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THE EFFECTS OF ANTIPURINES AND ANTIPYRIMIDINES ON THE GROWTH RATE AND NUCLEIC ACID SYNTHESIS IN *TRYPANOSOMA CRUZI*

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SUMMARY

The effect of several purine and pyrimidine analogs on the rate of adenine and uracil incorporation into nucleic acids of *Trypanosoma cruzi* have been investigated.

Likewise, *in vitro* effect of amethopterin, mitomycin C and 5-fluorouracildeoxyriboside on the growth of *T. cruzi* has been studied. The addition of any of these substances to a *T. cruzi* culture leads to the appearance of the known unbalanced growth picture, with aberrant morphological dividing forms of crithidiae.

INTRODUCTION

The requirements for rich media to grow *Trypanosoma cruzi in vitro* were well known soon after the discovery of this organism. Although research workers have succeeded in getting very convenient media to enable a production of large mass of trypanosomes, very little has been done in the direction to define the complex constituents of the culture media. Generally those constituents are an amino acids source like tryptose, peptone or similar substances, serum, hemoglobin or hematin and an organ infusion, such as liver, heart, brain. Such complex media although very good to get a great number of protozoa do represent a disadvantage when we want to study metabolic processes by the use of labeled precursors, such as amino acids, purines and pyrimidines as well as to study the effects of metabolic analogs of those substrates or cofactors highly concentrated in the medium as vitamins, purines, pyrimidines and amino acids analogs. These difficulties need to be overcome but very little has been done in the attempts to define the nutritional requirements of the flagellate. BONÉ & PARENT's [1] work, showing that stearic acid may replace the serum requirement for growing the flagellate, is a successfull and important contribution in the study of the nutrition of this parasite.

We have shown that the parasite has a very limited ability to synthesize purines and pyrimidines [5, 13]. In most media commonly employed these compounds are certainly furnished by liver or muscle infusions. The requirements for purines and pyrimidi-

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nes for *in vitro* growth of the organism can be a promising field of research in the attempts to find a chemotherapeutic agent against the flagellate. Previous works from this laboratory have already started the exploration in this field [2, 6, 11, 15].

In the paper to be presented, a new list of purine and pyrimidine analogs has been studied as inhibitors of nucleic acid synthesis of *T. cruzi*.

The effect of an antifolic (amethopterin) and of fluorouracil deoxyriboside (FUDR) on the growth rate and size distribution of the flagellate population will be presented. These agents establish what is known as unbalanced growth (COHEN & BARNER [3]), a biological situation that can be of importance in the aim to get an unviable population of *T. cruzi*. Mitomycin C, a potent inhibitor of DNA synthesis in *T. cruzi* (FERNANDES et al.[9]) exerts an even more remarkable picture of unbalanced growth, with curious alterations in the morphology of the protozoa in division.

MATERIAL AND METHODS

Trypanosoma cruzi used in these studies was obtained from cultures in LIT medium [8]. The organisms to be used in the experiments on nucleic acids biosynthesis were washed by centrifugation in 0.15M NaCl and resuspended in Ringer Krebs phosphate as described by CASTELLANI & FERNANDES [2].

Incorporation of uracil into nucleic acids pyrimidines ** — Each experimental flask contained 5×10^9 flagellates in a final volume of 3.6 ml of Ringer Krebs phosphate containing the inhibitor. The concentration of the inhibitor is indicated in the Table I and the uracil 2 C^{14} was added to give a final concentration of 0.8 μmole per ml (S.A. = 2.9×10^6 counts per minute per μmole). Each flask was incubated for 2 hrs. at 30°C with occasional shaking. The precipitation and hydrolysis of the nucleic acids, the chromatography of the nucleic acid pyrimidine nucleotides as well as the radioactivity measurements were carried out as previously described (REY & FERNANDES [13]).

TABLE I

Effect of antipyrimidines on the incorporation of uracil 2 C^{14} into nucleic acid pyrimidines of *T. cruzi*

Analogs added	Uridylic acid		Cytidylic acid	
	counts/min/μmole	% of inhibition	counts/min/μmole	% of inhibition
5 — hydroxyuracil	3,270	33	1.830	32
6 — azauracil	3,480	29	2,300	15
5 — bromouracil	3,500	29	2,150	20
4 — thiouracil	6,000	—	3,120	—
5 — aminouracil	5,600	—	3,500	—
5 — nitrouracil	4,830	—	3,000	—
None	4,900	—	2,700	—

The concentration of analogs was 2 μmoles per ml. The results were expressed as counts per min per μmole of the nucleotide, using as molar extintion coefficient of uridylic acid 10,000 and 6,200 for cytidylic acid.

