Rev. Inst. Med. trop. São Paulo
18(6):450-455, novembro/dezembro, 1976

UDC 593.161.13

DIFFERENCES IN THE SUSCEPTIBILITY OF *TRYPANOSOMA CRUZI* STRAINS TO ACTIVE CHEMOTHERAPEUTIC AGENTS

Z. BRENER, C. A. G. COSTA and C. CHIARI (1)

SUMMARY

In order to investigate differences in the *T. cruzi* susceptibility to active chemotherapeutic agents, groups of mice were inoculated with four *T. cruzi* strains and then treated with two nitrofuran and one nitro-imidazol derivatives. The criterion of cure was based on repeated fresh blood examination, hemocultures, immunosuppression of treated mice with gamma-rays, reinoculation and immunofluorescence tests. Evidence is being provided of marked differences in the susceptibility of several strains to active drugs. These differences have been detected with the three compounds and seem therefore to be related to biological characteristics of the strains rather than to the specific mode of action of the drugs.

INTRODUCTION

Animals inoculated with *T. cruzi* can be parasitologically cured through the administration of active compounds, especially nitrofurans, according to long-term schedules of treatment (BRENER [1]). Following this demonstration, a number of clinical papers from Chile and Argentina have reported presumable cures in humans with chronic or acute disease treated with nitrofurans [8, 15]. Those data, however, have not been confirmed in Brazil, where most patients still presented positive xenodiagnosis and serological tests after similar treatment [7, 13]. As differences in the biological behaviour of *T. cruzi* populations kept in the laboratory are so often detected, we decided to study the curative activity of some active compounds in groups of mice experimentally infected with different *T. cruzi* strains, to determine to what extent intra-specific variations may help to explain the reported discrepancies.

MATERIAL AND METHODS

T. cruzi strains — *CL* and *FL*, isolated from naturally infected *Triatoma infestans* collected in Rio Grande do Sul, South Brazil (BRENER [4]); *Y*, isolated from an acute human case (SILVA & NUSSENZWEIG [16]); *Berenice*, isolated from a chronic human case considered as the first patient described by Chagas (SALGADO et al. [14]). All strains have been maintained in mice, by repeated blood passages performed every 7 days for Y and *Berenice*, and every 8-10 for *CL* and *FL*.

Inoculation of animals — Male albino mice weighing 18-20 grams were inoculated by intraperitoneal route with 150,000 bloodstream forms. The method of collecting infected blood and estimating the number of parasites has been previously described (BRENER [2]).

Drugs used — a) 5-nitro-2-furaldehyde-semicarbazone (nitrofurazone); b) 3-methyl -4(5'-nitrofurfurylidene-amino)-tetrahydro-4 H-1,4-thiazine-1,1-dioxide (nifurtimox); c) N-benzyl -2-nitro-1-imidazoleacetamide (Ro 7-1051).

Schedules of treatment — The following groups of animals were submitted to treat-

Supported by the National Research Council, Brasil

(1) Departamento de Parasitologia, I.C.B., Universidade de Minas Gerais and Instituto Nacional de Endemias Rurais. 30.000 Belo Horizonte, Brasil

ment: a) 54 mice inoculated with *CL* strain and 48 inoculated with *Y* strain were treated with single daily doses of 100 mg/kg of nitrofurazone, p.o., for 25 consecutive days. b) 34 mice inoculated with *CL* strain, 33 inoculated with *Y*, 58 inoculated with *FL* and 60 inoculated with *Berenice*, were treated with nifurtimox, 100 mg/kg, p.o., for 20 days. c) 35 mice inoculated with *CL* strain and 30 inoculated with *Y* strain were treated with the 2-nitroimidazol compound, 100 mg/kg, p.o., for 20 days.

In all experiments, treatment started on the day after inoculation. In each group of inoculated animals 10 additional mice were taken as untreated controls and their infection confirmed by fresh blood examination on the 5th day after inoculation.

Assessment of drug activity

Fresh blood examinations — In all treated mice tail blood was microscopically examined for living flagellates over a period of 20-30 days beginning 10 to 15 days after treatment; animals showing at least 12 consecutive negative examinations, were submitted to hemoculture.

