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Evaluation of chemotherapy with benznidazole and nifurtimox in mice infected with *Trypanosoma cruzi* strains of different types*

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A test was made of the susceptibility of 30 strains of Trypanosoma cruzi *to chemotherapy with nifurtimox (Bay 2502) and benznidazole (Ro 7-1051). The strains had previously been classified as type I, II, or III according to their morphobiological and isoenzymic characteristics. Three type I strains, 14 type II strains, and 13 type III strains were studied. Mice were infected with 2×10^5 blood forms of these parasites and treated for 90 days with benznidazole or nifurtimox. All the surviving mice were submitted to parasitological tests (direct parasitaemia, xenodiagnosis, inoculation in new-born mice, and haemoculture) and serological tests (indirect immunofluorescence). As the latter remained positive in about 80% of the parasitologically negative animals, the cure rates were based on the more reliable parasitological tests. Type I strains displayed high susceptibility, type II strains showed medium to high susceptibility, and type III strains were highly resistant to both drugs. The fact that a particular strain type, with its own level of susceptibility, usually predominates in a given geographical area may explain the contradictory results after chemotherapy from different endemic areas.*

The results of specific treatment of patients with Chagas' disease from different countries (*1-5*) or from different geographical areas of the same country (*6-8*) have often been contradictory. Experimental investigations have shown a variety of chemotherapeutic responses by different strains of *Trypanosoma cruzi* to benznidazole and nifurtimox (*9-11*), depending on the biological behaviour of the strain (*12*). Drug resistance in some strains could be provoked by prolonged treatment (*13*) and tended to be stable in the subsequent passages. Cross-resistance has also been demonstrated (*14*). These experimental data indicate that for trials in humans with different chemotherapeutic drugs, the susceptibility of the parasite strain should previously be tested.

In the present study, strains of *T. cruzi* (from different geographical areas) that had previously been characterized into various types (*15*, *16*), according to their morphological behaviour in mice as well as their isoenzymic patterns (*17*), were tested *in vivo* with benznidazole and nifurtimox. Morphobiological characterization was undertaken following a previously described methodology (*16*), and the strains were then classified into types I, II, or III. Briefly, *type I* is characterized by a rapid course of the infection in mice, high levels of parasitaemia and mortality around the 9th and 10th day of infection, and predominance of slender forms and macrophagotropism during the acute phase of the infection; *type II* shows increasing parasitaemia from the 12th to the 20th day of infection, a low rate of mortality, a predominance of broad forms of the parasites, and myocardial tropism; *type III* shows a slow development of parasitaemia that reaches a high level 20-30 days after inoculation, a low mortality, and predominance of parasitism in skeletal muscles. Isoenzymic characterization has shown that strains of the same type show the same pattern with glucose-phosphate isomerase, phosphoglucomutase, aspartate aminotransferase, alanine aminotransferase and glucose-6-phosphate dehydrogenase (*17*). Strains from the same geographical area are all of one single type, with the same isoenzymic pattern. Therefore, it seems important to find out: (1) whether strains of the same type have the same susceptibility to chemotherapy; (2) the drug susceptibilities of several strains from different geographical areas; and (3) the variations in drug susceptibility of a significant number of strains from the same geographical area.

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MATERIALS AND METHODS

Strains of Trypanosoma cruzi

The strains from different geographical areas were collected through xenodiagnosis performed in patients with different forms of Chagas' disease. Two of the strains studied came from naturally infected bugs and from a silvatic host, respectively.

The strains isolated from human patients were identified as follows:

—seven strains from São Felipe (Bahia State, Brazil), classified as type II strains;

—six strains from Mambaí (Goiás State, Brazil), classified as type II;

—nine strains from Montalvania (Minas Gerais State, Brazil), classified as type III.

—three strains from different areas of Argentina: the *AWP* strain (from Buenos Aires), classified as type I; the *RA* strain (from San Luis), classified as type II; and the *CA-I* strain (from La Pampa), classified as type III.

