THE UN-INFECTIVITY OF THE PF CULTIVATED STRAIN OF TRYPANOSOMA CRUZI TO MICE. AN EVALUATION THROUGH A ONE YEAR PERIOD BY BLOOD CULTURES AND HISTOPATHOLOGY *

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Trypanosoma cruzi *of the cultivated* PF *strain when injected in mice per subcutaneous route, in adequate doses, is able to induce an efficient sterile immunization in the animals (for at least one year) as determined by whole blood cultures and histopathology.*

INTRODUCTION

It is well known that each antigen presented to the lymphoid system can react with a population of immunocompetent cells, variable with the antigen used (4).

The quantity and the route used for the antigen are also of importance, giving place to an immune response which may vary from optimal to no response at all (immunotolerant condition).

I had demonstrated (14) that 3 x 10^2 live parasites of the *PF* strain injected by subcutaneous route, with about 30 trypomastigotes, were able to give an efficient protection to 10g body weight mice, against a virulent infection of *Trypanosoma cruzi* that killed 80% of the control animals.

This work aims to demonstrate that the route and the dose of the live *PF* parasites are very important in obtaining a safe immune response, as applicable to any live vaccine.

MATERIAL AND METHODS

Albino mice from our Medical School Animal Facilities were used. 130 of them, all male, with an initial body weight of 10g were divided in groups of 10.

80 of these animals received by subcutaneous route 6 x 10^3 parasites of the *PF* strain raised in Warren medium (22), dialyzated in accordance with the Nakamura technique (20).

The culture medium was put into cellophane bags immersed in 1.5 volumes of Ringer-Hartmann solution (10) adjusted with NaOH sol. to pH 7.5.

The culture used as vaccine was 8 days old and the percentage of mobile forms was about 80% with 0.8% trypomastigotes, as determined in 1,000 parasites counted at random in Giemsa stained smears.

The Ringer solution with flagellates was centrifuged at 1,500 rpm for 15 minutes and the sediment suspended in sterile so-

* Presented to the X Congresso Brasileiro de Patologia. Curitiba, 05/09/74.
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Submitted to publication on 12-6-1974.

lution, pH 7.0 to give the above mentioned concentration.

The 50 other mice were kept as controls, under identical conditions.

30 days, later, 10 of the vaccinated animals and 10 controls were infected, per intraperitoneal route, with 5,000 parasites/g body weight. The infecting parasites were blood forms of the virulent *Y* strain, maintaned through seringe passages in baby mice.

Determinations of parasitemia by the Pizzi-Brener technique (6) were performed 8, 15, 30 and 60 days after the infection.

On the same day, 10 of the mice which were vaccinated 30 days earlier were anaesthetized by ether and killed by exsanguination (heart punction). The whole blood from each animal was distributed in 3 tubes with Warren medium, for culture.

After 30 days at 28ºC temperature, the cultures were searched for *Trypanosoma cruzi*, through microscopic examination of a drop of the medium. When negative, the examination was repeated at the end of 45 days. At this time the culture medium was centrifuged for 15 minutes at 1,500 rpm and the sediment examined.

45 days after the first vaccination the remaining 60 primed animals were boosted by subcutaneous route, with 5 x 10^5 *PF* parasites obtained by the same steps as for the first vaccine.

60, 90, 120, 270 and 360 days after the first vaccination the surviving mice, from each group of 10 were killed and the whole blood cultivated and examined as described previously.

The 7 remaining animals of the 360 days vaccinated group, and the only 3 surviving mice of the corresponding intact control group, were infected with blood forms of the virulent *Y* strain as mentioned for the first 30 days vaccinated mice.

Determinations of parasitemia were performed 8, 15, 30 and 60 days after the infection. 120, 270 and 360 days after the beginning of the experiment, the remaining control groups of normal intact mice were killed and the main internal organs saved for gross and microscopic examination and for comparison with its equivalent vaccinated groups.

From the majority of the animals used in this experiment fragments of tongue, eosophagus, lungs, heart, liver, spleen, adrenal glands, kidney, abdominal brown adipose tissue, urinary bladder, large and small intestine and skeletal muscle were fixed in 10% formalin solution. Paraffin sections of these different organs and tissues were stained by Hematoxylin & Eosin, Mallory phosphotungstic trichromic stain and by Periodic Acid Schiff stain.

Careful search for parasites was done in a mean of 12 sections of each of the afore mentioned organs and tissues.

A detailed description of the histological findings will be the subject of another paper.

