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DAMAGE OF *TRYPANOSOMA CRUZI* DEOXYRIBONUCLEIC ACID BY NITROHETEROCYCLIC DRUGS*

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Abstract—Kinetoplast DNA (*k*DNA) from *Trypanosoma cruzi* epimastigotes pretreated with the trypanocidal drugs nifurtimox (10 or 100 μM) or benznidazole (38 or 380 μM) showed an increased number of strand-breaks, as revealed by (a) trapping of alkali-denatured *k*DNA by nitrocellulose filters and (b) electrophoresis in an alkaline 2% agarose gel. Similar damage was observed in nuclear DNA (*n*DNA), as detected by centrifugation in an alkaline–sucrose gradient. DNA damage was reversible since reincubation in fresh medium for 24 hr restored filtration and electrophoretic and sedimentation patterns to normal.

Nifirtumix, a 5-nitrofuran derivative, and benznidazole, a nitroimidazole derivative, are the drugs most frequently used for the treatment of Chagas' disease [1]. Despite their clinical use, the mode of action of these drugs is not fully understood [2]. Tu and McCalla [3] reported that several nitrofurans are capable of causing single-strand breaks in bacterial DNA, the production of which is correlated with reduction of the nitro group and the mutagenicity of the drug. Similarly, nifurtimox and benznidazole are mutagenic in *Salmonella typhimurium*, *Escherichia coli* and *Klebsiella pneumoniae* [4] which implicates alterations in DNA. More topically, studies by Sims and Gutteridge [5] have proved that SQ 18506, a 5-nitrofuran, causes damage in the DNA of *Trypanasoma cruzi*, both in *n*DNA‡ and *k*DNA. The observations cited prompted us to investigate specific lesions in DNAs from *T. cruzi*, after treatment of the parasite with nifurtimox and benznidazole. The results described here show that, as expected, the nitroheterocyclic drugs induced strand-breaks in *k*DNA and *n*DNA. Interestingly enough, DNA damage induced by the nitroheterocyclic drugs was repaired after reincubation of the drug-treated epimastigotes in fresh culture medium for 24 hr.

MATERIALS AND METHODS

Organism and DNA extraction. T. cruzi (Tulahuen strain) was cultured for 3–4 days at 28° in a liquid medium made of brain–heart infusion (Difco), 37 g; hemin, 20 mg; fetal bovine serum, 40 ml; and water, to 1 litre [6, 7]. The cells were collected during exponential growth by centrifugation and resuspended in fresh warm medium at a concentration of 1×10^6 cells/ml. [^{3}H]Thymidine (10 μCi/ml) was added to the culture medium, and the epimastigotes were further cultured for 1–2 days. The cells were then collected by centrifugation at 5000 rpm for 10 min, washed twice, and resuspended in fresh medium to a concentration of 1×10^6/0.5 ml. After incubation, as indicated in Results, total DNA was extracted as described by Borst *et al.* [8]. *k*DNA was separated from *n*DNA by centrifugation at 15,000 *g* for 30 min at 4° [8].

Reagents. These were obtained from the following sources: [*methyl*-^{3}H]thymidine (5 Ci/mmole) from the Radiochemical Center, Amersham, U.K.; sodium dodecyl sulfate, Tris, EDTA, agarose (Type I, low electroendosmosis), sucrose, POPOP and PPO from the Sigma Chemical Co., St. Louis, MO, U.S.A.; nifurtimox from Bayer A.G., Leverkusen, Federal Republic of Germany; benznidazole from Hoffmann-La Roche Inc., Basel, Switzerland; and T7 phage DNA, φX174 phage DNA and HaeIII restriction endonuclease from Bethesda Research Laboratories Inc., Gaithersburg, MD, U.S.A. Other reagents were from previously mentioned sources [6, 7, 9]. Nifurtimox and benznidazole were dissolved in a 50% (v/v) dimethyl sulfoxide–ethanol mixture. The control samples received the corresponding volume of solvent.

