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EXPERIMENTAL CHEMOTHERAPY OF *TRYPANOSOMA CRUZI* INFECTION IN TISSUE CULTURE. A COMPARATIVE STUDY ON THE ACTION OF PRIMAQUINE, CARBIDIUM SULPHATE AND THE AMINONUCLEOSIDE OF STYLOMYCIN

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SUMMARY

Monolayer tissue cultures of trypsinized cells of a monkey heart strain with liquid nutrient medium were employed for studies on the experimental chemotherapy of *Trypanosoma cruzi* infection. The advantages of this technique for the evaluation of drug activity result from its providing means to follow the intracellular and extracellular development of the parasites in the cultures. Criteria were established for the quantitative evaluation of drug action, utilizing the following elements in the experimental and control groups: 1) number of parasites present in the liquid nutrient medium; 2) microscopical characteristics of fixed and stained preparations of parasitized cells, namely: a) abundance of parasites in the preparation; b) morphology of the parasites; c) frequency and aspect of the forms undergoing division; d) proportion of cells with trypanosomes to cells with leishmaniae.

According to the forementioned criteria, a comparative study was made of the effects of primaquine, of carbidium sulphate and of the aminonucleoside of stylomycin, in various concentrations used for different periods of time. Degenerative changes of the intracellular leishmaniae were particularly intense in the cultures treated with the aminonucleoside of stylomycin. Carbidium sulphate showed the most durable chemotherapeutic action, with definite evidence of alterations in the intracellular multiplication of the parasite. Primaquine did not seem to have any action against the intracellular forms, appearing to be effective only against the free flagellates.

INTRODUCTION

Of many different drugs tested against *Trypanosoma cruzi*, those with the highest activity "in vitro" and in experimental infections were shown to be some of the quinoline derivatives (GOBLE [4]), phenanthridine compounds (BROWNING *et al.*[1]), and a purine antagonist, namely the aminonucleoside fraction of an antibiotic, stylomycin (FERNANDES & CASTELLANI [2]; FERNANDES, PEREIRA & SILVA [3]).

Primaquine, the most effective of different aminoquinoline compounds tested (PIZZI [10]), has shown great activity against the blood forms of the parasite "in vitro" (RUBIO & PIZZI [11]). According to PIZZI's [10] observations in experimental infestions, the drug has no action against intracellular forms, the author assuming that the drug does not reach an effective concentration in the tissues where the parasites are located.

Carbidium sulphate, the most effective of the phenanthridine derivatives (LOCK [7]), has shown activity against the blood forms of the trypanosome "in vitro" (GOODWIN

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SILVA, L. H. P. da & KIRCHNER, E. — Experimental chemotherapy of Trypanosoma cruzi infection in tissue culture: a comparative study on the action of primaquine, carbidium sulphate and the aminonucleoside of stylomycin. Rev. Inst. Med. trop. São Paulo 4:16-28, 1962.

et al.[5]) or in cultures of heart explants from rat embryos (LOCK [7]). According to the latter author, a certain proportion of the intracellular forms presented degenerative alterations although the drug, in concentrations non-toxic for the culture cells, was unable to interrupt the intracellular cycle of the parasite.

The aminonucleoside fraction of stylomycin, which, as shown by FERNANDES and collaborators [2, 3, 14], exhibits intense action against the parasite, is an analogue of deoxyadenosine, with the following composition: 6-dimethylamino-9-(3'-amino-3'-deoxy- -D-ribofuranosyl) purine. FERNANDES & CASTELLANI [2], have shown that in the cultural forms of *Trypanosoma cruzi* the aminonucleoside inhibits the incorporation of adenine, thus acting as a purine antagonist.

As discussed by SILVA *et al.*[14] the tissue culture technique offers many advantages for studies of metabolism of the *Trypanosoma cruzi* and chemotherapy of the infection by this flagellate: the possibility of ready microscopical observation of the extracellular and intracellular forms either in live cultures or in stained preparations; the absence of nervous connections and humoral factors and the possibility of adding the drugs to be tested in precise concentrations. Employing the Carrell hanging-drop technique with the modifications suggested by MEYER & OLIVEIRA [8] for maintenance of *Trypanosoma cruzi* in chick embryo tissue culture, SILVA *et al.*[14] have shown that the aminonucleoside of stylomycin is effective in altering the process of mitotic division of the intracellular forms of the parasite, ultimately leading to its degeneration.

The technique of cultivating tissue fragments, utilized by LOCK [7] in a study on the action of carbidium sulphate involves some inconveniences, such as: 1) the tissue fragments cannot be fully visualized under the microscope, which prevents the observation of parasites in the central mass of the explants; 2) the semisolid nutrient medium does not allow a uniform concentration of the tested drug throughout the culture; 3) it is very difficult to obtain homogeneous samples of the culture in order to count the extracellular parasites, since they are irregularly distributed in the semisolid nutrient medium; 4) the uncontrolled individual variation of the number of flagellates present in each culture prevents adequate quantitative comparison among the different groups; 5) each hanging-drop culture of fragments requires a series of delicate and time-consuming manipulations, which burdens the simultaneous utilization of an adequate number of treatment and control cultures.

