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EVOLUTION OF SUBPATENT PARASITAEMIA IN Trypanosoma cruzi CHRONICALLY INFECTED MICE WITH THE HELP OF A CYCLOPHOSPHAMIDE AMPLIFICATION TRANSFER ASSAY.

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SUMMARY

We have evaluated the sensitivity of the classical blood subinoculation method, modified through cyclophosphamide treatment of transferred mice, for the detection of occult parasitaemias in **Trypanosoma cruzi** chronically infected mice. Besides its simplicity, the method was shown to be highly sensitive for both the "chronic" phase parasites (99% of chronic cases were shown to harbour occult parasitaemias) and for the acute phase parasites (**T. cruzi** could be detected in 53.8% of animals transferred with one Y strain parasite and in 20% of animals transferred with one CL strain parasite). Using acute phase bloodforms, the assay proved to be more sensitive than conventional subinoculation when dealing with the CL, but not the Y strain of the parasite. With the help of this parasite detection tool, we have studied during a one year period, the evolution of subpatent parasitaemias in a group of mice which survived through chemotherapy from lethal acute phase of **T. cruzi** infection. Cyclophosphamide transfer assay revealed occult parasitaemias in 100% of the chronic animals, nevertheless, continuous and discontinuous patterns of positivity were observed.

KEY WORDS: Trypanosoma cruzi; Infection in mice; Parasitaemia; Cyclophosphamide amplification transfer assay.

INTRODUCTION

Chagas disease can be frequently diagnosed while on the acute phase by simple microscopic blood examination, but in the chronic phase, when parasites are scanty in the peripheral blood, **Trypanosoma cruzi** demonstration is difficult.

The detection of **T. cruzi** in the chronic host has relied on xenodiagnosis[5], cultivation of blood in liquid media[7] or in cell culture[12], subinoculation to normal mice[3], and intracerebral inoculation to newborn mice[2, 13]. Although these techniques detect small **T. cruzi** numbers, their level of sensitivity for the detection of chronic phase parasitaemias remains obscure, mostly because of our ignorance about the magnitude and continuity of **T. cruzi** blood input in the chronic host. In this sense, little is known about parasitaemia levels in chronic Chagas; its evolution has been studied through xenodiagnosis in a group of chronic patients[6], but to our knowledge, studies to this respect are missing in the mouse model.

Recently we have been involved in studying the protective effects of immunotherapy in **T. cruzi** experimental mouse infection[9]. Protection, measured by classical enhancement of mean survival times and overall acute phase survival, required to be complemented with the assessment of parasitaemia levels in those animals escaping from lethal acute phase. With this purpose, and due to the complexity or inapplicability for the mouse model of some of the above mentioned techniques, we have employed a simple and sensitive method for detection of subpatent parasitaemias, based on a modification of the old subinoculation technique. This modification consists in treatment of blood

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transferred mice with the immunosuppressive agent cyclophosphamide, to prevent the establishment of a specific anti-**T. cruzi** immune response that might impose constraints in parasite amplification. Cy treatment in **T. cruzi** infection was previously shown to increase parasitaemias[1, 4, 10, 11, 15] and to reveal occult parasites in mice infected with **T. cruzi** stocks of low virulence[14]. Through a combination of Cy treatment with blood transfer we expected an increase in the sensitivity of the classic subinoculation method, while leaving the original mice unmanipulted, which would permit serial occult parasitaemia evaluations and other type of serial screenings.

In this report we present our results on the sensitivity of the cyclophosphamide-amplification transfer assay, and, data from a one-year subpatent parasitaemia study in chronic mice, which were prevented from death in the acute phase by treatment with the specific agent Benzonidazol.

MATERIAL AND METHODS

- **Mice:** A/Sn mice from our breeding colony at the Instituto Butantan.

- **Parasites:** Y and CL strain parasites of **T. cruzi**, maintained through passages in A/Sn mice.

