth of cultured mammalian cells, and consequently results in increase in the cell volume (Suzuki et al., 1968; Umezawa, 1975; Müller and Zahn, 1977). We also found that in trypanosomes bleomycin inhibited either nuclear DNA -synthesis or nuclear duplication, but had no effect on the kinetoplast, and thus induced anucleate forms. In addition, bleomycin increased the volume of the nucleus and cytoplasm of parasites by inhibiting cell duplication. Inhibition of cell duplication may occur in association with delay or inhibition of nuclear duplication, as shown in Fig. 5B.

Bleomycin and neocarzinostatin are an-



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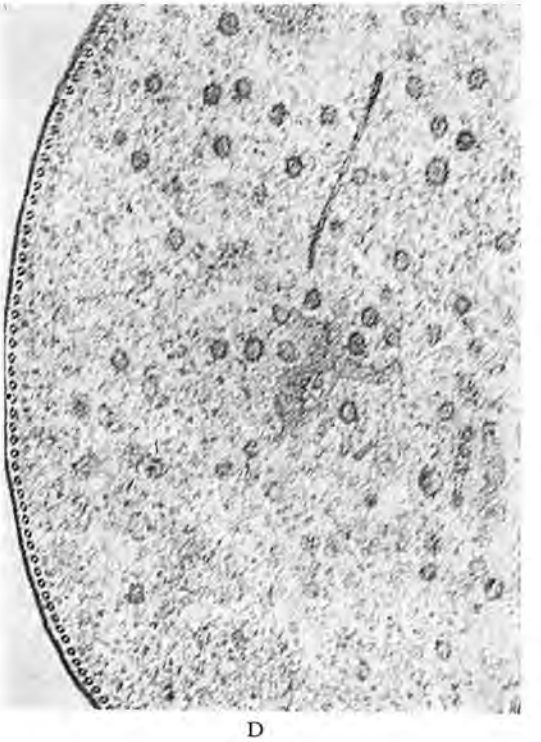


D

tineoplastic polypeptide antibiotics isolated from *Streptomyces* (Ishida et al., 1965; Umezawa et al., 1966) and they have similar effects on DNA in mammalian cells (Haidle et al., 1972; Kuo and Samy, 1978; Sim and Lown, 1978). The present experiments show that the effects of bleomycin on *T. gambiense* are similar to those of neocarzinostatin (Ono and Nakabayashi, 1976; 1978) in the following respects: both compound induce the anucleate form, disorder of cellular duplication, fragmentation of nuclear elements accompanied by disappearance of the nuclear membrane, mushroom-like enlargement of the flagellum, excess formation of pellicular microtubules, deformation of pellicular and axonemal microtubules, and the appearance of many round particles and electron dense structures.

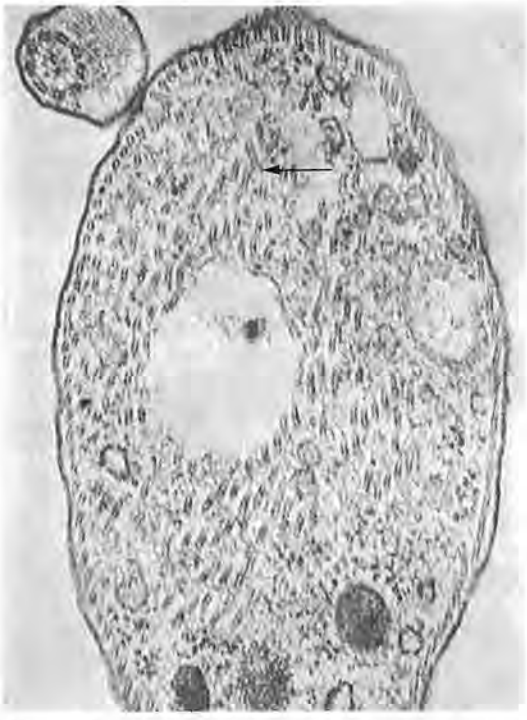


FIGURE 7. Electron micrograph showing excess formation of microtubules in a trypanosome 12 h after treatment with 10 mg/kg of bleomycin. Some microtubules appear to intersect one another (arrow). Pellicular microtubules are seen to be closely associated with each other. Similar changes are seen in Figs. 3C and D. ×52,400

Although bleomycin and neocarzinostatin have similar effects on trypanosomes, our electron microscopic study showed that bleomycin causes more severe alterations of the nucleus than neocarzinostatin. Reduction and fragmentation of chromatin, some residual fragmented nuclear elements, and deformation and disappearance of nuclear membrane were observed in the present study as in studies on mammalian cells by other workers (Ogawa and Onoé, 1969; Krishan, 1973). Therefore, anucleate forms may be produced by degeneration of the nucleus as well as by the inhibition of nuclear duplication recognized by light microscopy.