In order to eliminate the forementioned inconveniences, in the present work we utilized trypsinized monkey heart cells in monolayer cultures, employing a monophasic liquid nutrient medium. We have made a comparative study of the action of primaquine, carbidium sulphate and the aminonucleoside of stylomycin on *Trypanosoma cruzi* infection. Besides the quantitative estimation of drug activity, it was our purpose to develop standardized techniques which could be used for tissue culture studies on the chemotherapy of *Trypanosoma cruzi* infection.

MATERIALS AND METHODS

Tissue culture

The monkey heart cells of the strain isolated in 1954 by SALK & WARD [12] and maintained in our laboratory for two years was employed. The cells were cultivated in stationary tubes incubated at 37°C, with nutrient medium consisting of 70% Hanks saline solution, 10% of a lactoalbumin enzymatic hydrolysate (2.5% solution of Difco TC in Hanks saline) and 20% bovine serum, the final pH being adjusted to 7.2-7.4 with N/1 NaOH. The nutrient medium was changed every 2 to 3 days and subcultures were made with trypsinized cells whenever the proliferation was too intense and incipient cellular degeneration was observed, which occurred every 2 or 3 weeks.

Infection of the cultures

We utilized the "Y" human strain of *Trypanosoma cruzi* (SILVA & NUSSENZWEIG [13]) maintained in our laboratory for eleven years by mouse passages repeated every 8 to 14 days. All tissue cultures were in-

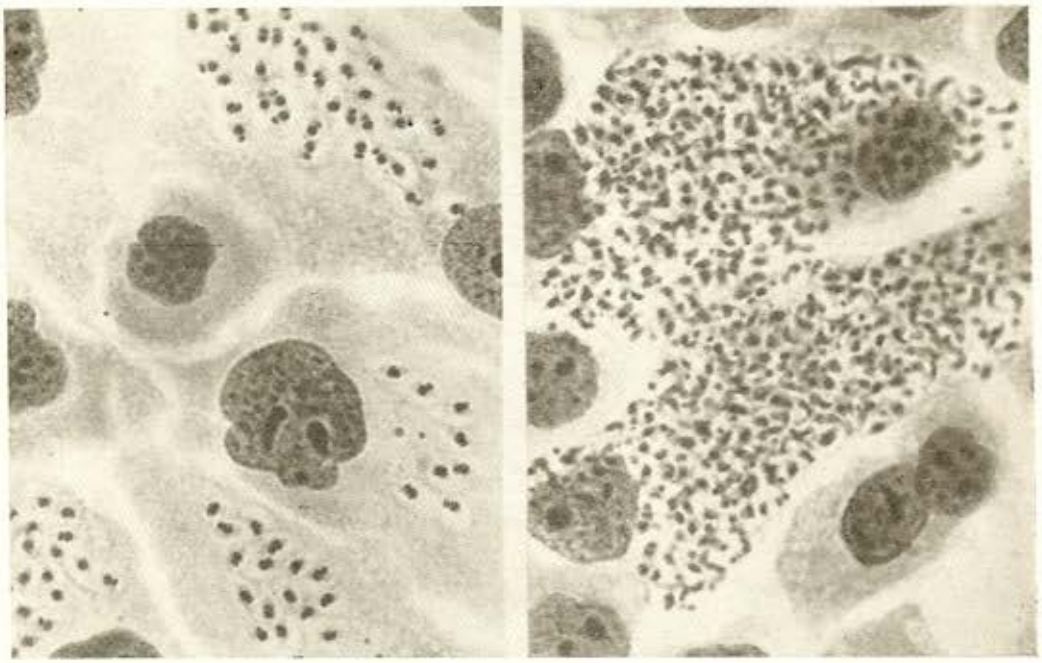
fected with cultural forms of the parasite on their 16th day of growth in MUNIZ & FREITAS [9] medium, where they were maintained for 3 passages after being isolated from mice.

For initial infection, about 3×10^6 flagellates were inoculated into each tube of tissue culture. Ten days later, abundant extracellular parasites as well as numerous intracellular leishmaniae, trypanosomes and intermediate forms could be observed.

which the flagellates were counted in Neubauer's hemocytometer under 400 × magnification.

Preparation of the cultures for the experiments on drug action

Tissue cultures from several tubes, infected or not, depending on the test group, were pooled and distributed into new tubes, all equipped with a flying 8 × 32 mm



Figs. 1 and 2 — Monkey heart tissue cultures infected winth *Trypanosoma cruzi* in control preparations, showing leishmaniae (Fig. 1) and trypanosomes (Fig. 2).

