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THERAPEUTIC ACTIVITY AND CRITERION OF CURE ON MICE EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA CRUZI*

Z. BRENER

SUMMARY

Observations performed through four years of successive passages in albino mice, using the "Y" strain of *Trypanosoma cruzi* confirm the possibility of maintaining the parasite virulence at a quite constant level, by adjusting the number of trypanosomes to the animals weight.

In experimental Chagas' disease, mortality and parasitemia are the best initial criteria for therapeutic activity. However, in treated animals with repeatedly negative fresh blood examination, other laboratory methods should be used to establish a dependable criterion of apparent cure. Among the methods used, subinoculation and xenodiagnosis appear the most appropriate. Reinoculation is very helpful, since the persistence of Chagas infection produces strong immunity to superinfections.

INTRODUCTION

The assessment, by blood examination, of therapeutic activity and of cure in experimental Chagas' disease is sometimes greatly handicapped, since the acute stage of the infection may be followed by a chronic phase in which parasites are reduced to submicroscopical levels. Negative blood examination, even when repeated, is not then a reliable sign of parasitological cure and indirect laboratory methods should be used to be sure that eradication has actually occurred.

This paper reports the experiments made to select the most dependable methods of assessment of cure and gives a description of the main techniques used.

MATERIAL AND METHODS

Importance of quantitative transmission of T. cruzi

Our four-year study of mice inoculated with the "Y" strain of *T. cruzi* led us to realize the possibility of maintaining the parasite virulence at a quite constant level, by adjusting the number of trypanosomes to the animals weight. This, however, will only be possible when experimental conditions are standardized so as to provide definite quantitative data (PIZZI [5]; PHILLIPS [4]). The number of parasites not being accurately estimated, the possibility of progressive deterioration of the strain patogenicity will arise (PHILLIPS [4]).

Counting of trypanosomes

A method based on Pizzi's technique [5] for counting trypanosomes was used. Five cubic millimeters of fresh blood taken from the mouse's tail, through a hemoglobin pipette, were compressed between a slide and a 22×22 mm cover-slip thus forming a single layer of blood cells. The number of microspical fields of the cover-lip had been previously estimated (under a 45× objective and a 10× ocular) as 3,500. The number of trypanosomes in the 5 mm^3 of blood was determined by scoring the parasites observed into 50 or 100 microscopical fields and, then, multiplying that number by 70 or 35, according to the case.

Instituto Nacional de Endemias Rurais — Centro de Pesquisas de Belo Horizonte.

Course of infection

Previous experiments demonstrated that mice weighing 18-20 grams and inoculated, by intraperitoneal route, with 50,000-100,000 trypanosomes, present quite homogeneous infection. Daily trypanosome counts provided the following pattern for the parasitemia: parasites appear from the 4th or 5th after inoculation, their number is markedly decreased on the 6th day, increases up to the 7th or 8th day, finally decreasing again around the 9th day. From the 10th on, the pattern of parasitemia is quite irregular (Fig. 1). These data are greatly helpful not only in the choice of the best time for the strain passage but also in the assessment of therapeutic activity.

Most infected animals die in the period from the 5th to the 20th day after inoculation, the highest mortality rates being observed around the 15th day (Fig. 2). Males die earlier than females but the general mortality rates are about the same with regard to both sexes and only a small number of infected animals will outlive 40 days. These characteristics related to mortality

