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Relationship between DNA methylation and cell proliferation in *Trypanosoma cruzi*

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5-Azacytidine treatment of *T. cruzi* epimastigotes in culture induces active cell proliferation. This effect was detected as an increase in the cell number and [^{3}H-methyl]thymidine incorporation into DNA. 5-Azacytidine does not alter other metabolic parameters. We have previously demonstrated that 5-azacytidine induces DNA hypomethylation in *T. cruzi*. Accordingly, we suggest that this chemical modification may be related to the control of *T. cruzi* cell division.

DNA methylation; 5-Azacytidine; Cell proliferation; *Trypanosoma cruzi*

1. INTRODUCTION

DNA methylation plays a role in the regulation of gene expression. Several studies have revealed that unmethylated genes can be expressed while methylated genes are inactive [1–4].

On the other hand, DNA hypomethylation can be induced if cells are grown in medium supplemented with 5-aza-C. This analog of cytosine differs from the canonical base only by the replacement of the 5-carbon atom by a nitrogen [5]. 5-aza-C is incorporated into DNA undergoing replication and inhibits the DNA (cytosine-5) methylase irreversibly, thus decreasing the m^{5}Cyt content in the newly replicated DNA strand [6]. The drug activates silent genes in a selective manner rather than causing a global increase in gene expression [7–9].

We have reported previously the presence of m^{5}Cyt in $_N$DNA of the parasitic protozoa *Trypanosoma cruzi*. We have also demonstrated that 5-aza-C treatment results in a decrease of methylated bases, suggesting that this analog induces DNA hypomethylation [10].

In this study, we investigated whether there is a correlation between DNA methylation and cell proliferation in *T. cruzi*. We tested 5-aza-C-treated cell cultures for several growth properties and found that this drug maintained cells in active proliferation while non-treated parasites enter the stationary phase. Other metabolic parameters tested were not altered.

Abbreviations: 5-aza-C, 5-azacytidine; m^{5}Cyt, 5-methylcytosine; $_N$DNA, nuclear DNA; FBS, fetal bovine serum; PBS, phosphate buffered saline; TCA, trichloroacetic acid; PCA, perchloric acid.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

T. cruzi epimastigotes (strain Tulahuen) were grown at 28°C in Diamond medium [11] supplemented with 2.5% FBS.

2.2. *5-aza-C treatment*

Cells cultured for 7 days were collected by centrifugation at 600 × *g* for 20 min, washed in PBS and resuspended in fresh culture medium. These cells were treated daily for 14 days with 5-aza-C (Sigma) freshly prepared in distilled water, at a final concentration of 10^{-5}, 10^{-6} or 10^{-7} M. Additionally, cell cultures, maintained continuously in exponential growth by dilution each 24 h with fresh medium supplemented with 2.5% FBS, were treated daily for 7 days with 5-aza-C at the same final concentrations.

Cell cultures grown without exposure to 5-aza-C were used as controls. Growth was followed by turbidity measurement in a nefelometer HF model DRT-100D and by cell number counting in a Neubauer chamber.

2.3. *[^{3}H-methyl]thymidine incorporation*

Aliquots of 1.5×10^6 cells taken from cultures in daily treatment with 10^{-6} M 5-aza-C and controls were incubated with 10 μCi/ml [^{3}H-methyl]thymidine (67 Ci/mmol, ICN Biochemicals), for 24 h at 28°C, at the exponential (day 5), the inflection (day 11) and the stationary (day 13) phases of growth. The incorporation was stopped by washing the cells twice in cold PBS. Afterwards, cells were broken by freezing at −70°C and treated with cold 5% TCA for 15 min. The TCA precipitate was washed twice in cold 5% TCA, once in 80% ethanol and once in ethanol:ether (3:1, v/v). Pellets were dissolved in 1 N NaOH and radioactive thymidine incorporation into DNA was measured in a Beckman liquid scintillation counter model LS100C.

2.4. *RNA and protein content*

Cells treated daily with 10^{-6} M 5-aza-C for 14 days and controls were sequentially treated with cold 5% TCA, 80% ethanol and ethanol:ether as described above. Nucleic acids were extracted from sediments in 5% TCA at 90°C for 20 min. After centrifugation at 1750 × *g* for 15 min RNA was measured in the supernatant by the Orcinol

method [12], and proteins were measured in the pellet by the technique of Lowry [13].

2.5. *Oxygen consumption*

Control and 5-aza-C-treated cells (10^{-6} M, 14 days) were collected by centrifugation and pellets were resuspended in 100 mM Tris buffer, pH 7.2, 52 mM NaCl. Oxygen consumption was measured in aliquots of 16×10^7 cells in a Gilson 5/6 oxigraph, following the procedure described by Lessler [14].