Hemocultures — 0.6-0.8 ml of citrated blood from the mice severed axillary vessels were inoculated into two tubes with "LIT" ("liver-infusion tryptose") liquid medium (CAMARGO [6]). The cultures were then kept at 28°C and examined for living flagellates at 30,40 and 50 days after blood inoculation. Hemocultures have been performed in 145 animals which had been treated according to the different schedules described above and presented negative fresh blood examinations. In each experiment we included as controls, hemocultures from mice showing, by fresh blood examination, persistence of bloodstream trypomastigotes after treatment. A total of 28 post-treated positive animals have been studied in this way. As controls also, another group of mice inoculated with *CL* strain and treated with nitrofurazone for 10 consecutive days was maintained in the laboratory for 6 months; hemocultures were performed in 9 of these animals which presented at least 10 repeated negative fresh blood examinations.

Immunosuppression by X-ray — In 15 animals of the group of mice inoculated with *CL* strain and treated with nitrofurazone, which presented persistent negative fresh blood examinations, the following procedure was used: the animals were irradiated with 560r from a RT Mueller -250 apparatus, which gives an average of 72 roentgen (r) per minute (250 kv, 15m A). A new series of 12 fresh blood examinations beginning 10 days after irradiation, and subsequent hemocultures have been performed in these animals. As controls, 4 animals treated with nitrofurazone in the same way, presenting bloodstream parasites after treatment were submitted to the same described sequence.

Reinoculation — 10 mice inoculated with "Berenice" strain and treated for 20 days with 100 mg/kg of nifurtimox (which provided with this strain a high percentage of cures as evaluated by fresh blood examinations and hemocultures; see *Results*), and presenting 15 negative fresh blood examinations after treatment, were reinoculated with 4,000 bloodstream trypomastigotes of the homologous strain per gramme of weight. A group of 10 normal mice of the same weight as well as a group of 4 mice treated according to the schedule above but showing persistence of infection, were reinoculated with the same inocula.

Immunofluorescent indirect test — This test was carried out with the sera of two groups of mice: a) 10 mice inoculated with *Berenice* strain, 6 with *CL* and 3 with *Y*, treated with one of the nitrofuran compounds (nifurtimox) and showing persistent negative fresh blood examinations after treatment; b) 15 mice inoculated with *Y* strain and 3 with *CL*, all of them having been treated but still presenting positive fresh blood examinations. In this test, *T. cruzi* culture forms and mouse fluorescein-labelled antiglobulin were used, and the reactions evaluated on a Zeiss — GF 25 ultra-violet lamp. The technique of this reaction has been described by CHIARI et al. [9].

RESULTS

Table I shows the results obtained in the different experiments with the three active compounds and groups of animals inoculated with the several strains. Marked differences

TABLE I

Percentage of cures in groups of mice inoculated with different *T. cruzi* strains and treated with active chemotherapeutic agents

Strains	Drug	Dose	No. treated/ no. cured mice	% cure
CL	Nitrofurazone	100 mg/kg, 25 x	54/32	59.2
Y	Nitrofurazone	100 mg/kg, 25 x	48/0	0.0
Berenice	Nifurtimox	100 mg/kg, 20 x	60/53	88.3
CL	Nifurtimox	100 mg/kg, 20 x	34/26	76.4
Y	Nifurtimox	100 mg/kg, 20 x	33/6	18.1
FL	Nifurtimox	100 mg/kg, 20 x	58/13	22.4
CL	2-nitroimidazol	100 mg/kg, 20 x	35/12	34.3
Y	2-nitroimidazol	100 mg/kg, 20 x	33/3	9.0

in the percentages of cure could be observed; moreover, with all three active drugs, the rates of cure in the groups of animals inoculated with *Y* strain were seen to be consistently lower than those in the group inoculated with *CL* strain. Even in the group of the *CL*-inoculated animals which had been treated with nifurtimox and submitted to gamma irradiation thereafter, no parasites could be detected either by repeated fresh blood examinations or by hemocultures performed at the end of the experiment. Two out of the 4 positive controls developed acute phases after irradiation and all four animals gave positive hemocultures, showing that X-irradiation enhances the course of infection and does not affect the parasite growth in culture.

Table II demonstrates the reliability of hemoculture method for detecting persistence of infection in groups of treated animals. Parasites were detected in all the 28 animals showing trypomastigotes in their bloodstream after treatment, and the 9 treated according to a non-curative schedule and which had undergone a long period of sub-patent parasitemia.