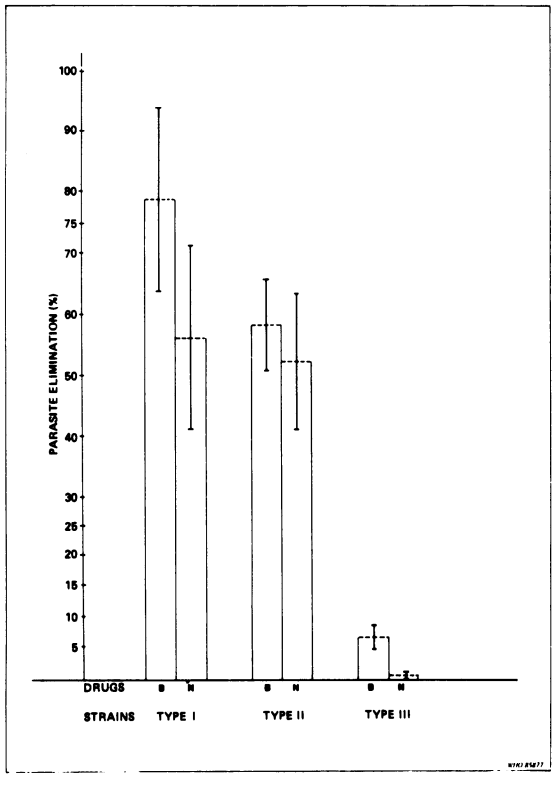

Fig. 1. Means and standard errors of the different cure rates for the three types of strains of *T. cruzi*, after treatment with benznidazole (B) and nifurtimox (N).

Strains isolated from non-human sources were:

—Bolivia strain, obtained from a naturally infected bug collected in a residence in Santa Cruz de la Sierra, Bolivia, and classified as type III;

—*PMN* strain, isolated by xenodiagnosis from a naturally infected silvatic guinea pig from Morada Nova (Ceará State, Brazil) and classified as type III.

Controls: The following previously tested strains were utilized as controls: (*a*) Y and Peruvian strains, prototypes of the type I strain (*15*), and (*b*) Colombian strain, prototype of type III strain (*15*).

The total number of drug-tested strains was thirty. All strains were maintained in the laboratory by serial passages in mice, with some intermediary passages in Hemiptera of the subfamily Triatominae.

Drug tests

For each strain of *T. cruzi*, 70 mice (average weight 18–20 g) were infected intraperitoneally with blood forms of the parasite, obtained from infected mice. The inoculum consisted of 2×10^5 trypomastigotes in 0.2 ml of citrated blood. The infected animals of each group were divided into three experimental subgroups: 25 mice were submitted to treatment with the nitrofuranic compound nifurtimox (Bay 2502); 25 mice were submitted to treatment with the nitroimidazolic compound, benznidazole (Ro 7-1051); and 20 mice remained as untreated infected controls.

Treatment schedules. Nifurtimox was administered initially for 4 days in daily doses of 200 mg/kg body weight, followed by daily doses (for 5 days a week) of 50 mg/kg body weight for a total of 90 days. Benznidazole was administered in daily doses (for 5 days a week) of 100 mg/kg body weight for 90 days.

Drug administration. Both nifurtimox and benznidazole were administered orally, by gavage, in a suspension made with 4% gum arabic in distilled water.

Evaluation of parasitological tests. From 30 to 90 days after treatment, the surviving animals from each experimental group were submitted to parasitological and serological tests. The parasitological tests consisted in looking for parasites in the peripheral blood by direct microscopic examination, and xenodiagnosis using five second-stage nymphs of *Rhodnius prolixus*, as well as intraperitoneal inoculation of the citrated blood in new-born mice followed by investigation of parasitaemia; after all these tests were performed, the surviving animals were sacrificed and their blood submitted to haemoculture in Warren medium.

Statistical analysis. The results of the parasitological tests in the animals treated with benznidazole

and nifurtimox were submitted to analysis by arithmetic means and standard errors of the mean.

Serological investigation. This was performed by the indirect immunofluorescence test according to Camargo (*18*), utilizing culture forms of *T. cruzi* as antigens, on only 20 of the 30 treated groups.

The criterion for cure was a negative result in the parasitological test.

RESULTS

Parasite clearance rates

The clearance rates of parasites with either benznidazole or nifurtimox are presented in Table 1 and Fig. 1. The rates for type 1 strains ranged from 50% to 100% (mean + S.E. = 79.0 ± 14.99) with

Table 1. Parasite clearance rates after chemotherapy with either benznidazole or nifurtimox in mice infected with *T. cruzi* strains of different types

Strain type and identification	Endemic area[a]	Parasite clearance rates (%)	
		Benznidazole	Nifurtimox
Type I:			
Y	Brazil (Saõ Paulo)	50	35
Peruana	Peru	87	47
AWP	Argentina	100	87
Type II:			
3-MAM	Mambaí	100	100
4-MAM	Mambaí	82	14.2
6-MAM	Mambaí	60	100
7-MAM	Mambaí	87.5	0
10-MAM	Mambaí	12.5	0
17-MAM	Mambaí	78	75
12-SF	São Felipe	100	100
17-SF	São Felipe	80	82.3
18-SF[b]	São Felipe	34	0
19-SF	São Felipe	28.5	94
21-SF	São Felipe	28.6	14.3
22-SF	São Felipe	38.5	25
24-SF	São Felipe	56.2	83.3
RA	San Luiz	31.2	42.8
Type III:			
1-MONT	Montalvania	9	5.8
2-MONT	Montalvania	0	0
6-MONT	Montalvania	0	0
7-MONT	Montalvania	7.1	0
8-MONT	Montalvania	0	0
9-MONT	Montalvania	0	0
11-MONT	Montalvania	20	0
14-MONT	Montalvania	12.5	0
17-MONT	Montalvania	12.5	0
CA-I	La Pampa	14.2	0
Bolivia	Santa Cruz la Sierra	0	0
PMN	Morada Nova	0	0
Colombiana	Colombia	16.7	0

[a] Mambaí is in Goiás State, São Felipe in Bahia State, Montalvania in Minas Gerais State, and Morada Nova in Ceará State — all in Brazil. San Luiz and La Pampa are in Argentina, and Santa Cruz La Sierra is in Bolivia.

