International Journal for Parasitology Vol. 23, No. 3, pp. 361–364, 1993
Printed in Great Britain

0020–7519/93 $6.00 + 0.00
Pergamon Press Ltd.

NUCLEAR AND KINETOPLAST DNA SYNTHESIS IN *TRYPANOSOMA CRUZI*, AUTORADIOGRAPHICAL STUDY WITH DNA POLYMERASE INHIBITORS

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(*Received* 21 *April* 1992; *accepted* 11 *September* 1992)

Abstract—ROJAS C., GUTIERREZ C., LITVAK S. and SOLARI A. 1993. Nuclear and kinetoplast DNA synthesis in *Trypanosoma cruzi*, autoradiographical study with DNA polymerase inhibitors. *International Journal for Parasitology* **23**: 361–364. DNA synthesis in epimastigote forms of *Trypanosoma cruzi* was studied autoradiographically by incorporation of [^{3}H]thymidine in the nuclear and kinetoplast DNA. Both DNA were heavily labelled. Three eukaryotic DNA polymerase inhibitors (aphidicolin, aracytidine, and dideoxythimidine) were chosen to study the nuclear and kinetoplast DNA synthesis *in vivo*. Inhibition was mainly observed with the nuclear DNA.

INDEX KEY WORDS: *Trypanosoma cruzi*; *in vivo* DNA synthesis; DNA polymerase inhibitors.

INTRODUCTION

Trypanosoma cruzi is a flagellated protozoon which causes Chagas' disease. This illness affects at least 24 million people in Central and South America (Walsh, 1984); an effective therapeutic treatment has not been found.

Considerable efforts have been made in order to understand *T. cruzi* DNA replication, particularly the process of DNA synthesis and its regulation as the parasites proceed through proliferative and non-proliferative cellular forms during their life cycle.

Biochemical characterization of the enzymes involved in DNA replication has proved to be difficult, especially in the case of less abundant enzyme species, because of enzyme instability and the high protease activity found in this parasite.

Alternatively *in vivo* DNA synthesis could be studied. Autoradiography, used for this purpose in several types of eukaryotic cells, seems particularly suitable for *T. cruzi* because of its morphological features. Organelles where DNA synthesis occurs, are large enough to be visualized by light microscopy. Nuclear DNA is distributed in 2.5 μm diameter spheres in interphasic cells and in dense plaques in mitotic cells (Solari, 1980). However, mitochondrial type DNA (kinetoplastic DNA) is a tightly packed network (1 μm long and 0.1 μm wide), with multiple interlocked DNA covalently closed, which represents 20–25% of total cellular DNA (Riou & Pautrizel, 1969). Previous studies of [^{3}H]thymidine incorporation into DNA *in vivo* led us to conclude that DNA synthesis was markedly inhibited by aphidicolin (Solari, Tharaud, Repetto, Aldunate, Morello & Litvak, 1983). This drug is a specific inhibitor of eukaryotic DNA polymerase α. However, this approach did not allow us to determine whether nuclear or kinetoplastic DNA synthesis were affected. Here we report autoradiographic studies concerning [^{3}H]thymidine incorporation into nuclear and kinetoplastic DNA of living epimastigotes in the presence of DNA polymerase inhibitors.

MATERIALS AND METHODS

The epimastigote form of the *Trypanosoma cruzi* Tulahuén strain was grown at 28°C in Diamond's medium, supplemented with 1% glucose and 10% fetal calf serum (Diamond, 1968). Cells were collected during the exponentially growing phase to a final concentration of 2–3 × 10^6 cells ml^{-1}. Experiments with DNA polymerase inhibitors were performed by preincubation of *T. cruzi* epimastigote suspension for 1 h at 28°C with the inhibitor in phosphate buffered saline, 10 mM-sodium phosphate pH 7.2, 135 mM-NaCl (PBS). This was followed by addition at zero time of 18 × 10^4 Bq [^{3}H]thymidine (s.a. 2.2 × 10^{12} Bq mmol^{-1}) (New England Nuclear, Boston, MA). The total incubation volume was 0.6 ml. After 2 h a 100 μl aliquot of the cell suspension, with or