Determination of k*DNA breakage.* Strand-breaks in *k*DNA were determined with a filter assay [10, 11] adapted to concatenated circular DNA. The assay measured the conversion of nicked or linearized double-stranded molecules into single-stranded DNA, after controlled alkali denaturation. Molecules having no single- or double-strand breaks remained attached to the *k*DNA network and could be washed from the filter with standard saline–citrate (SSC) buffer. The procedure was as follows: (1) to *k*DNA samples (50 μl; 10,000–20,000 dpm) there was added 150 μl of a solution containing 0.01% sodium

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‡ Abbreviations: *k*DNA and *n*DNA, kinetoplast and nuclear DNA respectively; nifurtimox, 3-methyl-4-(5′-nitrofurfurylideneamino)-tetrahydro-4*H*-1,4-thiazone-1,1′-dioxide; benznidazole, *N*-benzyl-2-nitro-1-imidazoleacetamide; SSC (1×), 150 mM NaCl/15 mM sodium-citrate; PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

dodecyl sulfate and 2.5 mM EDTA (pH 7.0); (2) after further addition of 200 μl of 0.3 M K_2HPO_4–KOH (pH 12.4), the reactions were left for 2 min at room temperature and then neutralized with 100 μl of 1 M KH_2PO_4–HCl (pH 4.0); (3) 200 μl of 5 M NaCl and 5 ml of buffer A (1 M NaCl, 50 mM Tris–HCl; pH 8.0) were added and the solutions were filtered through a Sartorius 0.2 μm nitrocellulose filter which had been presoaked with the same buffer; (4) the filters were washed twice with 2 ml of buffer A (the first buffer sample was used to rinse the tubes) and then with 20 ml of 0.3 M NaCl–0.03 M sodium citrate in a Petri dish, to remove the *k*DNA network; and (5) the filters were dried and placed into vials. Scintillation fluid (5 ml; 4 g PPO, 0.1 g POPOP, 1 l toluene) was added, and vials were counted for radioactivity in a liquid scintillation counter. To calculate the percent radioactivity trapped in the membrane filter, total radioactivity in each sample was measured before the alkali treatment. For this purpose, an aliquot of the original *k*DNA was layered on the nitrocellulose membrane, which was dried and counted for radioactivity without any further treatment.

For the electrophoretic determination of *k*DNA breakage, *k*DNA relative mobility through 2% (w/v) alkaline agarose was determined using a horizontal slab gel [12]. The bed size was 12 cm long, 10 cm wide and 0.5 cm deep and the electrophoresis buffer contained 30 mM NaOH and 2 mM EDTA. Bromophenol blue was added to *k*DNA samples (in electrophoresis buffer; 10,000–15,000 dpm) to a concentration of 0.025% before samples were loaded onto the gel. After 6 hr at 200 mA, the gels were cut with a slicer (Hoefer Scientific Instruments), and fragments were placed into vials and dried; scintillation fluid was added and they were counted as described above. Lengths of DNA fragments were determined by comparison with the electrophoretic mobilities of DNA fragments resulting from φX174 phage DNA, digested with HaeIII.

Determination of n*DNA breakage.* DNA sedimentation in an alkaline–sucrose medium was performed as described by Studier [13]. *n*DNA samples (10,000–15,000 dpm) were layered on a 5–20% (w/v) sucrose-gradient, in a medium containing sodium dodecyl sulfate (1 g/l), 0.9 M NaCl, EDTA (10 mg/l) and NaOH to pH 12.4. After centrifugation for 3 hr in the Spinco Centrifuge (SW65 rotor) at 36,000 rpm and 20°, samples (10 drops each) were taken from the bottom of the tube using a peristaltic pump. The fractions were layered on Whatman 3 MM paper disks soaked with 5% (w/v) trichloroacetic acid and, after drying, the disks were counted for radioactivity, as described above. The numbers of strand-breaks was calculated using Equation 1 where N, M_n, DNA-c

$$N = (M_n, \text{DNA-c}/M_n, \text{DNA-d}) - 1 \qquad (1)$$

and M_n,DNA-d, are the strand-break number, the average molecular weight of control DNA, and the corresponding value for the drug-treated DNA [14]. The molecular weight of DNA sedimenting fractions was determined by comparison with phage T7 DNA.