[b] Patient previously treated with benznidazole.

benznidazole and 35% to 87% (56.3 ± 15.72) with nifurtimox, while type II strains showed a wide variation from 0% to 100% with nifurtimox (52.2 ± 11.21) and from 12.5% to 100% with benznidazole (58.36 ± 7.89). The rates for type III strains varied from 0% to 20% in the animals treated with benznidazole (7.1 ± 2.1), and was 0% with nifurtimox in all but one case (0.45 ± 0.45).

Statistical analysis. The means of the results obtained with the three types of strains showed that type I strains were significantly more susceptible both to benznidazole and to nifurtimox when compared with type III strains (Fig. 1); type II strains showed an intermediate susceptibility with both drugs.

Serology

All animals that remained infected after treatment showed titres ranging from 1:10 to 1:80 in the indirect immunofluorescent test. A high proportion of parasitologically cured animals (82.0% of those treated with nifurtimox and 75.8% of those treated with benznidazole) gave positive results with maximum titres of 1:10.

DISCUSSION

Our results confirm previous experimental data and show clear differences in the chemotherapeutic response of the three types of strains of *T. cruzi* (*9–11*). These differences can be considered as another marker of the biological behaviour of these parasites: type I strains were highly susceptible, type II strains showed medium to high susceptibility, and type III strains were highly resistant to both drugs. The wide variation in response by type II strains is intriguing because, despite a marked sensitivity to both drugs, there were three cases that did not respond at all to nifurtimox while their response to benznidazole was medium to high. We cannot explain this discrepancy but previous exposure to one or both drugs may be a factor. Susceptibility can be influenced by previous treatment of the donor, as was the case with one of these three strains (18 SF, type II), which was isolated from a patient who had previously been treated with benznidazole and apparently developed a cross-acquired resistance to nifurtimox. Enhancement of resistance to the same drug (*13*) and the possibility of cross-resistance have been investigated and experimentally demonstrated (*14*). Another point is that the strains of type II identified in São Felipe (Bahia) and in Mambaí (Goiás) showed a wide variation in virulence and this may have influenced the therapeutic response. In our investigation the most virulent type I strains were the most susceptible to chemotherapy.

This study points out the importance of parasite strain as a factor in explaining the differences observed after treatment with nifurtimox in well-conducted clinical trials (*1, 3–5*). Evaluation of the efficacy of treatment was based on the absence of parasitaemia (negative xenodiagnostic test), even with persistently positive serology. In chronically infected patients, Cerisola et al. (*3*) in Argentina, Schenone et al. (*4*) in Chile, and Neves da Silva et al. (*8*) in South Brazil obtained a high percentage of negative results by xenodiagnosis, with positive serological findings. In Central Brazil (Minas Gerais), Cançado et al. (*1*) observed a zero or low percentage of negative parasitological tests, accompanied by positive serology, both with nifurtimox and with benznidazole (*2*), and claimed that only negative serology could indicate a cure. Rassi & Ferreira (*6*), after treating acute cases in Central Brazil (Goiás) with nifurtimox, found 40% were negative by xenodiagnosis but were positive serologically. Using benznidazole, Rassi (*19*) obtained negative results in parasitological tests in most of the cases but with persistently positive serology, while Cançado & Brener (*2*) reported 60% negative results by xenodiagnosis together with positive serological findings. Prata et al. (*7*) found differences in cure rate among patients from two geographically separate areas in Brazil—28.2% of patients from Bahia State were negative by xenodiagnosis, compared with 56.4% for the patients from Anapolis (Goiás).

Because a single type of strain may be predominant in a given geographical area, one should consider the possibility that this factor might be responsible for any therapeutic discrepancies. Therefore, the characterization and classification of the strain types in an area may help in the selection of a suitable drug for treatment. There was a clear indication that type I strains responded better to benznidazole than to nifurtimox. The resistant strains (type III) seemed to be more resistant to nifurtimox than to benznidazole. As for the type II strains, it may be necessary to test them individually since, as was shown, they disclosed a wide range of responses.