Chromatography was carried out in a column of Dowex 50 $H^+ \times 8$ (200 — 400 mesh) of 0.82 cm of diameter per 4 cm height. The column was eluted with 0.05N HCl, collecting 3 ml samples; uridylic acid came off in the first two tubes and cytidylic acid in the tubes 8, 9, 10 and 11.

** Radioactives uracil 2 C^{14} and adenine 8 C^{14} used in this investigation were products from New England Nuclear.

TABLE II

Effect of antipurines on the incorporation of adenine 8 C^{14} into nucleic acid purines of *T. cruzi*

Analogs added	Guanine		Adenine	
	counts/min/μmole	% of inhibition	counts/min/μmole	% of inhibition
2 — aminopurine	1,070	—	12,200	—
2 — mercaptopurine	527	51	14,000	—
5 — azaguanine	540	44	12,160	—
6 — isoguanine	1,220	—	16,800	—
6 — (1'-methyl-4'-nitro-5'-imidazolil) thiopurine	780	17	13,300	—
2 — amino-6-(1'-methyl-4'-nitro-5'-imidazolil) thiopurine	1,000	—	11,760	—
None	945	—	11,900	—

The concentration of the first two analogs was 2 μmole per ml and of others were around 0,1 to 0,2 μmole per ml. The chromatography was carried out in a column of Dowex 50 H^+ × 8 (200 — 400 mesh) of 0.82 cm of diameter per 4 cm height; the column was washed with 40 ml of 1N HCl; guanine was then eluted with 10 ml of 2N HCl; after washing with more 10 ml of the same acid, adenine was collected in 10 ml of 6N HCl. A molar extintion coefficient of 8,000 for guanine and 13,000 for adenine was used to express the concentrations of the purines.

Incorporation of adenine into nucleic acids purines — Each experimental flask contained about 7×10^9 flagellates in a final volume of 3.6 ml of Ringer Krebs phosphate containing the inhibitor at the concentrations indicated in Table II. Adenine 8 C^{14} was then added to give a final concentration of 0.5 μmole per ml (S.A. = 0.8×10^6 counts per min per μmole). Each flask was incubated at 30°C with occasional shaking, for 2 hrs. The separation and chromatography of the nucleic acid purines as well as the radioactivity measurements were carried out as previously (CASTELLANI & FERNANDES [2]).

Effect of amethopterin, FUDR and mitomycin C — The experiments to study the effect of amethopterin, FUDR and mitomycin C on the growth rate and size distribution of *T. cruzi* were carried out in LIT medium (FERNANDES & CASTELLANI [8]). After the centrifugation of a suitable volume of a logarithmic growing culture in LIT medium, the sediment was resuspended in fresh medium kept at 28°C in a measured volume in order ta start the experiment with 1.5×10^7 flagellates per ml. Amethopterin, mitomycin C or FUDR was then added to give the final concentrations indicated. The flasks were incubated at 28°C under shaking and the cells were counted in a Coulter electronic cell counter as previously shown (FERNANDES & CASTELLANI [8]) but using different thresholds to permit the evaluation of the size distribution of the cell population.

The organisms used for the stained preparation were from cultures of 5 days. Methylic alcohol fixation and Giemsa staining were used.

RESULTS

The effect of pyrimidine analogs on the rate of incorporation of uracil into nucleic acids pyrimidines of *T. cruzi* is shown in Table I. Only 5-hydroxy, 5-bromo and azauracil exert an inhibitory effect in spite of the high concentration of all analogs studied.

In Table II are shown the data on the effect of purine analogs on the rate of incorporation of adenine into nucleic acids purines. No inhibitory effect on the rate of incorporation of adenine into nucleic acids adenine was detected. However 2-mercaptopurine and 5-azaguanine do inhibit the conversion of adenine to guanine compounds, as shown by the low rate of incorporation of the labeled precursor into the nucleic acids guanine.