- **Occult parasitaemia detection assay:** Blood (aprox.0.2 ml) from the ophthalmic plexus of individual "chronic" mice, recovered in sodium citrate 3.8%, was injected i.p. into one or two normal A/Sn mice. Two days later, transferred mice received a single i.p. dose of cyclophosphamide 200 mg/kg (Enduxan, Pravaz, São Paulo). Starting one week after blood transfer, recipients were screened every two or three days for direct parasitaemias using blood from the tail vein.

- **Chronic animals:** These were Y strain or CL strain infected A/Sn mice surviving the acute phase of the disease:

a) spontaneously (circumstance which was very rare, as expected from the extreme sensitivity of A/Sn mice to the infection with a low number of **T. cruzi** parasites).

b) after a single oral dose (1/gr/kg) of the specific chemotherapy agent Benzonidazol (Rochagan, Roche), given one day before establishment of the peak of parasitaemia.

c) after immunoprophylaxis with BCG (Onco-BCG, Instituto Butantan, São Paulo), given in a single i.v. dose of 2 mg/mouse, in different time protocols (from day -35 to day 0, in relation to infection); or with 0.25 ml of Immune Mouse Serum (IMS) from A/Sn mice chronically infected with **T. cruzi**), given i.v., 24 hours before parasite challenge.

- **Sensitivity of the Cy amplification transfer assay to evaluate parasitaemia.** The sensitivity was established quantifying the appearance of parasitemia in groups of mice infected with known low numbers of **T. cruzi** and subsequently treated with Cy. Control groups where Cy treatment was omitted were set up in paralell.

RESULTS

Evaluation of the assay's sensitivity with acute phase parasites

Mice were infected with 1 or 5 parasites of the Y strain, or with 1,5 or 10 parasites of the CL strain, treated at day 2 with cyclophosphamide, and screened for parasitaemias in the subsequent days. All parasite inocula were carried out in 1 ml volumes, to minimize the risk of mice receiving disparate parasite numbers. Our results show that 7 out of 13 mice infected with one Y-**T. cruzi** (53.8%), and 10 out of 10 mice infected with 5 Y-**T. cruzi** (100%), yielded positive parasitaemias; this occurred 10.4 days after infection (range 9-13). For the CL strain, infection with one parasite turned positive in 20% of mice (2 out of 10); using 5 CL-**T. cruzi**/mouse, positivity occurred in 33% (6 out of 18), and with 10 CL-**T. cruzi**/mouse, 100% (10 out of 10) showed positive amplification. CL parasitaemias turned positive at days 10 to 14, although in two cases (11%) this did not occur until day 17. Cy treatment did not change the sensitivity of the transfer method when assaying the Y strain of **T. cruzi**. On the other hand, detection of parasitaemia for infections with the CL strain (10 bloodforms/mouse) increased from 70% (14 out of 20) in control groups to 100% in Cy treated animals. Moreover, the Cy assay turned to be far more convenient than the conventional subinoculation method when dealing with low virulence variants (see discussion).

Evaluation of the assay's sensitivity for "chronic phase" parasites

After the evaluation of the assay's sensitivity, we proceeded to screen acute phase survivors for subpatent parasitaemia. This was important considering that whole blood transfer would carry over to recipients the specific anti-**T. cruzi** antibodies present in the chronic host. Also, because "chronic" phase parasites could behave differently in the assay than those acute phase parasites used to evaluate the sensitivity of the amplification method.

Transferred blood was from mice surviving an acute infection with **T. cruzi** trypomastigotes, spontaneously, after drug treatment, or after immunoprophylaxis with immune mouse serum or BCG. All these mice presented direct positive parasitaemia while on the acute phase, turned negative afterwards, and remained as such up to the day of the transfer assay (performed at different times from day 80 to day 346).

As shown in Table I, most of the acute phase survivors had subpatent parasitaemias revealed by the cyclophosphamide assay. From a total of 102 mice showing positivity upon transfer, 86 (84.3%) were revealed positive from the first screening, 12 required two or three screenings to result in positivity, and the remaining four required four to six tests to reveal occult parasites. Mouse registered as negative corresponds to a chronic animal that died after three negative Cy transfer assays.