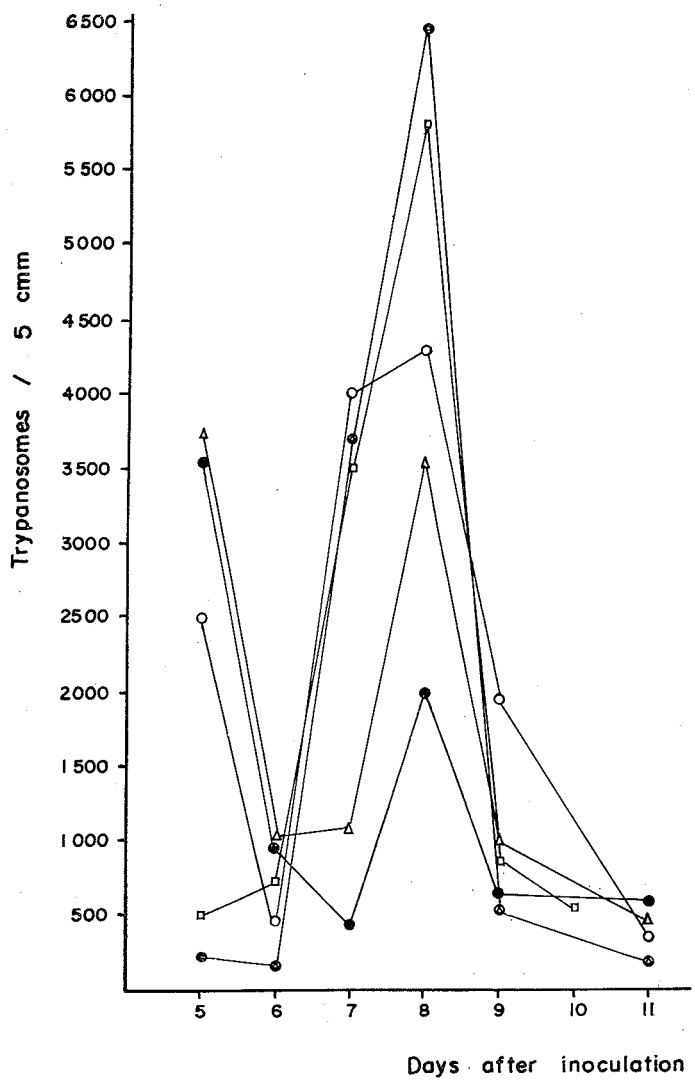

Fig. 1 — Pattern of parasitemia in 5 mice inoculated intraperitoneally with 75,000 trypanosomes ("Y" strain).

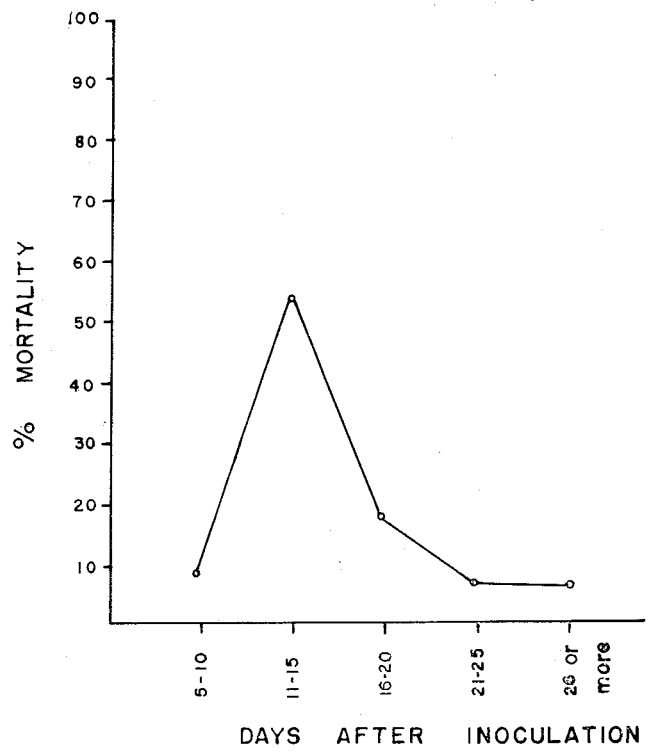

Fig. 2 — Mortality among 300 mice inoculated intraperitoneally with about 50,000 trypanosomes ("Y" strain).

have remained relatively stable for over 4 years of successive transfers in mice. Temporary exacerbation of decrease in virulence were controlled by adequate modifications of the number of trypanosomes and weight of the animals.

Experimental therapeutics

Drug activity is sooner and much more easily detected when it is administered either on the day of inoculation or on the day following it. For screening purposes, mice weighing 18-20 grammes are inoculated with 50,000-100,00 blood forms, 5 animals being used for each drug. The administration of drugs begins on the day after inoculation and doses corresponding to about 1/5 of the LD_{50} are given for 10 consecutive days. On the 5th day after inoculation the number of trypanosomes in 5 mm^3 of blood is determined as described above. On the 8th day, when the number of parasites in the inoculated animals is generally higher, a new counting is performed. Comparison of the data thus obtained with those from the controls is generally quite sufficient for a good evaluation of the drug activity. Daily records of the mortality rates must be kept so that a clear picture of such activity may be provided.