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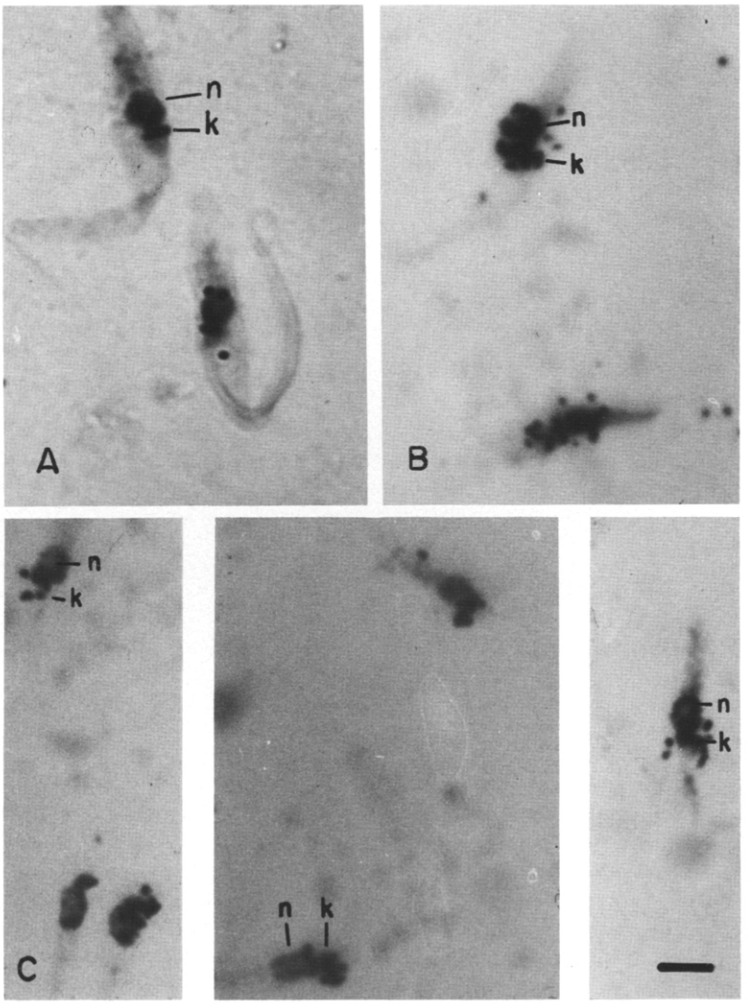

FIG. 1. Distribution of [^{3}H] thymidine in nuclear and kinetoplast DNA *Trypanosoma cruzi* (A) with and without silver grains over the nuclei (n) and kinetoplast (k); (B) *Trypanosoma cruzi* incubated in the absence of inhibitor; (C) *Trypanosoma cruzi* incubated in the presence of aphidicolin 100 μg ml^{-1} (three different experiments). Scale bar, 4 μm.

without inhibitor, was taken and precipitated with 1 ml of 10% cold trichloroacetic acid (TCA). Radioactivity incorporated into acid-insoluble material was determined by means of filtration on GF/A glass fiber filters (Whatman, Maidstone, Kent) and counting in a liquid scintillation system. For autoradiographic processing a 300 μl aliquot was taken in parallel, and the labelling reaction was followed by centrifugation of the samples on an Eppendorf bench centrifuge. Briefly, parasites were resuspended in culture medium to a final concentration of 1 × 10^6 cells ml^{-1} and smeared on subbed slides. These were fixed with methanol–acetic acid solution as described by Baserga & Malamud (1969), incubated in 5 N-HCl at room temperature for 20 min and coated with NTB 2 emulsion (Kodak, Rochester, NY). After 2 or 3 days exposure, samples were developed and stained with 4% Giemsa stain. Photomicrographs were taken in a Leitz Orthophan microscope at a magnification of × 1600. In this condition a good resolution between kinetoplast and nuclei is obtained which allows the quantification of [^{3}H]thymidine incorporation in both DNA cellular organelles.

RESULTS

Autoradiography was used to detect the incorporation of [^{3}H]thymidine into the nuclear and kinetoplastic DNA of culture epimastigotes. Figure 1 shows the distribution of silver grains on labelled parasites incubated in the absence and presence of aphidicolin. Parasites incubated in the absence of aphidicolin (Fig. 1B)

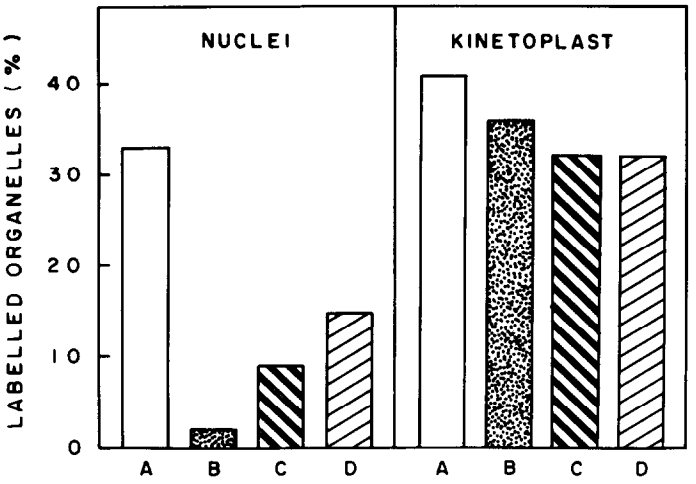

FIG. 2. Inhibition of nuclear and kinetoplast DNA synthesis by DNA polymerase inhibitors. *Trypanosoma cruzi* epimastigote suspensions were preincubated for 60 min at 28°C in PBS (A), aphidicolin 100 μg ml^{-1} (B), dideoxythimidine 200 μM (C), aracytidine 200 μM (D) and incubated with [^{3}H]thymidine in the presence or absence of inhibitor for 120 min. Counting of silver grains was performed in selected regions of the slide where the background was negligible. Results are expressed as % of parasites having one to seven silver grains over the organelle. The total number of oganisms counted in each experiment (100%) was A, 315; B, 292; C, 448; and D; 436.