Evolution of parasitamias in T. cruzi infected chronic mice

The studies shown in the precedent section indicated that with certain "chronic" mice, subpatent parasitaemias could only be demonstrated after several cyclophosphamide amplification tests. This could indicate that the parasitaemia level in these animals was of an order magnitude, that would only occasionally permit the transference of a **T. cruzi** parasite number sufficient to reach the assay's sensitivity threshold. Alternatively, the parasitaemia fluctuation seen with certain mice, could reflect the discontinuity of **T. cruzi** blood input in these animals.

While the two possibilities are not mutually exclusive, we thought that a systematic follow-up of chronic parasitaemias would give us a parasitaemia evolution picture that would favor one of the above explanations. With this purpose we have performed a one-year study in 20 **T. cruzi**-infected A/Sn mice treated with Benzonidazol to prevent death on the

TABLE I

Detection of Occult Parasitaemias in Acute **T. cruzi** Infection Survivors using the Cyclophosphamide Amplification Transfer Assay.

Mechanism of survival	**T. cruzi** infection strain	Infection dose (ip) parasite nº	Treatment	positive mice/ total mice
Spontaneous	Y	100	none	1/1
"	CL	10	none	3/3
Chemotherapy	Y	200000	Benz. day6	10/10
"	Y	5000	Benz. day7	10/10
"	Y	100	Benz. day10	5/5
"	CL	1000	Benz. day13	6/6
Immuno Prophylaxis	Y	100	IMS day(-1)	17/18
"	Y	400	BCG	42/42
"	CL	400	BCG	8/8
			TOTAL:	102/103

acute phase. Two different protocols of chronification were used: infection with 200.000 **T. cruzi** (Y) followed by treatment with Benzonidazol (1 g/kg of body weight) at day 6, and infection with 5.000 Y parasites and treatment with the specific agent at day 7.

We found (Table II) that all 20 animals had positive parasitaemias. From these, 13 always yielded positive transfers. With respect to the remaining 7 giving discontinuous parasitaemias, there were mice that accumulated negative tests during a certain period of time, pattern that would support a discontinuous parasite blood input, and others, showing a more randomic distribution of positive and negative transfers, that would favour a continuous parasitaemia of lower magnitude.

DISCUSSION

The overall simplicity of the cyclophosphamide amplification transfer assay permits processing of blood from a large number of chronic mice; this represents a clear advantage over classical techniques that require delicate manipulation and/or preisolatioin step. It also represents an improvement over xenodiagnosis, where triatomid maintenance, animal discomfort and eventual appearance of alergic reactions limits its use. The assay can be used in a semiquantitative fashion through transfer of defined blood volumes. Besides this, follow-up of parasitaemias in subinoculated animals, together with eventual survival of transferred mice (death normally occurred 20 to 30 days after Cy transfer) permits detection and eventual isolation of **T.**

TABLE II

Occult parasitaemias in **T. cruzi** infected mice, chronified after Benzonidazol treatment.

Infection dose (**T. cruzi**)	Benzonidazol treatment	Occult Parasitaemias at days:								Positive Transfers
		81	95	115	184	200	234	272	346	Total Transfers
200 000	day 6	+					+		+	3/3
"	"	+				+		+	+	4/4
"	"	+				+			+/+*	4/4
"	"	+				+			+/+	4/4
"	"	+				+		+	+	4/4
"	"	+				+		+	+	4/4
"	"	+				+		+	+/+	5/5
"	"	+				+		+	+	4/4
5 000	day 7	+				+			+/+	4/4
"	"	+				+		+	+/+	5/5
"	"	+				+		+	+/+	5/5
"	"	+				+		+	+/+	5/5
"	"	+				+			+	3/3
200 000	day 6	-	+			+		-	+/+	4/6
"	"	-		-/-	-/+	-		+	+	3/8
5 000	day 7	+				+		-		2/3
"	"	-	+			+			+/+	4/5
"	"	-		-/-	-		+	+	+/+	4/8
"	"	+				+	-	-	+	3/5
"	"	+				+	-	-	+/-	3/6

* The presence of two symbols at a particular time point expresses the results of a test where two mice were subinoculated.

cruzi chronic variants with unusual low proliferative and/or virulence patterns (this occurred with some of the chronic mice included in this report: data not shown). This virulence-related information would be completely ignored by axenic or cellular culture **T. cruzi** detection methods.