Criteria of cure

Although the assessment of drug activity based on the reduction of parasitemia and of mortality can be easily done, the true interpretation of repeatedly negative blood examination from treated animals is, however, rather difficult, since we are never sure whether eradication has actually occurred or whether we are dealing with a chronic non-patent infection. The following techniques were then used to establish a dependable criterion of cure in experimental Chagas' disease:

Fresh blood examination: a drop of blood from the mouse's tail was carefully examined between slide and cover-slip under 450× magnification. At least 100 microscopical fields were examined once or twice a day, daily or every other day.

Blood sub-inoculation: mice were killed about 1 or 2 months after treatment and 0.4-0.6 ml of citrated blood, collected from the severed axillary artery, was inoculated, intraperitoneally, in two young mice. From the 5th day of inoculation on, fresh blood

TABLE 1

Results of sub-inoculation in animals treated with different drugs and presenting repeatedly negative fresh blood examination.

Nº	Treatment	Negative blood examination (nº of days)	Sub-inoculation	Pre-patent period in the sub-inoculated animals (days)
1	Carbidium sulphate, 15 mg/kg, 10×	20	Positive	11
2	Carbidium sulphate, 15 mg/kg, 10×	18	Positive	11
3	Carbidium sulphate, 15 mg/kg, 10×	25	Positive	5
4	Carbidium sulphate, 15 mg/kg, 10×	25	Positive	9
5	Carbidium sulphate, 15 mg/kg, 10×	25	Positive	9
6	3024, 1 mg/20 g, 10×	40	Positive	10
7	3024, 1 mg/20 g, 10×	40	Positive	12
8	3024, 1 mg/20 g, 10×	40	Positive	12
9	3024, 1 mg/20 g, 10×	40	Positive	12
10	3024, 1 mg/20 g, 10×	31	Positive	11
11	Nitrofurazone, 100 mg/kg, 20×	30	Positive	10
12	Nitrofurazone, 100 mg/kg, 20×	30	Positive	10
13	Nitrofurazone, 100 mg/kg, 20×	40	Positive	10
14	Nitrofurazone, 100 mg/kg, 20×	60	Positive	11
15	Nitrofurazone, 100 mg/kg, 20×	90	Positive	10
16	Nitrofurazone, 100 mg/kg, 20×	90	Positive	12
17	Nitrofurazone, 100 mg/kg, 50×	105	Positive	14

examination was performed once or twice a day, daily or every other day, for a period of 6 weeks at least.

Blood culture: blood from treated animals was inoculated into Noeller's culture medium and the culture was frequently examined for, at least, 30 days after inoculation. Tubes with Noeller's medium inoculated with blood from positive untreated animals were also used in each experiment.

Xenodiagnosis: one or two months after treatment, the mice were anesthetized and 4 triatomine nymphae were allowed to feed on them until they were dead from acute anemia. After 45 to 50 days, the bugs were carefully examined for trypanosomes.

Histological examination: histological sections of the heart of treated animals were stained with hematoxylin-eosin and carefully examined for leishmanial forms.

Re-inoculation: some of the treated animals were re-inoculated, at different periods after treatment, with about 4,000 blood parasitic forms per gram of weight; daily counts of trypanosomes were performed so that a new acute phase of the disease might be detected.

TABLE 2

Comparative study of sub-inoculation, culture and search for leishmania in myocardium, in mice treated with different drugs and presenting repeatedly negative fresh blood examination.