The same maintenance technique was used as for nonparasitized cells, except for the addition of centrifuged and washed uninfected cells to the infected ones, whenever subcultures were made; equal parts of both kinds of cells were mixed and the mixture distributed into twice as many tubes so as to maintain approximately the same number of cells in each culture.

At any desired moment, the number of extracellular parasites could be established with accuracy: the liquid nutrient medium was removed to a separate tube and, after homogenization, samples were taken in coverslip, on which the cell monolayer did also grow. After certain periods, the coverslips were replaced by new ones, and those with adhering cells were fixed and stained for microscopical examination.

The trypanosomes present in the nutrient medium of each infected culture tube were counted and those tubes with very discrepant numbers of flagellates were discarded. The cultures to be used were classified according to the observed numbers. These tubes were then distributed in equal sets and in such a manner that in each set the entire range of intensity of the infection was represented

and each had the same average number of trypanosomes per tube. These sets were distributed at random as control or experimental groups. For the determination of drug toxicity, non-infected cultures were employed.

Drugs used

The primaquine employed was obtained from the Winthrop Laboratories as tablets of "Neo-Quipenyl" (primaquine-diphosphate). Each tablet contained 26.3 mg of 8(4-amino-1-methylbuthylamino)-6-metoxiquinoline diphosphate, corresponding to 15 mg of primaquine base. The tablets were ground in Hanks saline solution, the resulting suspension being centrifuged for removal of the excipient. The carbidium sulphate, provided in crystalline form by the Wellcome Laboratories of Tropical Medicine, of London, was initially dissolved in distilled water whereas the aminonucleoside of stylomycin, supplied by the Lederle Lab. Division, American Cyanamid Co., was dissolved in Hanks saline solution. All solutions were sterilized by filtration in Seitz and further dilutions were prepared aseptically.

Before incubation with nutrient medium containing the drug to be tested, the tissue cultures were washed with Hanks saline solution containing the drug in the same concentration, in order to avoid a reduction in drug concentration due to residual nutrient medium in the culture tubes.

Criteria for the evaluation of drug action

a) *Toxicity for the culture cells*

During drug treatment, the cultures were incubated at 37°C and examined daily up to the 8th or 14th day. Cellular growth was observed in 6 to 10 culture tubes for each of various drug concentrations and the untreated control groups. This observation was complemented by examination of the fixed and stained material on the coverslips. The toxic action of the drugs was evaluated by the presence of morphological alterations indicating cellular degeneration.

b) *Chemotherapeutic action*

The cultures were daily observed and every 2 to 6 days samples of nutrient medium were taken, in which the free flagellates were counted as described above. In the course of the experiments, whenever flagellates could not be detected in the nutrient medium, periodical sampling of this medium was replaced by direct microscopical examination, under 400 × magnification, of the entire cellular film contained in the culture tubes, which was repeated every 24 to 48 hours.

The activity of the tested drugs against intracellular forms was estimated by periodical examination of fixed and stained coverslips. The fixation and staining techniques adopted were the following: the coverslip was aseptically removed from the culture tube, washed by immersion in Hanks saline solution, fixed in Bouin's solution, stained by Giemsa's (Merck) and mounted in synthetic resin.

In the microscopical examination of coverslips, the following elements were taken into account: a) frequency of parasitized cells; b) morphology of the parasites; c) frequency and aspect of dividing forms of the parasites; d) relative frequency of cells harboring trypanosome and leishmania forms.

The frequency of parasitized cells, observed under 400 × magnification, was expressed as follows:

+++ — Numerous parasitized cells in each microscopical field;

++ — A few parasitized cells present in almost all microscopical fields;

+ — Rare parasitized cells in the whole preparation.

In order to assess the relative frequency of trypanosome and leishmania-harboring cells, several microscopical fields were examined under 1000 × magnification until 150 leishmania-infected cells were found; the number of trypanosome-infected cells counted in the same fields was recorded.

RESULTS

Toxicity of the drugs for the culture cells

Primaquine was toxic in the concentration of 20 μg per ml of nutrient medium; in the concentration of 10 μg/ml it did not affect the cells even after a 14 day treatment for the cultures did not exhibit any degenerative alterations and presented the same rate of growth as the controls.

Due to the poor solubility of carbidium sulphate in the nutrient medium, the highest concentration used was 3 μg/ml, which did not show any evidence of toxicity for the culture cells.

The aminonucleoside of stylomycin could be safely employed up to the concentration of 9 μg per ml of nutrient medium.

Chemotherapeutic action of primaquine

Primaquine was used in the concentration of 10 μg per ml of nutrient medium for periods of 2, 4, 6 and 14 days. The numbers of free flagellates present in the nutrient medium during treatment and for periods up to 31 days after drug removal, are shown in Table I.