RESULTS

The results of this experimental work can be summarized as follows:

1) all the animals gave negative blood cultures from 30 to 360 days after vaccination (Table I);

2) in spite of this negativity, mice challenged at 30 and 360 days after vaccination were protected (Tables II and IV);

3) in all the vaccinated groups the heart weight was equal, or inferior, to that of the respective normal control (Table III);

4) the spleen weight of the 120 and 270 days vaccinated mice was greater than that of the respective intact control animals (Table III);

5) no gross alterations were observed in the heart, oesophagus, large and small intestine of the 120, 270 and 360 days vaccinated animals when compared with the respective normal intact groups (Figs. 1 and 2);

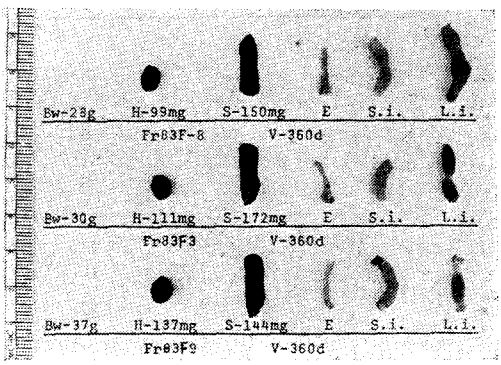

Fig. 1 — Organs of 360 days vaccinated mice showing the lighter, the intermediate and the heavier heart. No gross alterations are seen in the hollow visceras.

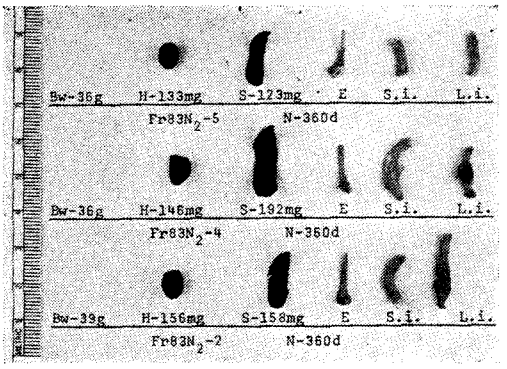

Fig. 2 — Organs of normal intact mice with the same age of those shown in the Fig. 1, ordered by the heart weight.
H = heart (ventricular mass); S = spleen; E = esophagus; S.i. = small intestine and L.i. = large intestine.

6) no parasites were found in the histological sections examined;

7) in all vaccinated animals a small hepatic accumulation of histiocytes, lymphocytes, plasmocytes and sometimes neutrophils was observed, but this lesion was seen, in small proportion, in normal intact mice too.

DISCUSSION

The greater effectiveness of live or attenuated vaccines demonstrated by the greater degree of protection and the longer duration of the immunity is well known, although the enhancement of this efficacy is not yet clear (3).

The dose-response relationship between antigen concentration and the resultant immune response is also described in all modern textbooks of Immunology.

The development of immunological tolerance by "low dose" or "high dose" of antigens has been the subject of several publications (13; 11, 5, 9; 19) and must be considered every time we intend to induce an immune response in a determined animal species with a specific antigen, particulary if the antigen is a live micro-organism.

In a previous paper, as already mentioned, I demonstrated that subcutaneous injection of 3 x 10^2 parasites of the *PF* strain was able to induce an efficient protection in mice against a challenge that killed 80% of the control mice (14).

Later on with the use of immunosuppressive drugs I confirmed the non virulence of the mentioned *Trypanosoma cruzi* strain (16; 17).

Chiari (7) in a comparative study of different *T. cruzi* strains, injected in 86% of their 65 mice, 14g body weight, from 2 millions to 100 millions of live *PF* parasites per intraperitoneal route.

It must be emphasized that the investigator (7) makes reference only to the number of trypomastigotes but it represents only 5% of the total parasites injected in each animal (8).

Epimastigotes are so antigenic as trypomastigotes (12; 18) and cannot be neglected in an experiment like this.

30 days after those massive trypanosomose injections Chiari (7) finds no patent parasitemia, refers to no mortality, presents no histologic findings and obtains a mean of 60% of positive blood cultures.

Considering only the animals inoculated with the *PF* strain whose blood was cultivated in LIT medium (Tables V and VI of the Chiari's paper (7) one can see that 2 millions of intraperitoneal injected parasites gave 75% of positive blood cultures (3/4), the same percentage obtained with the intraperitoneal injection of 100 millions of the same parasites (15/20).

So it is admissible to consider another cause than the infectivity of the parasites as responsible for the positivity of the blood cultures. In consequence of the enormous amount of parasites injected and the route employed, the most probable argument must be the development of an immunotolerant state.