Nº	Treatment	Sub-inoculation	Culture	Parasites in cardiac tissue
1	Carbidium sulphate, 15 mg/kg, 10× ..	Positive	Negative	Negative
2	Carbidium sulphate, 15 mg/kg, 10× ..	Positive	Negative	Negative
3	Carbidium sulphate, 15 mg/kg, 10× ..	Positive	Positive	Negative
4	Carbidium sulphate, 15 mg/kg, 10× ..	Positive	Positive	Negative
5	3024, 1 mg/20 g, 10×	Positive	Positive	Negative
6	3024, 1 mg/20 g, 10×	Positive	Positive	Negative
7	3024, 1 mg/20 g, 10×	Positive	Positive	Negative
8	3024, 1 mg/20 g, 10×	Positive	Negative	Negative
9	3024, 1 mg/20 g, 10×	Positive	Negative	Negative
10	3024, 1 mg/20 g, 10×	Positive	Negative	Negative
11	Nitrofurazone, 100 mg/kg, 20×	Negative	Negative	Negative
12	Nitrofurazone, 100 mg/kg, 20×	Negative	Negative	Negative
13	Nitrofurazone, 100 mg/kg, 20×	Negative	Negative	Negative
14	Nitrofurazone, 100 mg/kg, 20×	Positive	Positive	Negative
15	Nitrofurazone, 100 mg/kg, 20×	Positive	Negative	Negative
16	Nitrofurazone, 100 mg/kg, 20×	Positive	Negative	Negative
17	Nitrofurazone, 100 mg/kg, 20×	Negative	Negative	Negative
18	Nitrofurazone, 100 mg/kg, 20×	Negative	Negative	Negative
19	Nitrofurazone, 100 mg/kg, 20×	Negative	Negative	Negative
20	Nitrofurazone, 100 mg/kg, 20×	Positive	Positive	Positive
21	Nitrofurazone, 100 mg/kg, 20×	Positive	Positive	Negative
22	Furazolidone, 100 mg/kg, 50×	Negative	Negative	Negative
23	Furazolidone, 100 mg/kg, 50×	Positive	Positive	Positive
24	Furazolidone, 100 mg/kg, 50×	Positive	Positive	Negative
25	Furazolidone, 100 mg/kg, 50×	Negative	Negative	Negative
26	Furazolidone, 100 mg/kg, 50×	Negative	Negative	Negative
27	Nitrofurazone, 100 mg/kg, 20×	Negative	Negative	Negative
28	Nitrofurazone, 100 mg/kg, 20×	Positive	Positive	Positive
29	Nitrofurazone, 100 mg/kg, 20×	Negative	Negative	Negative
30	Nitrofurazone, 100 mg/kg, 20×	Positive	Positive	Negative
31	Nitrofurazone, 100 mg/kg, 20×	Positive	Negative	Negative
32	Nitrofurazone, 100 mg/kg, 20×	Positive	Positive	Negative
33	Nitrofurazone, 100 mg/kg, 20×	Negative	Negative	Negative

RESULTS

Table I shows that, in mice treated by different schedules, it was possible to detect active infection through sub-inoculation, even when the result of fresh blood examination had been steadily negative for many weeks.

A comparative study of sub-inoculation, culture and histological examination was carried out on unselected mice treated by different schedules (Table II). For this purpose the mice were killed and their blood inoculated in two young mice and also into, at least, two cultures tubes. Sub-inoculation was positive in 63.6% of the trated mice, while blood culture was positive in only 39.4% of them, the difference between the two methods being, then, statistically significant ($P<0.05$). Histological examination provided very poor results.

Table III shows the results from a comparative study between xenodiagnosis and sub-inoculation carried out on two groups of, respetively, 32 and 57 unselected animals treated by different schedules. Both methods were found to give apparently similar results.

(BRENER [1]). Table IV shows that, at least in mice treated with nitrofurazone from the day after inoculation on, challenging infection with 4,000 trypanosomes per gram of weight gave origin to parasitemias following the acute phase pattern. Since mice in the chronic non-patent stage of the disease are immune to new infection, the absence of immunity in the challenged mice is a quite reliable sign of parasitological cure. In mice treated from the 1st and 5th day after inoculation with carbidium sulphate and even so not parasitologically cured, the challenging infection was not followed by high parasitemia which demonstrates strong immunity and active chronic infection (BRENER [3]).

DISCUSSION

Although repeated blood examinations may detect some cases of chronic experimental Chagas' disease, the constant absence of parasites is not, on the other hand, a reliable sign of eradication of the disease. In general, when fresh blood of treated animals is not found positive for trypano-

TABLE 3

Comparative study between xenodiagnosis and sub-inoculation as methods of laboratory control of cure in treated mice.