In relation to its sensitivity, the cyclophosphamide assay seems to be as good (if no better) for detection of acute phase parasites as any of the classical methodologies[7]. For chronic parasites it revealed similar efficiency (99% positivity) than assays such as hemoculture in LIT medium[8] or cell tissue culture[12].

On the other hand, when using conventional high virulence strains (Y or CL), we found that the Cy transfer assay showed identical or slightly higher levels of sensitivity than the conventional subinoculation method. Nevertheless, upon transfer of very small blood volumes from mice infected with low virulence stocks, the Cy transfer assay turned out to be far more sensitive; this was repeteadly observed with a variant of the CL strain, isolated in our laboratory from a CL strain infected chronic mouse, that upon blood transfer yielded no positive parasitaemias unless recipient mice were treated with cyclophosphamide (data not shown).

With respect to the parasitaemias evolution, two groups were dissected: first one, that included 65% of the animals, in which all subinoculations gave positive results; and a second one which gave discontinuous results, and that included animals with a high number of negative tranfers (5 negative transfers out of 8 total transfers), and, others with nearly continuous positivity (one negative transfer out of 5 subinoculation assays). These results represent to our knowledge the first description on the evolution of chronic parasitaemias in the mouse model.

Our data are compatible with the results obtained with 14 untreated human chagasic patients[6], where a similar variation in the percentage of positive xenodiagnosis tests was observed. Variation according to these authors went from a 0% of positive tests founded in one individual, up to 100% positivity founded in another one. Nevertheless, the differences in the assay employed, as well as major differences in the protocols, such a host difference, polymorphism of the natural infection strains vs the uniformity of the Y strain in our experiments, unknown infection doses in the human cases, etc.., impedes any correlation besides the referred observed variation.

Our results can not explain the discontinuous levels of parasitaemia observed in some chronic mice, because the distribution of negative transfers were randomic for some mice, and accumulated in a certain period of time for others. While a more extensive study is needed to clarify this problem, we think that the assessment of parasitaemia evolution in the mouse model can be of inestimable value, not only for screening the efficiency of chemotherapy or immunetherapy protocols, but for the understanding of the natural chronic human disease.

RESUMO

Evolução das parasitemias subpatentes na infecção crônica experimental pelo Trypanosoma cruzi **através do ensaio de subinoculação modificado pelo tratamento com ciclofosfamida.**

Avaliamos o potencial do ensaio clássico de subinoculação, modificado pelo tratamento com ciclofosfamida dos animais receptores, na detecção de parasitemias ocultas em camundongos com infecção crônica pelo **Trypanosoma cruzi.**

O ensaio, além de simples, mostrou ter uma alta sensibilidade; assim, utilizando-se parasitas da fase aguda, o tratamento com ciclofosfamida revelou parasitemias em 53,8% dos animais infectados com um tripanosoma da cepa y, e em 20% dos animais infectados com um tripanosoma da cepa CL. O tratamento com ciclofosfamida aumentou a sensibilidade do ensaio de subinoculação nas infecções pela cepa CL, e resultou em igual sensibilidade quando utilizada a cepa Y. Nos camundongos de fase crônica, obtidos a partir de diversos esquemas de imunoprofilaxia (BCG, soro de camundongo imune) ou quimioterapia, o ensaio revelou parasitemias ocultas em 99% dos animais.

Auxiliados pelo método da subinoculação-ciclofosfamida estudamos no espaço de um ano a evolução das parasitemias ocultas em um grupo de camundongos infectados que sobreviveram à fase aguda pelo tratamento com Benzonidazol. O ensaio revelou parasitemias ocultas em 100% dos animais. Entretanto, padrões contínuos e

discontínuos de positividade puderam ser detectados.

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