DNA of the kinetoplast differs from that of the nucleus in buoyant density, forming satellite DNA on ultracentrifugation in a CsCl density gradient (Du Buy et al., 1965; Ono et al., 1971). There are reports that the proportion of kinetoplast DNA in total cell DNA is 8–10% in Trypanosomatidae. (Riou and Pautrizel, 1969; Simpson and Berliner, 1974). In our previous (Ono et al., 1971) and present studies we obtained values of 9% and 7%, respectively. In trypanosomes after treatment with 80 μg/ml of bleomycin in vitro, however, incorporation of ^{3}H-thymidine into nuclear DNA decreased, while

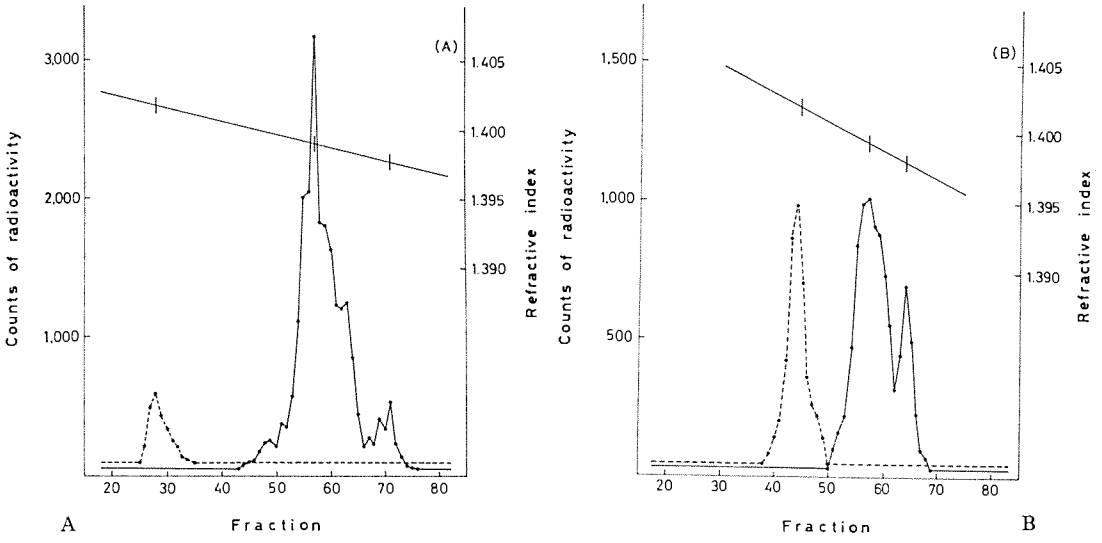
A B

FIGURES 8A and B. Electron micrographs showing disorder in arrangement of axonemal microtubules in trypanosome 5 h after a second treatment with 10 mg/kg of bleomycin, 7 h after the first treatment (10 mg/kg of bleomycin). ×50,600 ×45,600

incorporation into kinetoplast DNA was not changed. Therefore, the proportion of kinetoplast DNA in total cell DNA increased to 22%. Thus, bleomycin inhibits DNA synthesis in the nucleus, but not in the kinetoplast. This result shows that the sensitivities of the nucleus and kinetoplast to bleomycin differ, clearly supporting the results of morphological observations.