RESULTS

Effects on k*DNA. T. cruzi k*DNA is composed of several 39 kbp circular DNA molecules (maxi-circles) and thousands of 1.40 kbp circular DNA molecules (mini-circles) [15, 16]. The results in Table 1 show that pretreatment of *T. cruzi* epimastigotes with drugs stimulated *k*DNA cleavage in every case. Drug concentrations were selected on the basis of values producing half-maximal inhibition of growth [6, 7, 9], the greater effect being obtained with 100 μM nifurtimox. Comparison of *k*DNA damage and drug concentration values shows that nifurtimox was far more effective than benznidazole. Interestingly enough, removal of drug and reincubation of epimastigotes in fresh culture medium for 24 hr, allowed repair of *k*DNA strand-breaks (Table 1).

Table 1. Strand-breaks in *k*DNA after incubation of *T. cruzi* epimastigotes with trypanocidal drugs*

Drug (μM)	Trapping of *k*DNA in filter (%) of DNA deposited on the membrane	
	Immediately after drug treatment	24 Hr after reincubation in fresh medium
None	5.4 ± 0.1	4.5 ± 0.6
Nifurtimox (10)	20 ± 1.3	5.0 ± 0.2
(100)	47 ± 2.4	7.0 ± 1.2
Benznidazole (38)	10 ± 0.4	4.0 ± 0.5
(380)	35 ± 1.4	3.0 ± 0.3

* Epimastigotes cultured in standard medium supplemented with [^{3}H] thymidine were washed, resuspended in fresh medium, and reincubated with drugs as stated above, for 3 hr at 37°. After reincubation, *k*DNA was extracted, subjected to alkali denaturation, and filtered through the nitrocellulose membranes. Trapping of *k*DNA in membranes was measured before and after denaturation. Other experimental conditions were as described under Materials and Methods. The values represent the average of three independent measurements, each one in duplicate (mean ± S.D.). Radioactivity in *k*DNA was characteristic of each epimastigote sample and, therefore, only the relative variation values are presented.

Complementary evidence for drug-induced *k*DNA breakage was obtained by gel-electrophoresis. Concatenated DNA molecules obtained from control epimastigotes were not able to migrate through the gel channels because of their relatively large size, but linearized, *k*DNA molecules resulting from drug-induced strand-breaks migrated as indicated by the radioactivity peak centered in fraction 17 (Figs. 1 and 2). The height of the peak was related to the drug concentration. Radioactivity in fractions 3–7 suggests some contamination with *n*DNA, which did not interfere, however, with the demonstration of *k*DNA breakage. Calculation of DNA fragment length yielded a value of 100 bp, well below that for *k*DNA mini-circles [15]. In good agreement with the results in Table 1, washing of epimastigotes free of drug and reincubation in fresh medium for 24 hr restored the normal electrophoretic pattern (Figs. 1 and 2).

Effects on n*DNA*. To establish whether the trypanocidal drugs also affected the *n*DNA, strand-breaks were investigated in the latter. Figure 3A shows the sedimentation profile of *n*DNA from control and nifurtimox-treated epimastigotes. The control *n*DNA yielded a single, symmetric, fast-sedimenting peak containing 80% of total radioactivity in the gradient, while *n*DNA From epimastigotes exposed for 3 hr to 100 or 10 μM nifurtimox produced broad peaks and did not travel as far down as the control sample. *n*DNA damage was completely repaired after incubation of epimastigotes in a nifurtimox-free medium for 24 hr (Fig. 3B). Figure 4