TABLE 1—INHIBITION OF TOTAL DNA SYNTHESIS BY DNA POLYMERASE INHIBITORS IN *T. cruzi* EPIMASTIGOTES

Inhibitor	Concentration	% Inhibition
Aracytidine	200 μM	30
Dideoxythymidine	200 μM	45
Aphidicolin	100 μg ml^{-1}	80

DNA synthesis was measured by [^{3}H]thymidine incorporation into DNA as described in the Materials and Methods. An average of 120,000 c.p.m. 10^{-6} parasites was obtained without DNA polymerase inhibitor.

showed as much silver grains over the nuclei as in the kinetoplast. However, when parasites were incubated with aphidicolin, the silver grains over the nuclei disappeared and those over the kinetoplast remained (Fig. 1C). A six-fold greater level of [^{3}H]thymidine incorporation was observed when labelling experiments were performed in PBS instead of the culture medium (not shown).

Deoxyribonucleotide derivatives are well known *in vitro* DNA polymerase inhibitors. They fail to act *in vivo* due to the high charge density that hinders their transfer through biological membranes. Unphosphorylated nucleosides have to be used when studying their *in vivo* effect. Aracytidine, dideoxythymidine and aphidicolin were assayed producing a strong decrease in total DNA synthesis (Table 1).

Autoradiography proved able to provide valuable information on the site where known mammaliam eukaryotic DNA polymerase inhibitors act in *T. cruzi* epimastigotes. As shown in Fig. 2, aphidicolin, dideoxythymidine and aracytidine exerted a strong inhibition on nuclear DNA synthesis. The major decrease in [^{3}H]thymidine incorporation was produced by aphidicolin in nuclei, while any drug tested had almost no effect on kDNA synthesis. The smaller inhibition observed with aracytidine, a nuclear DNA polymerase inhibitor, as well as aphidicolin, correlated well with the total DNA synthesis measurements.

None of the drugs tested showed a significant effect on kDNA synthesis, although dideoxythymidine is known to be a typical inhibitor of higher eukaryote mitochondrial DNA polymerases.

DISCUSSION

Autoradiography allowed us to distinguish between the incorporation of [^{3}H]thymidine into the nuclear and kinetoplastic DNA of cultured epimastigotes. Labelling was performed with exponentially growing cultures, thus providing 100% of cells involved in the cell cycle. Nuclear DNA synthesis is known to occur during the S phase of the cell cycle. However the

information concerning the synchronism with kinetoplast DNA synthesis suggests that the kinetoplast is labelled before the nucleus. Similar results were obtained by others (Cosgrove & Skeen, 1970; Santos & Oliveira, 1988).

Results obtained by us suggest that both nuclear and kinetoplast DNA are synthesized during approximately the same period of the cell cycle, since a relatively short labelling time (2 h) provides a similar number of cells bearing silver grains in nuclei (33%) and kinetoplasts (41%). This information indicates that phase S of this *T. cruzi* strain occupies around 33% of the whole cycle which is 25 h. The result correlates with those obtained by others on *T. cruzi* clones with high growth rates (Dvorak, 1984). It is also remarkable that nuclear and kinetoplast DNA were labelled to a similar extent in these experimental conditions in spite of the fact that the nuclear DNA content can be two to three times larger than kinetoplast DNA (Dvorak, Hall, Crane, Engel, McDaniel & Uriegas, 1982). These results could be attributed to a difference in the rate of replication in both organelles.

DNA polymerase inhibitors were tested at relatively high doses taking into account the fact that the nucleoside precursors should be enzymatically phosphorylated into the corresponding nucleoside triphosphate within the cells. Aphidicolin displayed a dramatic inhibition in nuclear DNA synthesis, with almost no effect on [^{3}H]thymidine incorporation into kinetoplastic DNA. These results are in apparent discrepancy with those obtained *in vitro* since none of the DNA polymerase activities detected in epimastigote extracts are sensitive to aphidicolin (Rojas, Venegas, Litvak & Solari, 1992). It is not possible to discard the fact that specific DNA substrates are required to detect this enzyme form or that it is unstable compared with other DNA polymerases. As in the case of aphidicolin, both aracytidine and dideoxythymidine depict a preferential but lesser inhibition of nuclear DNA synthesis. The effect of dideoxythymidine cannot easily be explained, since this drug has been reported as a particularly effective inhibitor of the mitochondrial DNA polymerase from mammals (Krokan, Shaffer & De Pamphilis, 1979).

Autoradiography seems to be a useful tool for investigating whether a particular DNA polymerase inhibitor affects nuclear or kinetoplastic DNA synthesis in whole *T. cruzi* cells.

Acknowledgements—This investigation received financial support from the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, Universidad de Chile and from Fundación Andes. The authors are grateful to José Navarro for his help with the photomicrographs.

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