Treatment	Xenodiagnosis		Sub-inoculation	
	Nº	Positive	Nº	Positive
Nitrofurazone, 100 mg/kg, 50×	20	1	45	2
Furazolidone, 100 mg/kg, 50×	5	0	5	2
Nitrofurazone, 100 mg/kg, 20×	7	3	7	4
Total	32	4 (12.5%)	57	8 (14.0%)

Mice treated with 50 consecutive doses of nitrofurazone from the day after inoculation on, had consistently negative blood examination and many other signs, including negative xenodiagnosis and sub-inoculation, strongly suggest the cure of the animals

somes in the first thirty days after administration of a suppressive drug, it will very seldom be positive in subsequent examination. The existence of actual parasitism, in such cases, however, can be demonstrated by xenodiagnosis and sub-inoculation, as

TABLE 4

Value of reinoculation as criterion of cure: results of reinoculation in animals treated with carbidium (15 mg/kg, 10×) starting on the day after inoculation and the 5th day of infection, and in animals treated with nitrofurazone (100 mg/kg, 53×) starting on the day of inoculation. In the animals treated with carbidium and presenting positive fresh blood examination there was no acute phase after reinoculation. The data represent number of trypanosomes in 5 mm³ of blood.

Nº	Treatment	Days after reinoculation									Days after inoculation							
		5	6	8	10	12	15	20	25	30	4	5	6	7	8	9	15	20
1	Carbidium sulphate	—	—	—	—	—	—	—	70	—	—	—	—	70	—	—	—	70
2	Carbidium sulphate	—	—	—	—	—	—	—	70	140	—	—	—	—	—	70	70	—
3	Carbidium sulphate	—	—	—	—	—	—	—	—	70	—	70	70	—	—	—	—	—
4	Carbidium sulphate	1540	1190	1820	70	—	—	—	—	—	—	70	—	—	—	—	—	—
5	Carbidium sulphate	8540	980	1680	70	—	—	—	—	—	70	—	70	—	—	—	—	—
6	Carbidium sulphate	4480	3080	2310	140	140	—	70	—	—	—	—	—	—	—	—	—	—
7	Carbidium sulphate	5600	3010	2100	420	—	—	—	140	—	—	—	—	—	—	70	—	—
8	Nitrofurazone	—	—	—	—	—	—	—	—	—	420	3990	140	910	1610	350	...	...
9	Nitrofurazone	—	—	—	—	—	—	—	—	—	210	1540	140	280	560	70	...	...
10	Nitrofurazone	—	—	—	—	—	—	—	—	—	1190	3920	210	490	6160	2800	...	...
11	Nitrofurazone	—	—	—	—	—	—	—	—	—	210	3510	770	140	490	140	...	...
12	Nitrofurazone	—	—	—	—	—	—	—	—	—	840	4900	210	4270	10190	980	...	...

mentioned above. The method to be followed will depend on the practical conditions of the laboratory where the experiments are to be performed.

Considering the good results obtained by sub-inoculation, we decided to find out whether the negative recipients that had received the sub-inoculated blood were really free from parasites or whether they had very slight parasitemia, not detectable by microscopical examination. For this purpose some of those mice were inoculated with 4,000 blood trypanosomes per gram of weight and soon presented high parasitemia, which indicated absence of immunity and existence of active chronic infection.

It should be pointed out that mice treated, from the 5th day of inoculation, with the long term schedule of nitrofurazone, presented very low parasitemia when challenging infections were performed 1 and 3 months after treatment. Challenging infections carried out 5 and 7 months after treatment were, however, followed by higher parasitemia, which indicates slow progressive decrease of the acquired immunity (BRENER [1]). These basic facts reported above should be taken into consideration whenever the assessment of cure by re-inoculation is intended.

RESUMO

Atividade terapêutica e critério de cura no camundongo experimentalmente infectado com Trypanosoma cruzi.

Observações realizadas através de 4 anos de passagens sucessivas em camundongos albinos, empregando a amostra "Y" do *Trypanosoma cruzi*, confirmaram a possibilidade de estabilização da virulência do parasito através do ajuste entre o número de tripanosomas do inóculo e o pêso do animal inoculado.

Em experiências de terapêutica, a mortalidade e a parasitemia representam os melhores critérios de atividade de drogas. Entretanto, em animais tratados e com exame de sangue a fresco repetidamente negativos outros métodos de laboratório necessitam ser usados para a evidenciação da presença de tripanosomas. Entre os métodos empregados, a subinoculação e o xenodiagnóstico foram os mais eficazes. A cultura deixou de revelar inúmeros casos de infecção crônica e o exame histopatológico do coração e outros órgãos revelou-se o mais precário dos métodos. A reinoculação fornece subsídios importantes já que a persistência da infecção chagásica condiciona o aparecimento de forte imunidade a superinfecções.

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