On the 2nd day of treatment the numbers of extracellular trypanosomes decreased in all experimental groups. However, when the drug was removed either on the 2nd or on the 4th day, those numbers quickly raised and became comparable to figures observed in the control groups. When the treatment lasted 6 days the numbers of trypanosomes decreased until the 14 th day, subsequently raising to the initial level. When the cultures were treated during 14 days, the flagellates disappeared from the nutrient medium by the 12th day, which, however, did not mean sterilization of the cultures, since trypanosomes were again observed on the 45 th day.

Table II summarizes the observation of intracellular forms of *Trypanosoma cruzi* in cultures submitted to treatment with primaquine and in the corresponding control cultures. The experimental cultures differed from the controls in the frequency of parasitized cells, which was somewhat lower in the latter cultures. Concerning the ratio of trypanosome-harboring cells to leishmania-harboring cells, cultures examined after after 2, 4, 6 and 8 days of treatment showed figures comparable to those observed in

TABLE I

Action of primaquine upon extracellular forms of *Trypanosoma cruzi* in tissue culture.

(Drug concentration: 10 μg per ml of nutrient medium).

Groups	Initial number of tubes *	Duration of treatment (days)	Average number of free flagellates per mm³ of nutrient medium — Days											
			0	2	4	6	8	10	12	14	18	22	28	45**
I Control	6	—	316	313	444	464	312	306	805	599	361	839	476	...
II Experimental	6	2	352	155	324	324	439	396	926	490	405	...	...	...
III Experimental	6	4	435	189	315	393	216	234	328	241	490	...	...	...
IV Experimental	6	6	330	210	239	224	123	68	55	30	99	179	333	...
V Experimental	6	14	393	189	298	157	27	2	—	—	—	—	—	+

* On the 12th day subcultures were made and the initial number of tubes in each group was duplicated.

** Between the 28th and the 45th day, the entire cellular film of the culture tubes was microscopically examined every 1-2 days and showed no free flagellates.

\+ Cultures showing a few flagellates.

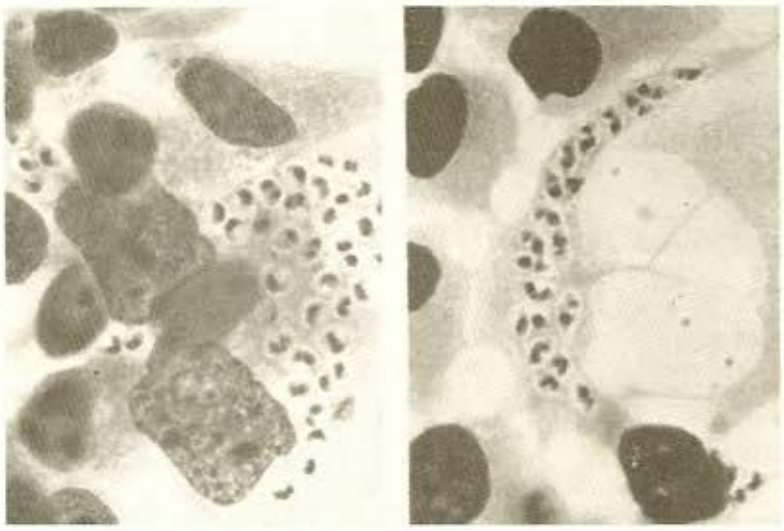
the controls, namely 15 to 34 per cent. After 14 days of treatment no intracellular parasites were found; the drug was removed and the cultures continued to be negative, as revealed by direct microscopical examination of the tubes, repeated every 24 to 48 hours. On the 45th day (Table II) a few parasitized cells were again observed in those cultures, and the forementioned ratio had reached the same level as in the control cultures.

TABLE II

Action of primaquine upon intracellular forms of *Trypanosoma cruzi* in tissue culture.

Cultures	No. of days from beginning of experiment to coverslip fixation	Number of coverslips	Frequency of parasitized cells	Average no. of trypanosome-harboring cells per 100 leishmania-harboring cells	Presence of vacuolated leishmaniae
Control	2	3	+++	19	—
Control	4	3	+++	22	—
Control	6	3	+++	13	—
Control	8	3	+++	17	—
Control	14	4	+++	16	—
Control	45	6	+++	18	—
Experimental ..	2	3	++	15	+
Experimental ..	4	3	+++	34	+
Experimental ..	6	2	++	27	+
Experimental ..	8	2	++	18	+
Experimental ..	14	2	—	—	—
Experimental ..	45 *	6	+	16	+

* Drug suppressed on the 14th day.
+++ Numerous parasitized cells in each microscopical field (400 ×).
++ A few parasitized cells present in almost all microscopical fields (400 ×).
\+ Rare parasitized cells in the whole preparation (400 ×).



Figs. 3 and 4 — Monkey heart tissue cultures infected with *Trypanosoma cruzi* and treated with primaquine, showing leishmaniae containing a large vacuole.