Krishan (1973) reported that bleomycin inhibits cell multiplication, but does not affect either the mitotic apparatus or the formation of spindle microtubules. However, we found that treatment with bleomycin caused disorder in the arrangement of axonemal and pellicular microtubules and aberrant increase of pellicular microtubules. Morevoer, in previous work we found that neocarzinostatin, vinblastine and colchicine, which are known to affect microtubular structures in cultured mammalian cells, induced the anucleate form in *T. gambiense* (Ono and Nakabayashi, 1978; 1979). However, we never observed disorganization of the microtubules in trypanosomes treated with chemicals inducing the dyskinetoplastic form (Ono and Inoki, 1971; 1973; 1975). These findings further support the suggestion in our previous report (Ono and Nakabayashi, 1978) that chemicals inducing the anucleate form affect nuclear duplication through the microtubule-nucleus system of trypanosomes and induce the anucleate form, unlike chemicals inducing the dyskinetoplastic form.

From our results (Inoki, 1956; Ono and Inoki, 1971; 1973; 1975; Ono and Nakabayashi, 1978; 1979), many of the chemicals that interact with DNA and have a therapeutic effect in experimental trypanosomiasis, can be divided into two groups: One group, including the antineoplastic agents bleomycin and neocarzinostatin, causes morphological alterations in the nucleus and microtubules, but no



FIGURES 9A and B. Analysis of whole cell DNA extracted from bleomycin-treated and untreated trypanosomes by CsCl density gradient centrifugation. *T. gambiense* DNA labeled with ^{3}H-thymidine. (●—●) *Micrococcus lysodeikticus* DNA (ρ=1.731 g/ml) labeled with ^{14}C-thymidine as density marker. (●---●) Ultracentrifugation was carried out 40,000 rpm for about 46 h at 5°C using a Beckman #65 rotor.

FIGURE 9A. Whole cell DNA extracted from untreated trypanosomes labeled with ^{3}H-thymidine for 2.5 h. Figure 9B. Whole cell DNA extracted from bleomycin-treated trypanosomes. Trypanosomes that had been incubated with 80 μg/ml of bleomycin for 2 h in vitro, were cultivated with ^{3}H-thymidine for 2.5 h after removal of bleomycin.

apparent changes in the kinetoplast and induces the anucleate form. The second group, including p-rosaniline, hydroxystilbamidine, ethidium bromide and furazolidon, inhibits kinetoplast duplication and causes ultrastructural deformation of the kinetoplast without affecting the nucleus or microtubules of trypanosomes and consequently induces the dyskinetoplastic form. The antitumor agent mitomycin-C inhibits DNA synthesis and proliferation of both prokaryotic and eukaryotic cells. As already reported (Ono and Inoki, 1974), however, mitomycin-C did not induce either the dyskinetoplastic or anucleate form of trypanosomes, and had no therapeutic effect on infected mice. But, clones of both *T. gambiense* and *T. evansi* obtained by repeated treatments with mitomycin-C were not affect by p-rosaniline or ethidium bromide which have marked effect in inducing the dyskinetoplastic form of trypanosomes, although the clones were not exposed to these drugs. Therefore, a new classification may be necessary for drugs such as mitomycin-C.

Aberrant enlargement of the flagellum was observed. The materials observed in the flagellum seemed to be derived from the cytoplasm, as shown in Fig. 4. Metabolites may accumulate in the cytoplasm of trypanosomes treated with bleomycin, because bleomycin does not inhibit synthesis of RNA or protein, even at concentrations that inhibit DNA synthesis and cell duplication almost completely (Müller and Zahn, 1977). Therefore, the aberrant enlargement of the flagellum may be caused by the selective inhibiting effect of bleomycin on macromolecule synthesis in trypanosomes. We have observed enlargement of extracellular flagellum in *T. gambiense* treated with neocarzinostatin that is similar to bleomycin in respect of effect on macromolecule synthesis in cell (Ono and Nakabayashi, 1976). Moreover, we have reported, in trypanosomes treated with vinblastine or colchicine enlargement of extracellular flagellum is observed with the appearance of paracrystals and paraxial rod -like structures. These findings suggest that unusual metabolites may accumulate in the flagellum of trypanosomes.

In *T. gambiense* after treatment with bleomycin, many round particles and electron dense structures are seen in cytoplasm; these electron dense structures were referred to as electron dense areas in the previous report on trypanosomes treated with neocarzinostatin (Ono and Nakabayashi, 1976). These are thought to differ morphologically from the ribosomal aggregation seen in *Trypanosoma rhodesiense* treated with suramin, tryparsamide or mapharside (Macadam and Williamson, 1974), but their exact nature is not known.

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