The effect of amethopterin, FUDR and mitomycin on the growth rate of *T. cruzi* is shown in Fig. 1. The concentration of amethopterin and even FUDR necessary to bring a deep degree of growth inhibition is relatively high. This is certainly due to the presence of folic (folinic) acid and thymidine in the culture medium.

The inhibitory picture by the above mentioned drugs is related to cell multiplication. Growth in size of individual flagellates does occur. This is shown in Fig. 2, where a size distribution picture of the control and drug inhibited flagellate population can be seen. Flagellates counted in threshold 10 indicate small and normal size flagellates, and those counted in threshold 50 are large size flagellates, found in small number in normal culture. In threshold 30 are counted medium size flagellates. In normal culture of any growth phase about 90% of the population are counted in thresholds 10 and 30 (differential counting). In exponential growth phase equal values for each of the above thresholds are found, while at the plateau about two thirds of the value are found in threshold 10 and one third in threshold 30.

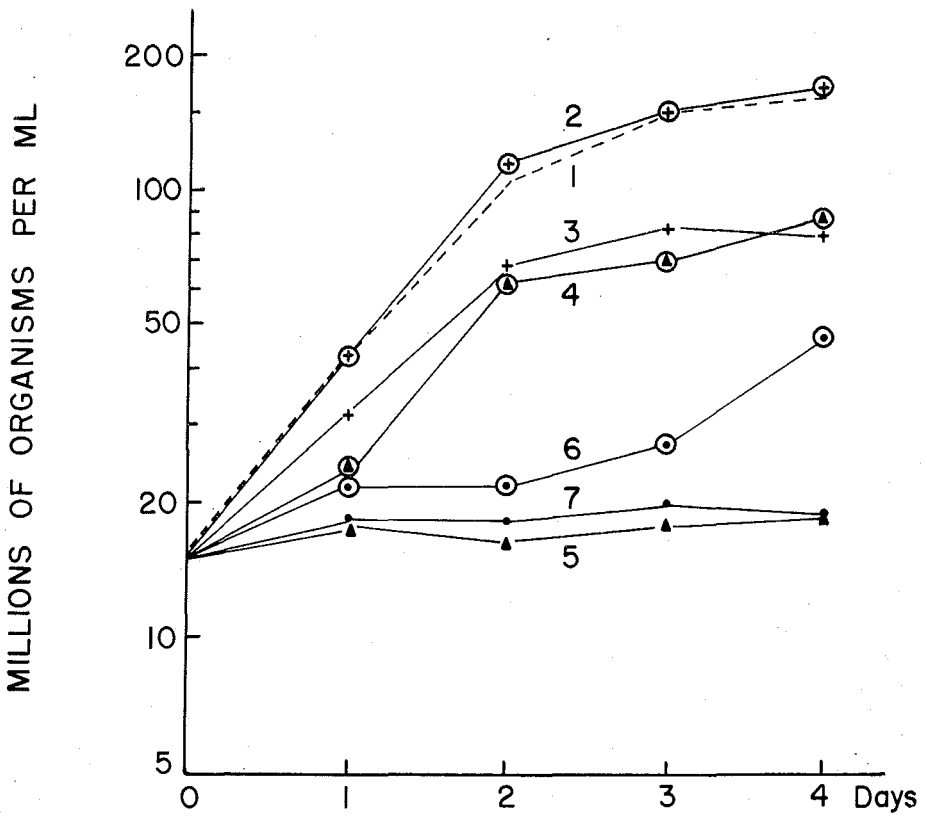

Fig. 1 — Effect of FUDR, amethopterin and mitomycin C on the growth rate of *T. cruzi*. Curve 1 represents the control. The other curves are respectively: amethopterin 10^{-5}M (curve 2) and 10^{-3}M (curve 3); mitomycin 1 μg/ml (curve 4) and 10 μg/ml (curve 5); FUDR 10^{-4}M (curve 6) and 10^{-3}M (curve 7).