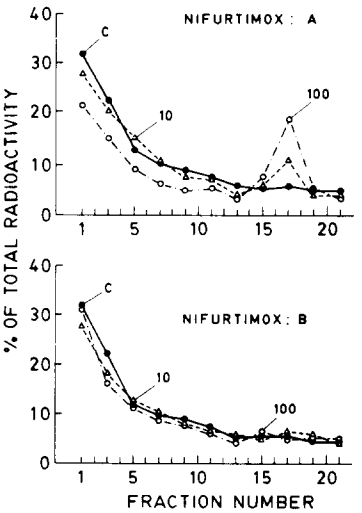

Fig. 1. Electrophoretic pattern of *k*DNA from nifurtimox-treated *T. cruzi*. (A) Epimastigotes cultured in standard medium supplemented with [^{3}H]thymidine were washed, resuspended in fresh medium, and reincubated with nifurtimox for 3 hr at 37°. After reincubation, *k*DNA was extracted and subjected to gel electrophoresis; the gel was sliced and the fragments were counted for radioactivity. Other experimental conditions were as described under Materials and Methods. Nifurtimix concentration (μM) is indicated by the figures near the lines; C indicates control sample. Fraction 1 is the origin of the run. (B) Control and nifurtimox-treated epimastigotes were washed, resuspended in fresh medium, and incubated for 24 hr at 37°; *k*DNA was extracted and processed as described above.

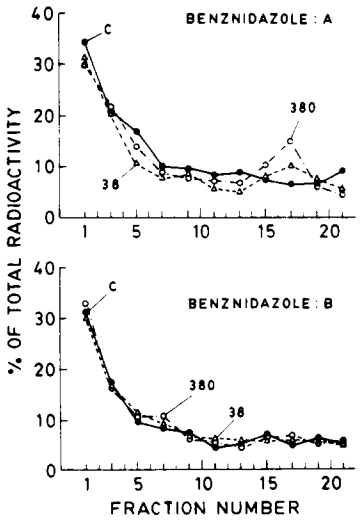

Fig. 2. Electrophoretic pattern of *k*DNA From benznidazole-treated *T. cruzi*. Experimental conditions were as in panels A and B of Fig. 1, except for the drug; benznidazole concentration (μM) is indicated by the figures near the lines; C indicates control sample.

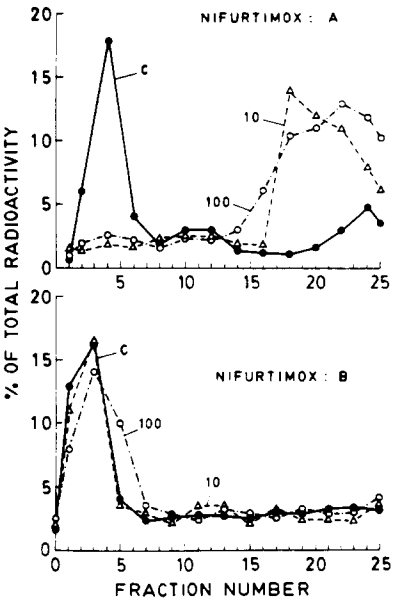

Fig. 3. Sedimentation pattern of *n*DNA from nifurtimox-treated epimastigotes. (A) Epimastigotes cultured in standard medium supplemented with [^{3}H]thymidine were washed, resuspended in fresh medium, and reincubated with nifurtimox for 3 hr at 37°. After reincubation, *n*DNA was extracted and subjected to ultracentrifugation analysis. Fractions were collected and counted for radioactivity. Other experimental conditions were as described under Materials and Methods. Fraction 1 is the bottom of the gradient. Nifurtimox concentration (μM) is indicated by the figures near the lines; C indicates control sample. (B) Control and nifurtimox-treated epimastigotes were washed, resuspended in fresh medium, and reincubated for 24 hr at 37°. *n*DNA was extracted and processed as above. Fraction 0 is the bottom of the gradient.