In our study, we considered the obtaining of negative results in the parasitological tests as an indication of cure. Persistently positive results in the immunofluorescence test in the period 30–90 days after treatment were not taken into account in the final evaluation. The significance of a persistently positive result in the different serological reactions (complement fixation, haemagglutination, and indirect immunofluorescence test) has not yet been clarified. Even the search for lytic antibodies, which was recently claimed to be a very important test to

confirm the presence of infection with *T. cruzi* (*20*, *21*), can fail to give a positive result in animals that still show parasitaemia after treatment (*22*). Therefore, the parasitological tests, when performed several times, are at present the best available indicator of cure after chemotherapy.

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RÉSUMÉ

ÉVALUATION DE LA CHIMIOTHÉRAPIE PAR LE BENZNIDAZOLE ET LE NIFURTIMOX CHEZ DES SOURIS INFESTÉES PAR DIFFÉRENTS TYPES DE SOUCHES DE *TRYPANOSOMA CRUZI*

Le traitement de la maladie de Chagas par les antiparasitaires donne des résultats contradictoires selon les auteurs. On a montré de façon expérimentale que les différentes souches de *Trypanosoma cruzi* présentent une sensibilité variable à la chimiothérapie.

Dans l'étude rapportée ici, on a mis à l'épreuve 30 souches de *T. cruzi*, classées en trois types (I,II ou III) selon leur comportement morphobiologique chez les animaux d'expérience et leur profil iso-enzymatique (pour les enzymes GPI, PGM, ASAT, ALAT, G6PD) et, isolées dans diverses régions géographiques, afin de déterminer leur réponse à la chimiothérapie par le nifurtimox (Bay 2502) et le benznidazole (Ro 7-1051). Les souches de type I — caractérisées par la rapidité de l'infestation chez la souris, une parasitémie et une mortalité élevées vers le 9^{e} ou 10^{e} jour, la prédominance de formes minces et un macrophagotropisme au cours de la phase aiguë de l'infestation — sont représentées dans la présente étude par la souche Y (São Paulo, Brésil), la souche AWP (Argentine) et une souche péruvienne. Les souches de type II — avec lesquelles on observe une parasitémie qui augmente du 12^{e} au 20^{e} jour de l'infestation, un faible taux de mortalité, une prédominance des formes larges et un tropisme myocardique — sont représentées par 7 souches de São Felipe (Bahia, Brésil), 6 souches de Mambaí (Goiás, Brésil) et la souche RA (Argentine). Les souches de type III — caractérisées par une parasitémie à installation lente, atteignant des niveaux élevés 20 à 30 jours après l'inoculation, une faible mortalité, la prédominance des formes larges et un tropisme envers les muscles squelettiques — sont représentées par 9 souches de Montalvania (Minas Gerais, Brésil), la souche CA-I (Argentine), la souche PMN (Ceará, Brésil), une souche bolivienne ainsi qu'une souche colombienne.

On a procédé à des épreuves thérapeutiques expérimentales chez des souris infestées par inoculation intrapéritonéale de 2×10^{5} parasites du stade sanguicole. Pour chaque souche, on a infesté 70 souris (pesant entre 18 et 20 g), réparties en trois groupes: 25 traitées au benznidazole, 25 au nifurtimox et 20 non traitées servant de témoins. Les deux médicaments ont été administrés par voie orale pendant 90 jours aux doses suivantes: 100 mg/kg de poids corporel par jour pour le benznidazole; 200 mg/kg de poids corporel pour les quatre premières doses de nifurtimox, puis 50 mg/kg de poids corporel par jour. L'évaluation des taux de guérison a été effectuée 30 à 90 jours après la fin du traitement par des épreuves parasitologiques (parasitémie directe, xénodiagnostic, inoculation à des souriceaux nouveau-nés et hémoculture) et des épreuves sérologiques (immunofluorescence indirecte). Les résultats ont montré une grande sensibilité des souches de type I aux deux médicaments (50 à 100% de guérison avec le benznidazole et 35 à 87% avec le nifurtimox). Celles de type II présentaient une sensibilité moyenne à élevée et très inégale selon les souches. Enfin, les souches de type III ont manifesté une forte résistance aux deux médicaments (avec un taux de guérison de 0 à 20%). Les épreuves d'immunofluorescence sont restées constamment positives chez une grande partie des animaux (environ 80%) pour lesquels les épreuves parasitologiques étaient négatives. Les épreuves d'immunofluorescence ont donné des résultats durablement positifs chez une forte proportion (environ 80%) des animaux pour lesquels les épreuves parasitologiques étaient négatives. On a donc adopté comme critère de guérison la négativation des épreuves parasitologiques, attestant de l'élimination totale des parasites à la suite du traitement.

Vu que, dans une région géographique donnée, il y a en général prédominance d'un type de souche particulier, ayant un seuil de sensibilité qui lui est propre, cela pourrait expliquer les résultats contradictoires de la chimiothérapie dans les différentes régions d'endémie.

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