The presence of a large vacuole extending to about one third of the leishmaniae was observed in treated cultures (Figures 3 and 4). This aspect was never found in control preparations, but, on the other hand, it was neither constant in the treated cultures nor did it show any relation to the duration of treatment.

Chemotherapeutic action of carbidium sulphate

Carbidium sulphate was tested in the concentrations of 3 μg and 1.5 μg per ml of nutrient medium, during 15 and 21 days. The numbers of flagellates present in the nutrient medium during the treatment and for periods up to 61 days after drug removal, are shown in Table III.

centration of 1.5 μg/ml, became negative on the 17th day of treatment and positive again 11 days after drug removal. Used in the same concentration but during 21 days, the drug apparently sterilized the cultures by the 15th day, since up to the 61st day after treatment suppression, no flagellates could be detected. In the concentration of 3 μg/ml apparent sterilization also resulted, with 15 days as well as with 21 days of treatment.

Table IV summarizes the observation of intracellular forms of *Trypanosoma cruzi* in cultures submitted to treatment with carbidium sulphate and in the corresponding control cultures.

In the control group the frequency of parasitized cells was always high until the

TABLE III

Action of carbidium sulphate upon extracellular forms of *Trypanosoma cruzi* in tissue culture.

Groups	Drug concentration (μg/ml)	Initial number of tubes*	Duration of treatment (days)	Average number of free flagellates por mm³ of nutrient medium — Days											
				0	2	4	6	8	13	15	17	19	21	26	33	82**
I Control	—	6	—	376	318	1767	1433	875	654	621	2058	1471	1138	6166	2021	...
II Experimental	3	3	15	357	373	1400	650	167	—	—	—	—	—	—	—	—
III Experimental	3	3	21	373	427	1583	900	67	2	—	—	—	—	—	—	—
IV Experimental	1.5	3	15	343	543	1333	717	250	3	2	—	—	—	+	...	...
V Experimental	1.5	3	21	380	550	1067	800	167	14	—	—	—	—	—	—	—

* On the 13th day subcultures were made and the initial number of tubes in each group was duplicated.

** Between the 33rd and the 82nd day, the entire cellular film of the culture tubes was microscopically examined every 1-2 days and showed no free flagellates.

\+ Cultures showing a few flagellates.

The number of trypanosomes decreased between the 4th and the 6th day, falling sharply after the 8th day of treatment; experimental cultures became negative between the 13th and the 17th day, while controls, in spite of some fluctuation, always kept a high number of parasites.

The experimental group treated during 15 days with carbidium sulphate in the con-

45th day, and the ratio of cells parasitized by trypanosomes to cells parasitized by leishmaniae varied from 8 to 18 per cent.

On the 8th day the cultures treated with 3 μg/ml carbidium sulphate exhibited a lower proportion of trypanosome-harboring cells than the control cultures; 2 days later no intracellular trypanosomes were found any more, in spite of the fact that for the

TABLE IV

Action of carbidium sulphate upon intracellular formes of *Trypanosoma cruzi* in tissue culture.

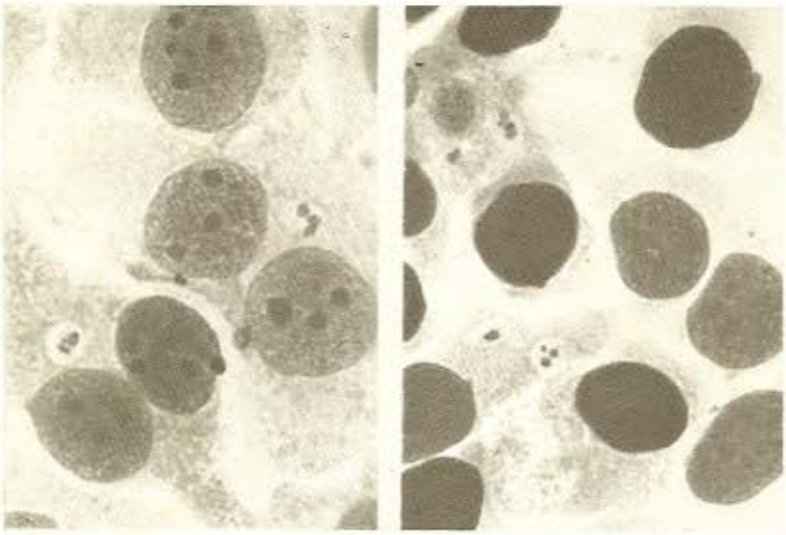
Cultures	No. of days from beginning of experiment to coverslip fixation	Number of coverslips	Frequency of parasitizid cells	Average no. of tryponosome-harboring cells per 100 leishmania-harboring cells	Presence of degenerative alterations in leishmaniae
Control	8	3	+++	12	—
Control	10	1	+++	8	—
Control	15	4	+++	16	—
Control	21	6	+++	18	—
Control	45	4	+++	16	—
Exp. 3 μg/ml	8	3	+++	4	+
Exp. 3 μg/ml	10	3	+++	—	+
Exp. 3 μg/ml	15	6	++	—	+
Exp. 3 μg/ml	21	3	+	—	+
Exp. 3 μg/ml	45 *	8	—	—	—
Exp. 1.5 μg/ml	8	3	+++	9	+
Exp. 1.5 μg/ml	10	3	+++	1	+
Exp. 1.5 μg/ml	15	3	++	—	+
Exp. 1.5 μg/ml	21	6	+	—	+
Exp. 1.5 μg/ml	45 *	4	—	—	—