Figure 1 shows the parasitemia curves in the groups of animals inoculated with "Berenice" strain, treated with nifurtimox, and presenting repeated negative fresh blood examinations as well as in the normal controls. A typical acute phase, quite similar to that of the controls was observed in the treated group after the challenge infection, strongly suggesting that the original parasitism had been eradicated. In the group of four animals showing persistence of infection, no acute phase was detected after reinoculation.

Figure 2 shows the results obtained with the immunofluorescent test in the two groups of treated mice presenting, respectively, negative and positive fresh blood examina-

TABLE II

Results of hemocultures in groups of mice inoculated with *T. cruzi* and treated with active drugs according to non-curative schedules

No. animals	Fresh blood examination	Positive hemocultures
28 (*)	+	28
9 (**)	—	9

(*) Treated during 25 — 30 days with a nitrofuran compound and presenting positive fresh blood examination after treatment.

(**) Treated for 10 days, kept for 6 months in the laboratory and presenting at least 10 repeated negative fresh blood examination.

tions after treatment. Among the 19 mice with persistent negative fresh blood examinations, 8 presented negative IMF tests, 5 had an 1:5 positive test, 5 showed positivity at 1:10 and only one animal provided a 1:20 positive test. In the group presenting bloodstream forms after treatment not a single negative IMF test could be observed and most sera showed positive reactions in high dilutions.

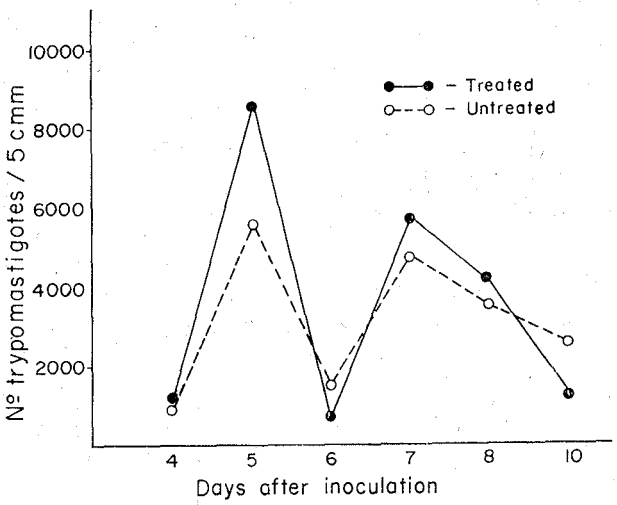

Fig. 1 — Curves of parasitemia in animals inoculated with "Berenice" strain, treated with nifurtimox and reinoculated with the homologous strain as well as in untreated control animals.

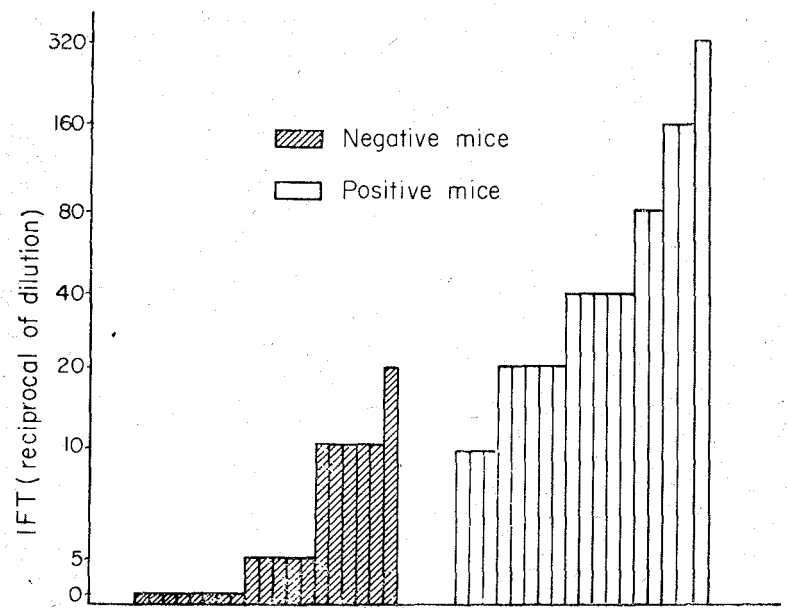

Fig. 2 — Results of immunofluorescent tests in groups of treated animals with positive and persistently negative fresh blood examinations.