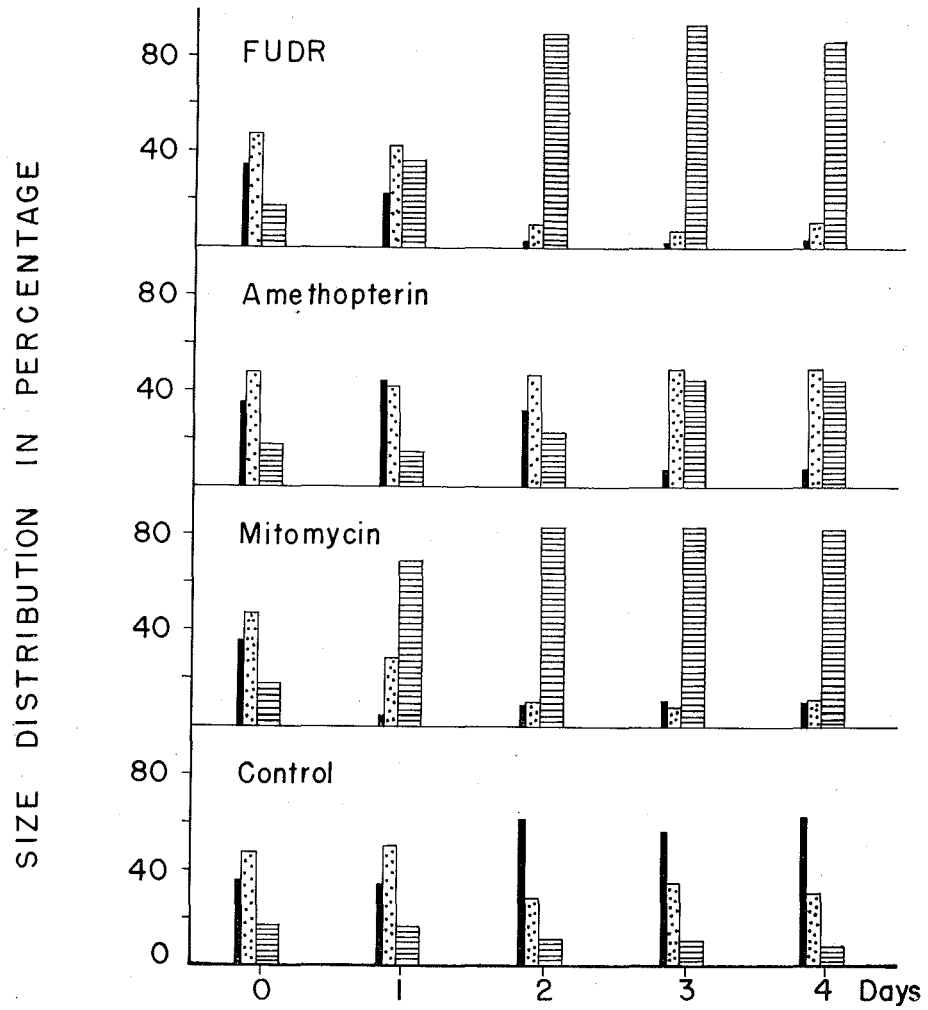

Fig. 2 — Effect of FUDR, amethopterin and mitomycin C on the size of *T. cruzi*. The concentrations of the drugs in LIT medium are: FUDR 10^{-4}M, amethopterin 10^{-3}M and mitomycin 10 μg/ml. The size distribution refers to differential counts recorded dayly by counting at threshold 10, 30 and 50. The black columns show the percentage of small flagellates while the intermediary and the widest columns show the proportion of medium size and large (including giants) flagellates.

On the other hand the size distribution picture of the population cultivated in the presence of the drugs is completely different from the control. With FUDR and mitomycin, 80 to 90% of the population consist of large crithidiae, counted in threshold 50. With amethopterin, a less marked alteration is found. All these crithidae are alive and slow motile organisms.

In stained preparation this size alteration can be nicely confirmed as can be seen in Fig. 3. The difference in size between the control flagellates and those exposed to the mitomycin is so marked that most of the flagellates counted in threshold 50 of the control culture (about 10%) are probably insoluble particles from the medium and not the giant crithidiae of the FUDR or mitomycin cultures.