* Drug supressed on the 21st day.
+++ Numerous parasitized cells in each microscopical field (400 ×).
++ A few parasitized cells present in almost all microscopical fields (400 ×).
\+ Rare parasitized cells in the whole preparation (400 ×).

subsequent 7 days abundant intracellular leishmania forms were still present. Similar findings resulted from the observation of cultures treated with the lower concentration of the drug.

In both experimental groups there were leishmaniae showing nuclear degenerative aspects of pyknosis, karyorrhexis and karyolysis (Figures 5 and 6), and sometimes mitotic alterations as suggested by a higher frequency of binucleated forms than observed in control cultures. Cells parasitized simultaneously by normal and altered leishmaniae could be found, as well as cells containing only normal leishmaniae. For comparison purposes, leishmania and trypanosome forms in control cultures are shown in Figures 1 and 2.

After 21 days of treatment with 3 μg/ml, as well as with 1.5 μg/ml, the frequency of parasitized cells was very low and the leshmaniae observed in those cells were always degenerated, in numbers never higher than 10 for each cell.



Figs. 5 and 6 — Monkey hearth tissue cultures infected with *Trypanosoma cruzi* and treated with carbidium sulphate, showing leishmaniae with nuclear degeneration.

Chemotherapeutic action of the aminonucleoside of stylomycin

The aminonucleoside of stylomycin was used in the concentration of 9 μg per ml of nutrient medium, during 4 or 8 days. The numbers of flagellates present in the nutrient medium during the period of the treatment and for periods up to 10 days after durg removal, are shown in Table V.

The number of extracellular flagellates, on the 4th day of treatment with the aminonucleoside of stylomycin, was reduced to one half, and on the 6th day the flagellates were not found in the nutrient medium.

In the concentration used, however, the drug did not eliminate the infection, since when it was discontinued flagellates reappeared in the nutrient medium after 4 days in

TABLE V

Action of aminonucleoside of stylomycin upon extracellular forms of *Trypanosoma cruzi* in tissue culture.

(Drug concentration: 9 μg per ml of nutrient medium).

Groups	Initial number of tubes	Duration of treatment (days)	Average number of free flagellates por mm³ of nutrient medium								
			Days								
			0	2	4	6	8	10	12	14	16
I Control	9	—	136	337	1212	1694	2678	2333	3170	...	...
II Experimental	3	4	126	120	57	—	7	11	30	135	...
III Experimental	6	8	118	110	42	—	—	—	—	—	+

\+ Cultures showing a few flagellates.

the group submitted to the shorter treatment and after 8 days in the other groups.

Table VI summarizes the observation of intracellular forms of *Trypanosoma cruzi* in cultures submitted to treatment with the aminonucleoside of stylomycin and in the corresponding control cultures. In the control group the frequency of parasitized cells was always high, and the ratio of cells parasitized by trypanosomes to cells parasitized by leishmaniae varied from 13 to 23 per cent. In the treated cultures, the intracellular parasites became scarce after the 4th day and consisted almost exclusively of leishmaniae, for the trypanosomes had practically disappeared.

DISCUSSION

In order to interpret the results obtained, it is important to bear in mind the life cycle of *Trypanosoma cruzi* in tissue cultures, a problem carefully studied by H. MEYER *et al.* in several papers (see MEYER & OLIVEIRA [8]). Those authors demonstrated that the parasite has an intracellular cycle of 4 to 5 days, multiplication occuring in the first 3 or 4 days, as leishmaniae; transformation of leishmaniform elements to trypanosomes and subsequent emergence from the host cells take about 12 to 24 hours.

On the basis of those findings, it is estimated that in a tissue culture that has been

TABLE VI

Action of the aminonucleoside of stylomicin upon intracellular forms of *Trypanosoma cruzi* in tissue culture.