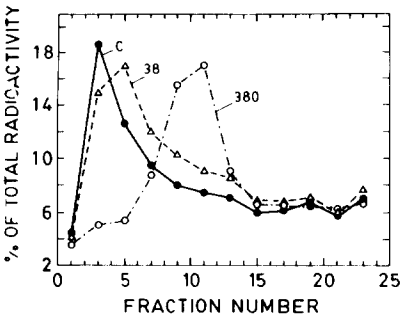

Fig. 4. Sedimentation pattern of *n*DNA from benznidazole-treated epimastigotes. Experimental conditions were as in Fig. 3A, except for the drug; benznidazole concentration (μM) is indicated by the figures near the lines; C indicates control sample.

shows the results obtained with benznidazole. It is to be seen that drug treatment modified DNA, which sedimented at a slower rate than the control sample. In contrast to the nifurtimox experiment, the effects of different concentrations of benznidazole could be clearly distinguished. Reincubation of drug-treated epimastigotes in fresh medium for 24 hr restored the normal sedimentation pattern (experimental data omitted). Calculation of strand-break numbers in *n*DNA samples from drug-treated epimastigotes yielded the values presented in Table 2. Nifurtimox was, again, more effective than benznidazole (far more than in Table 1).

DISCUSSION

The results in Tables 1 and 2 show that treatment of *T. cruzi* culture forms with nifurtimox and benznidazole produced strand-breaks in nuclear and kinetoplast DNA. It is obvious that there was much more damage to *n*DNA (Figs. 3 and 4) than to *k*DNA (Figs. 1 and 2), but the reason for such a difference is not apparent. Significant DNA breakage was obtained with nifurtimox concentrations about those existing in blood after treatment of rats with pharmacologically effective doses of drug (10–20 μM; Ref. 17). The effect of nifurtimox is in good agreement with previous observations with the nitrofuran SQ 18506 by Sims and Gutteridge [5]. To explain the mechanism of DNA breakage, it is worth recalling that (a) redox-cycling of nifurtimox generates in *T. cruzi* the nitrofuran anion and O_2^- [6, 7, 9], and (b) the parasite is an organism deficient in enzymatic defences against oxygen damage, particularly catalase [18, 19]. Accordingly, DNA breakage after incubation of *T. cruzi* with nifurtimox may be attributed to one or any combination of parent drug, drug anion or O_2^- (or metabolites of it, such as H_2O_2 and $OH^\cdot$ [20–23]). Gutteridge *et al.* [2] have suggested, however, that the trypanocidal action of nitroheterocyclic drugs in *T. cruzi* does not involve oxygen radicals, but inhibition of nucleic acids and protein synthesis. Nifurtimox and benznidazole are, in fact, inhibitors of DNA, RNA and protein biosynthesis in *T. cruzi* [24, 25], while only nifurtimox generates oxygen radicals in the parasite [6, 7, 9, 26]. On this basis, it seems reasonable to assume that the trypanocidal effect of drugs acting on *T. cruzi* may implicate several mechanisms, including (a) direct inhibition of biosynthetic reactions and (b) breakage and degradation of macromolecules by drug-generated radicals.

Incubation of *T. cruzi* damaged epimastigotes in fresh culture medium allowed complete reparation of both *n*DNA and *k*DNA. The results in Tables 1 and 2 and also in Figs. 1–3 suggest the occurrence of one-substrate synthesis, which in other organisms depends on DNA polymerase β [27]. Repair of damaged DNA may involve biochemical mechanisms, such as mediated excision followed by resynthesis of DNA in the damaged area [28, 29]. In this connection, it seems worth recalling that the 5-nitrofuran derivative SQ 18506 is an inhibitor of *T. cruzi* DNA polymerase [5]. However, it is also possible that, in the presence of the nitroheterocyclic drugs, the repair capacity of the parasite would be surpassed by the extent of DNA damage and, as a result of this, strand-break number would increase. The repair of *T. cruzi* DNA breakage is in good agreement with the observation that the cure of animals treated with nitrofurans depends more on the duration of treatment than on the total dosage of these drugs, and that interruption of treatment, even for short periods of time, prevents the parasitological cure [17].

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Table 2. Strand-breaks in *n*DNA after incubation of *T. cruzi* epimastigotes with trypanocidal drugs*

Drug (μM)	Strand-breaks (number/10^6b)	
	Immediately after drug treatment	24 Hr after reincubation in fresh medium
Nifurtimox (10)	32	0
(100)	65	0
Benznidazole (38)	0.1	0
(380)	1.4	0

* Experimental conditions were as in Figs. 3 and 4. The values represent the average of two independent measurements.

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