DISCUSSION

Comparative studies on the sensitivity of *T. cruzi* strains so far have not been extensively performed. A limiting factor to those studies is the difficulty of stablishing reliable endpoints with animals inoculated with strains often markedly different in their biological characteristics such as parasitemia curves and death rates (BRENER [5]). Such studies should use, therefore, parasitological cures as evaluation criteria instead of elusive suppressive activity. Administration of active compounds, however, often gives origin to sub-patent infections, with bloodstream forms hardly detectable in the treated animals; it is then necessary in these experiments, to distinguish between actual parasitological cures and chronic sub-patent infections resulting from treatment failures. Fresh blood examinations are usefull, chiefly when strains extremelly well adapted to the vertebrate hosts are used. In this case the few remaining parasites which survive treatment are apparently enough to build up a population liable to be detected by fresh examination. Nevertheless, to be sure that parasitological cures are really occurring, animals with persistent negative fresh examinations should be submitted to several laboratory procedures usually employed to uncover sub-microscopic parasitemias. Hemoculture in "LIT" medium has been selected in our experiments for this purpose and has proved to be a sensitive and reliable method, since it provided positive results in all treated but non-cured animals used as controls. This is in agreement with the data of NEAL [12], who demonstrated that as few as 10 trypomastigotes are enough to initiate a culture, whereas more than 600 forms are necessary to provide positive xenodiagnosis.

Besides those direct parasitological methods, the development of acute phase in animals submitted to a treatment and challenged with bloodstream forms from the homologous strain is a further evidence of cure, since it is well known that animals with chronic infection develop a strong acquired resistance against challenge infections [2]. The occurrence of negative or low-titre positive immunofluorescent tests in treated animals presenting repeated negative fresh blood examination is also suggestive of parasitological cure. The general concordance of results with the several methods and the steady negative fresh blood examinations and hemocultures of treated animals even after the use of an active immunosuppressive agent as gamma rays which often induces acute phases in chronically *T. cruzi* infected animals (BRENER & CHIARI [5]) strongly suggests that a reliable criteria of cure has been attained in the present experiments.

No reasonable explanation for the different rates of cure has so far been provided. There seems to be no apparent relationship between any biological characteristics of the strains in the vertebrate and the reported differences in drug sensitivity. Strains *CL* and *FL*, for instance, which behave in a rather different way as regards treatment, have been isolated at the same time in South Brasil from naturally-infected vectors and although *FL* has demonstrated to be somewhat more virulent, no striking differences between both parasite populations have been detected [4]. Moreover, strain *Y* and *Berenice* which presented significant differences as regards treatment show many biological similarities in experimentally infected animals (BRENER [4]).

Mutant bacteria lacking a nucleotide-dependent enzyme which is essential for the reduction of the nitro-group of nitrofurans and its chemotherapeutic action (effected by macromolecules-binding and damage of DNA) have been detected (reviewed by MCCALLA & VOUTSINOS [11]). The possibility of a similar mode of action, involving reductase-containing strains, which could explain differences in susceptibility to nitrofurans, has not been demonstrated in protozoa. On the other hand, existence of specific drug receptors is difficult to envisage at this stage, but further investigations with labelled compounds could help to explain the phenomenon.

Despite the small numbers of animals used in their experiments, HABERKORN & GONNERT [10] have also noticed a wide range of sensitivity to nifurtimox among 8 different *T. cruzi* strains. More recently, ANDRADE et al. observed marked differences in the susceptibility of mice inoculated with two strains and treated with nifurtimox for 150-170 days (SONIA ANDRADE, 1975, per-

sonal communication). Our data, obtained with large numbers of animals and different active compounds, emphasize the need of investigating any presumptive curative drugs in groups of animals inoculated with several *T. cruzi* populations.