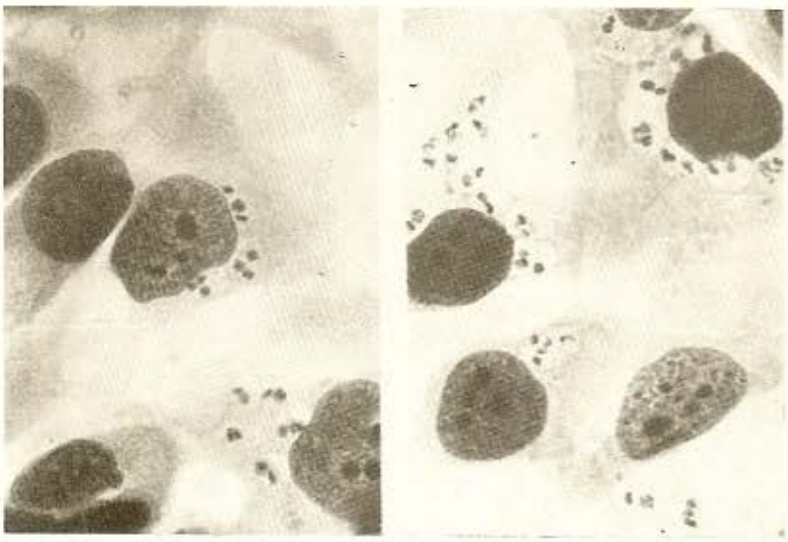
(Drug concentration: 9 μg per ml of nutrient medium).

Group	No. of days from beginning of experiment to coverslip fixation	Number of coverslips	Frequency of parasitized cells	Average no. of tryponosome-harboring cells per 100 leishmania-harboring cells	Presence of degenerative alterations in leishmaniae
Control	4	3	+++	23	—
Control	8	3	+++	13	—
Control	12	3	+++	19	—
Experimental ..	4	3	++	2	+
Experimental ..	8	3	+	—	+
Experimental ..	12 *	3	+	—	...

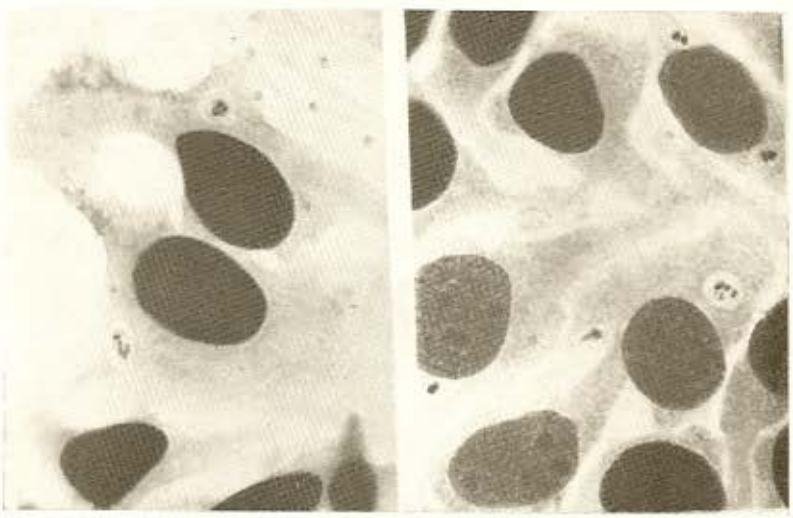
* Drug supressed on the 21st day.
+++ Numerous parasitized cells in each microscopical field (400 ×).
++ A few parasitized cells present in almost all microscopical fields (400 ×).
+ Rare parasitized cells in the whole preparation (400 ×).

Mitotic disturbances of the intracellular forms, as suggested by the presence of numerous binucleated leishmaniae, were very frequent. Degenerated leishmaniae, presenting alterations varying from pyknosis and karyolysis to complete disintegration of the parasites, were frequent since the 4th day of treatment (Figures 7 to 10).

infected with *Trypanosoma cruzi* for several days, at any given moment about 10 to 30 per cent of the parasitized cells are filled with trypanosome forms and 70 to 90 percent with leishmaniform elements. We have actually observed these proportions, which also correspond to the figures obtained in the control preparations of the pre-



Figs. 7 and 8 — Monkey heart tissue cultures infected with *Trypanosoma cruzi* and treated with the aminonucleoside of stylomycin, showing binucleated leishmaniae and leishmaniae with nuclear degeneration.



Figs. 9 and 10 — Monkey heart tissue cultures infected with *Trypanosoma cruzi* and treated with the aminonucleoside of stylomycin, showing completely lysed leishmaniae. In Fig. 9 a binucleated leishmania is also seen.

sent work (see Tables II, IV and VI). A decrease in the proportion of trypanosome-harboring cells would indicate some disturbance in the development of the leishmaniae, which are prevented from reaching the ultimate trypanosome stage. An increase in the relative proportion of cells containing trypanosomes, on the other hand, would suggest that penetration of flagellates in new cells was not occurring, while development of forms already present inside the cells was proceeding normally.