3. RESULTS

Fig. 1 shows the effect of 5-aza-C on cell proliferation of *T. cruzi* cultures. It can be seen that this treatment maintains the cells in active proliferation, preventing them from entering stationary phase. This activation was detected either as an increase in turbidity or cell number.

In Table I, results of [^{3}H-methyl]thymidine incorporation into DNA in different phases of the growth curve of 5-aza-C-treated and control *T. cruzi* epimastigote cultures are shown. It can be seen that at the exponential phase (5–6 days of culture), [^{3}H-methyl]thymidine incorporation into DNA was nearly the same for control and treated cells. However, at the inflection (11–12 days of culture) and at the stationary (13–14 days of culture) phases of the growth curve, there is a higher incorporation of [^{3}H-methyl]thymidine into DNA in cultures exposed to the drug than in control cells, reaching a 6-fold increase at day 14. These results suggest that 5-aza-C-treated cells maintain an active DNA synthesis and do not enter the G0 state.

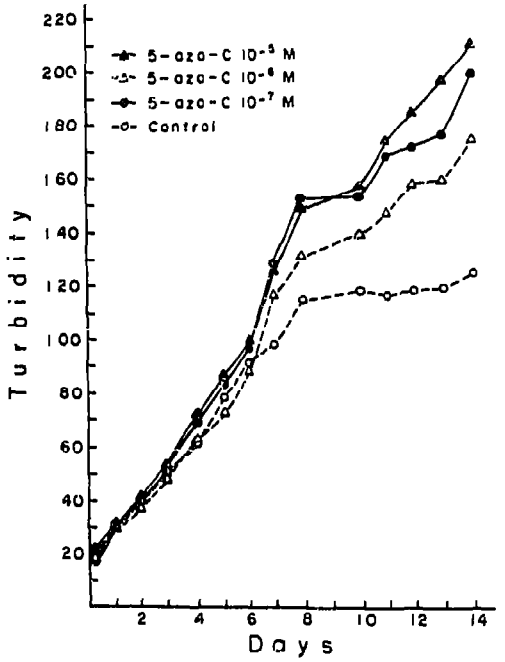

Fig. 1. Effect of 5-aza-C on *T. cruzi* epimastigotes proliferation. Cultures of *T. cruzi* in Diamond medium supplemented with 2.5% FBS were treated daily for 14 days with 10^{-5}, 10^{-6} or 10^{-7} M 5-aza-C. Cultures without exposure to the drug were used as controls.

Table I

Effect of 5-aza-C on the incorporation of [^{3}H-methyl]thymidine in *T. cruzi* epimastigotes DNA

Culture (days)	[^{3}H-methyl]thymidine per 10^6 cells (cpm)		Treated/controls
	Control	5-aza-C	
6	386404 ± 12116	366571 ± 63527	0.95
12	64305 ± 4867	142924 ± 5588	2.22
14	16649 ± 3677	97924 ± 4547	5.88

Cultures of *T. cruzi* epimastigotes in Diamond medium with 2.5% FBS were treated daily for 14 days with 10^{-6} M of 5-aza-C. At days 5, 11 and 13, aliquots from treated and untreated cultures were centrifuged, resuspended in conditioned medium up to 1.5×10^6 cells per ml and incubated for 24 h with 10 μCi of [^{3}H-methyl]thymidine. Results are the average of triplicate experiments.

In agreement with the previous results, Fig. 2 shows that *T. cruzi* cultures maintained at the logarithmic growth phase and simultaneously exposed to 10^{-5}, 10^{-6} or 10^{-7} M 5-aza-C, present a higher growth rate than control cells.

In Table II results of the analysis of RNA and protein contents in *T. cruzi* cells treated and not with 5-aza-C are shown. Clearly this drug does not affect the RNA and protein content per cell.

A considerable increase (nearly 54%) in oxygen consumption was detected in *T. cruzi* cells treated with 5-aza-C as compared with control cells, which probably could be correlated with the active DNA synthesis and cell proliferation shown in Table I and Figs. 1 and 2, respectively.

4. DISCUSSION

The present study demonstrated that 5-aza-C treatment prevents *T. cruzi* epimastigote cultures from entering stationary phase. This effect was detected by an

Table II

Effect of 5-aza-C on O_2 consumption, and RNA and protein contents in *T. cruzi* epimastigotes

	Content per cell (pg)		O_2 consumption per 10^6 cells (n Atom g × min^{-1})
	RNA	Protein	
Control	0.657 ± 0.038	4.38 ± 0.09	0.0575 ± 0.0025
Treated	0.697 ± 0.001	4.27 ± 0.07	0.0889 ± 0.0009

RNA and protein contents were determined in cultures treated daily with 10^{-6} M of 5-aza-C for 14 days and in untreated controls. RNA was measured by the Orcinol technique [12] using tRNA from yeast as standard. Proteins were determined by the method of Lowry [13] using bovine serum albumin as standard. Oxygen consumption was determined in aliquots of 16×10^7 cells in 100 mM Tris buffer, pH 7.2, 52 mM NaCl.