RESUMO

Diferenças na suscetibilidade de cepas de Trypanosoma cruzi *a agentes quimioterápicos ativos*

Com a finalidade de se investigar possíveis diferenças de suscetibilidade de cepas de *T. cruzi* a agentes quimioterápicos ativos, grupos de camundongos inoculados com quatro diferentes cepas do parasita foram tratados com dois derivados nitrofuranos e um nitro-imidazol. O critério de cura foi baseado em repetidos exames de sangue a fresco, hemoculturas em meio LIT ("liver-infusion tryptose"), imunosupressão de animais tratados por raios gama, reinoculação e teste de imunofluorescência. Diferenças significativas da porcentagem de curas em grupos de animais inoculados com as diferentes cepas foram observadas com os três medicamentos empregados.

REFERENCES

1. BRENER, Z. — Atividade terapêutica do 5-nitro-2-furaldeido-semicarbazona (nitrofurazona) em esquemas de duração prolongada na infecção experimental do camundongo pelo *Trypanosoma cruzi*. *Rev. Inst. Med. trop. São Paulo* **3**:43-49, 1961.

2. BRENER, Z. — Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*. *Rev. Inst. Med. trop. São Paulo* **4**:389-396, 1962.

3. BRENER, Z. — Observações sobre a imunidade a superinfecções em camundongos experimentalmente inoculados com *Trypanosoma cruzi* e submetidos a tratamento. *Rev. Inst. Med. trop. São Paulo* **4**:119-125, 1962.

4. BRENER, Z. — Comparative studies of different strains of *Trypanosoma cruzi*. *Ann. Trop. Med. Parasit.* **59**:19-26, 1965.

5. BRENER, Z. & CHIARI, E. — The effects of some immunosuppressive agents in experimental chronic Chagas' disease. *Trans. Roy. Soc. Trop. Med. Hyg.* **65**:629-636, 1971.

6. CAMARGO, E. P. — Growth and differentiation in *Trypanosoma cruzi*. I — Origin of metacyclic trypanosoma in liquid media. *Rev. Inst. Med. trop. São Paulo* **6**:93-100, 1964.

7. CANÇADO, J. R.; MARRA, U. D.; LOPES, M.; MOURÃO, O.; FARIA, C. A. F.; ALVARES, J. M. & SALGADO, A. A. — Toxicidad y valor terapéutico del Bay 2502 en la enfermedad de Chagas crónica en três esquemas posológicos. *Bol. Chileno Parasitol.* **24**:28-32, 1969.

8. CERISOLA, A. — Imunodiagnóstico da Doença de Chagas. Evolução sorológica de pacientes com doença de Chagas. *Rev. Inst. Med. trop. São Paulo* **12**:403-411, 1970.

9. CHIARI, C. A.; MAYRINK, W. & MAGALHÃES, P. A. — Reação de imunofluorescência indireta no controle de tratamento da leishmaniose tegumentar americana. *Rev. Inst. Med. trop. São Paulo* **15**:298-303, 1973.

10. HABERKORN, A. & GONNERT, R. — Animal experimental investigation into the activity of nifurtimox against *Trypanosoma cruzi*. *Arzneimittel-Forschung* **22**:1570-1581, 1972.

11. MC CALLA, D. R. & VOUTSINOS, D. — On the mutagenicity of nitrofurans. *Mutation Research* **26**:3-16, 1974.

12. NEAL, R. A. — Superiority of the culture technique over xenodiagnosis for the detection of trypanosomes in Chagas disease. *Proc. Ninth Inter. Congress Trop. Med. Malaria* **1**:56, 1973.

13. RASSI, A. & FERREIRA, H. — Tentativa de tratamento específico da fase aguda da doença de Chagas com nitrofuranos em esquemas de duração prolongada. *Rev. Soc. Brasil. Med. Trop.* **5**:235-262, 1972.

14. SALGADO, J. A.; GARCEZ, P. N.; OLIVEIRA, C. A. & GALIZZI, J. — Revisão clínica atual do primeiro caso humano descrito da doença de Chagas. *Rev. Inst. Med. trop. São Paulo* **4**:330-337, 1962.

15. SCHENONE, H.; CONCHA, L.; ARAUDA, R.; ROJAS, A. & ALFARO, E. — Experiencia terapéutica con el Bay 2502 en la infección chagásica crónica del adulto. Importancia del uso adequado del xenodiagnóstico. *Bol. Chileno Parasitol.* **24**:66-69, 1969.

16. SILVA, H. L. P. & NUSSENZWEIG, V. — Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Folia Clin. et Biol.* **20**:191-207, 1953.

Recebido para publicação em 8/9/1975.