The results of countings of extracellular parasites in the nutrient medium must be adequately interpreted. Since this medium was renewed every 2 to 3 days, most of the

free flagellates present were then removed, and therefore the countings revealed mainly the trypanosomes produced in the preceding 48 to 72 hours. In the presence of a drug specifically effective against extracellular forms of the parasite, the number of flagellates in the liquid nutrient medium could remain practically unaltered for several days, progressively decreasing as the intracellular forms were exhausted. Were the drug effective against intracellular forms, the decrease in the number of free flagellates in the medium would be more precocious, and the cultures would sooner become negative.

Comparative studies of drug activity against *Trypanosoma cruzi* infection, according to the methods and criteria established in our experiments, which include the observation of degenerative alterations in the intracellular forms of the parasite, present advantages over studies utilizing experimentally infected animals, in which evaluating criteria such as parasitemia, survival time and histopathological alterations require a more complex interpretation. Obviously, experimental animal infection would be indispensable in a later phase, when therapeutical application is intended.

Of the three drugs tested, carbidium sulphate showed the most durable chemotherapeutic action, since trypanosomes disappeared from the cultures between the 13th and the 17th day of treatment and did not reappear during the whole period of observation, which was extended for 61 to 67 days after the drug was withdrawn. The microscopical examination of stained coverslips from cultures treated with carbidium sulphate revealed the gradual disappearance of intracellular trypanosomes, even when parasitism by leishmaniae was still relatively frequent, which indicated a disturbance in the development of the leishmaniae, preceding their degeneration.

Concerning the degenerative alterations observed in the intracellular forms of the parasite, although quite definite and frequent in the cultures treated with carbidium sulphate, they were considerably more intense in the cultures treated with aminonucleoside of stylomycin, which produced disturbances in the mitotic process and the same progressive degenerative changes described by SILVA *et al.*[14]. The latter drug also caused, after 6 days of treatment, a temporary disappearance of trypanosomes from the nutrient medium, parallel to the disturbance in the development of intracellular leishmaniae indicated by their almost exclusive presence in the infected cells. However, used in the concentration of 9 g/ml during 8 days, the drug did not suppress the infection and free flagellates reappeared in the nutrient medium on the 16th day.

The toxicity of the aminonucleoside, quite intense for the used strain of monkey heart cells, prevented us from increasing its concentration in the present experiments. In cultures of chick embryo heart cells infected with *Trypanosoma cruzi*, SILVA *et al.*[14] were able to use the drug in a concentration 10 times greater during 4 days. The higher toxicity of the aminonucleoside for cell strains such as the one presently employed could be foreseen in view of the known action against certain tumor cells of purine antagonists, especially the aminonucleoside of stylomycin (see HUTCHINGS[6]). The inconvenience, for studies of this kind, of cell strains susceptible to the toxic effect of purine antagonists might possibly be prevented by the use of trypsinized cells recently isolated from normal organs.

In what regards primaquine, our experiments did not supply data indicating an effect against the intracellular forms. Only vacuolation of parasitary elements in certain preparations was observed, but these alterations did not proceed as degenerative processes. Furthermore, the abundance of intracelluar parasitism and the relatively high proportion of trypanosome-harboring cells to leishmania-harboring cells, indicated that the drug activity was limited to the extracellular forms.

RESUMO

Quimioterapia experimental de infecção pelo Trypanosoma cruzi *em cultura de tecido. Estudo comparativo da ação de primaquina, sulfato de carbidium e do aminonucleosídio da estilomicina.*

Para estudos de quimioterapia experimental de infecção pelo *Trypanosoma cruzi*, em-

pregaram-se culturas de células tripsinizadas de coração de macaco em camada monocelular. Esta técnica apresenta vantagens para a avaliação da atividade de drogas por proporcionar meios que permitem acompanhar o desenvolvimento intracelular e extracelular dos parasitos nas culturas. Estabeleceram-se critérios para a avaliação quantitativa da ação de drogas, utilizando os seguintes elementos nos grupos experimentais e contrôle: 1) número de parasitos presentes no meio nutriente líquido; 2) características microscópicas de preparações fixadas e coradas de células parasitadas, a saber: a) abundância de parasitos na preparação; b) morfologia dos parasitos; c) freqüência e aspecto das formas em divisão; d) proporção de células com tripanosomas para células com leishmânias.

Segundo êsses critérios, fez-se um estudo comparativo dos efeitos da primaquina, do sulfato de carbidium e do aminonucleosídio da estilomicina, em concentrações diversas e por diferentes períodos de tempo. Alterações degenerativas de leishmânias foram particularmente intensas nas culturas tratadas com o aminonucleosídio da estilomicina. O sulfato de carbídium apresentou a ação quimioterápica mais duradoura, com evidências definidas de alterações na multiplicação intracelular do parasita. A primaquina não demonstrou ter qualquer ação sôbre as formas intracelulares do parasito, parecendo eficaz apenas contra os flagelados livres.

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