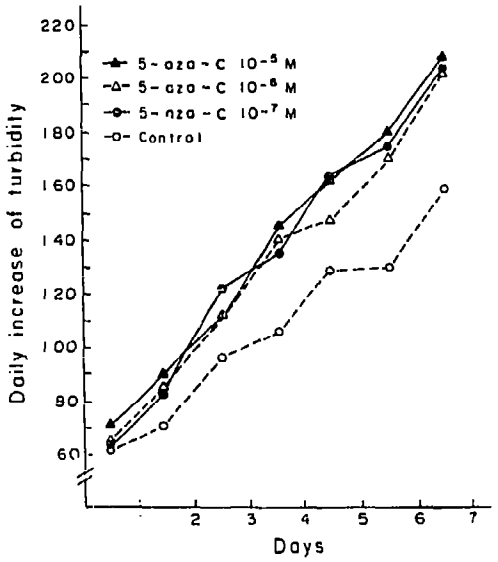

Fig. 2. Effect of 5-aza-C on a *T. cruzi* epimastigotes culture maintained at exponential growth. Cultures of *T. cruzi* epimastigotes in Diamond medium supplemented with 2.5% FBS were kept at the logarithmic phase of growth by daily dilution in fresh medium in the presence of 10^{-5}, 10^{-6} or 10^{-7} M of 5-aza-C. Cultures without exposure to the drug were used as controls.

increase in [^{3}H-methyl]thymidine incorporation into DNA as well as in the cell number in cultures treated with the drug. These results do not agree with those reported previously, which showed that 5-aza-C treatment decreased [^{3}H-methyl]thymidine (and deoxiadenosine) incorporation into DNA of L1210 leukemia cells and of ascitic cells isolated from leukemic mice [15]. Similar results were found using sea urchin embryos [16] and isoproterenol-stimulated salivary glands [17]. Moreover, 5-aza-C was cytotoxic toward L1210 cells growing in culture [15] and retarded the cellular division rate in sea urchin embryos [16].

We have previously demonstrated that the same 5-aza-C treatment as reported here also results in a decrease of the m^{5}Cyt content into $_N$DNA [10]. Therefore, we propose that 5-aza-C induces DNA hypomethylation and reactivation of specific genes associated with cell proliferation. Thus, this event may be at least partially controlled by DNA methylation which may function as a first level control of gene activation.

Regarding the relationship between DNA methylation and cell proliferation, decreased levels of DNA methylation have been found in a number of tumor cells and tumor cell lines, and in regenerating tissues, where an increased level of DNA synthesis was associated with a decreased m^{5}Cyt content of DNA [18,19]. Interestingly, 5-aza-C-treated *T. cruzi* epimastigote cultures do not enter the stationary phase, resembling the behavior of transformed cells. Probably, arrest in cell division requires methylation at specific location(s) in DNA.

No toxic effect of 5-aza-C treatment was observed. On the contrary, thymidine incorporation and O_2 consumption increased after 5-aza-C treatment as compared with control, untreated cells. The increase in oxygen consumption in 5-aza-C-treated cells may reflect a more active metabolism in those cells as compared with controls, probably due to the maintenance of the proliferative state.

Moreover, protein and RNA contents per cell remain unaffected by the drug. Similar results were obtained when [^{3}H]uridine incorporation into RNA and [^{3}H] leucine into total proteins were studied (data not shown). Furthermore, light microscope examination revealed that motility and morphology were unaffected in treated cells.

There is agreement between our data on protein content and those of Fernandes and Castellani for the Y strain of *T. cruzi* measured by the same method [20]. However, there is divergence between the reports for RNA content. Thus, Fernandes and Castellani found an RNA content of 0.157 pg per cell at the stationary phase of growth, which is considerably smaller than our values. We believe that the procedure used for these authors for RNA extraction is responsible for this difference. They used the Ogur and Rosen method [21] which employed treatment with cold 1 N PCA for extracting RNA, which may not be adequate for complete extraction of the RNA [22]. These authors also used the Orcinol technique for RNA determination.

The relation between RNA and protein contents of *T. cruzi* parasites at the stationary phase was nearly 1:7. This result differs significantly from that estimated for bacteria and mammalian cells (1:2.5 and 1:16, respectively [23]) and may be a characteristic trait of these protozoa.

Considering our previous results and those reported here, we conclude: (i) *T. cruzi* $_N$DNA is methylated in cytosine; (ii) 5-aza-C induces $_N$DNA hypomethylation; (iii) cells in which $_N$DNA is hypomethylated do not enter the stationary phase but remain in active cell proliferation; and (iv) 5-aza-C does not present any toxic effect.

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