Another feature in Fig. 3 we want to call attention refers to the remarkable alterations among the division forms of the flagellates. The control cultures show trypanosomes and crithidiae with typical aspects. The dividing

forms of these crithidiae show two nuclei, two kinetoplasts and two flagella or differ of such forms by presenting one nucleus or one nucleus and one flagellum. Otherwise, in the flagellates cultivated in the presence of mitomycin C a deep morphological alteration is found, besides the size increase. These alterations affect mainly the dividing forms and can be seen in Fig. 3 and summarized in few words:

1) Commonly, the kinetoplast is much bigger than the normal and no crithidia presents divided kinetoplast even when two equal or unequal flagella are found.

2) Dividing forms present partially or almost completely divided cytoplasm even when only one nucleus is found. Several dividing forms present posterior and not anterior cytoplasm fission and often two, three or even more posterior cytoplasm fissions of variable lenght are found.

3) Rarely double nuclei are found. Frequently one nucleus is found even in those forms showing a partially divided cytoplasm.

These aberrant morphological forms are found in smaller degree with FUDR and even less with amethopterin.

Finally, in control cultures a variable proportion of crithidiae differentiate to the metacyclic forms. In this experiment this proportion was high, 10 to 20% of metacyclic forms being found. However, with mitomycin and FUDR no differentiation did occur.

Fig. 3 (A) — Control flagellates in culture during 5 days in LIT medium, showing normal dividing forms of crithidiae. 880 ×.

Fig. 3 (B) — Flagellates in culture during 5 days in LIT medium containing 10 μg per ml of mitomycin C. Enormous flagellates showing abnormal dividing forms of crithidiae and nuclear, kinetoplast and flagellum alteration. 880 ×.

Fig. 3 (C) — Enormous flagellates showing abnormal dividing forms of crithidiae and abnormal cytoplasm fission, after 5 days in LIT medium containing 10 μg per ml of mitomycin C. 880 ×.

DISCUSSION

The search for a chemotherapeutic agent against Chagas' disease has been a very disappointed field of work. In spite of the known dependence of rich media to grow, *Trypanosoma cruzi* when anchored within the host cells appears highly resistant to any kind of substances. Purine, pyrimidine and nucleic acids inhibitors constitute the largest pool of effective metabolic inhibitors against microorganisms and even cancer cells. However, *T. cruzi* remains resistant to the tried agents from this area as shown by NAKAMURA & JAMES [12] and the present investigation. However, the most hopeful area for this inhibitors trials, is just the nucleic acids field, as shown by HEWITT et al.[10] and in papers from this laboratory [2, 6, 7, 8, 9, 11, 13, 15]. These refer to the chemotherapeutic point of view as well as the possibility of getting non virulent strain of *T. cruzi* or organisms devoid of multiplication capacity (FERNANDES et al.[7, 9]).

The picture of unbalanced growth clearly obtained by the action of mitomycin C and FUDR deserves comments. The sluggish giant organisms show such a high proportion of aberrant morphological forms that their genetic structure could be deeply affected. These abnormal dividing forms may indicate possibly an irreversible inhibition of the reprodutive ability and/or infective capacity of the whole population. If this is true a vaccination aproach as tried with actinomycin treated flagellates preparation (FERNANDES & CASTELLANI [7]) must be tried. Similar aberrant forms are found in bacteria [4] and mammalian cells [14] as result of mitomycin action. In the present investigation, the efficient blockage of the kinetoplast division appears to suggest more detailed study of mitomycin in order to understand better the flagellate division. Questions of several natures could be answered. As examples we could ask whether there is a message from the macronucleus to the kinetoplast in order this micronucleus can divide. Whether the control of flagellum formation and development is related with some stable messenger ribonucleic acid originated from deoxynucleic acid of the kinetonucleus as logically expected or from the macronucleus that seems less affected by mitomycin.

RESUMO

Efeitos de antipurinas e antipirimidinas no crescimento e na síntese de ácidos nucléicos do Trypanosoma cruzi

Os Autores estudaram o efeito de diversos análogos de purina e pirimidina na incorporação de adenina e uracila nos ácidos nucléicos do *Trypanosoma cruzi*.

Também foi estudado o efeito *in vitro* da ametopterina, mitomicina C e 5-fluoruracila desoxiribosídeo no crescimento do *Trypanosoma cruzi*. A adição de qualquer uma destas drogas à cultura de *Trypanosoma cruzi*, conduz ao aparecimento do conhecido crescimento não balanceado, com formas de critídia em divisão, com morfologia aberrante.

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