It is apropriate to call attention, once more, to the fact that the existence of a trypanosome positive blood culture, particulary in these circumstances, is no acceptable evidence of its pathogenicity. This must involve: 1) the rate of infection (injected animals/infected animals); 2) the duration of the prepatent period; 3) the intensity of the parasitemia; 4) the duration of the acute phase; 5) the rate of mortality and 6) the degree of tissular parasitism (21).

Hungerer (12) injecting epimastigotes of *T. cruzi* into thymusless nu/nu mice got positive blood cultures with no other evidence of infection.

It is the subject of long observation and of experimental data too, that the antigens, specially live micro-organisms, are

used in a narrow range of doses to have the least harmful effect possible on the patent. When the immunologists recommend 3 x 100mg of BCG by oral route to a child or 50mg per intradermic route, the pediatrician must not use 3,000g or 500mg respectively because the results would surely be disastrous. Every internist knows the danger of emploing, even in optimal doses, live vaccines during the use of immunodepressive drugs or in the cases of infectious disease.

Another point in the work of Chiari (7) on which I would like to make some remarks is that concerned with the culture medium.

He states that the LIT medium gives better results than the Warren medium. In an unpublished work, Rosa Ribeiro and myself, working with 60 albino mice injected by subcutaneous route with 5 x 10^7 *PF* parasites, made cultures of blood and macerate of heart, spleen and liver in both media.

The cultures were done in groups of 10 animals from 15 to 120 days after vaccination.

We got only positive cases in the Warren medium (8%) and 4 in the LIT (7%). 3 of the positive cases were obtained in the 15 day of vaccination. One case was detected by the LIT medium and not by the Warren but on the other hand 2 were positive in the Warren and negative in the LIT medium.

Better results were seen by Albuquerque et als (1) from the Warren than the LIT, comparing the results of human chronic cases of Chagas disease.

So I did not find any advantages in substituting the Warren by the LIT medium.

CONCLUSIONS

From the data of this experiment and of several others (14; 18) I can conclude that the *PF* cultivated strain of *Trypanosoma cruzi*, when given by the appropiate route, in adequate doses (as it is also done in all other live vaccines), is able to induce an efficient sterile immunization in mice against a challenge with a virulent strain of the same parasite. This immunity remains for a long time-one year at least-for mice.

No megas could be detected in the long term vaccinated animals whose internal organs, except the spleen in two groups, were similar or smaller than those of the intact normal mice of the same age, maintained under the same food and ambiental conditions.

RESUMO

Trypanosoma cruzi *da cepa* PF *quando injetado em camundongos, por via subcutânea, em doses adequadas, produz imunidade eficiente e duradoura (um ano pelo menos), sem que se tenha podido identificar infecção ou infecção-doença por meio de hemoculturas e estudos histopatológicos.*

O Autor tece considerações sobre resultados aparentemente discrepantes obtidos por outro pesquisador (7-8), concluindo pela impropriedade do método utilizado por este para obter um estado de imunidade esteril.

Corroborando suas observações e experimentações anteriores, o Autor conclue pela eficácia da cepa referida como meio de se obter imunização ativa contra a tripanosomose americana experimental.

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TABLE I — Results from inoculation in mice, by subcutaneous route, of *PF* parasites. Three blood cultures tubes per each animal.

Days after vaccination	number of animals	1st dose 6 x 10^3 parasites s. c. route	2nd dose 5 x 10^5 parasites s. c. route	Blood culture (Warren medium) (Animal positive/inoculated)		Histopathology Parasites in tissues	Obs.
				after 30 days	after 45 days		
30	10	10		0/10	0/10	—	
60	10	10	10	0/10	0/10	—	
90	10	10	9	0/9	0/9	—	1 died 4 days after 1st vaccine
120	10	10	9	0/7	0/7	—	1 died 6 days 1 died 85 days 1 died 90 days after 1st vaccine
270	10	10	9	0/9	0/9	—	1 died 1 day after 1st vaccine
360	10	10	9	0/8	0/8	—	1 died 6 days 1 died 360 days after 1st vaccine
Total	60	60	46	0/53	0/53	0/53	

TABLE II — Parasitemia and mortality rate from vaccinated * and control mice infected, intraperitoneally, with 5,000 parasites/g, "Y" virulent strain of *Trypanosoma cruzi*.

Mice		Parasites in 5mm³ of blood — Days after infection				Obs.
		8	15	30	60	
1	vaccinated	140	280	0	0	
2	"	0	0	0	0	
3	"	3,080				Died
4	"	525	210	0	0	
5	"	480	2,345	0	0	
6	"	525	525	0	0	
7	"	4,375	2,170	0	0	
8	"	315	0	0	0	
9	"	630				Died
10	"	480	0	0	0	
MEDIAN		502	245	0	0	
Mortality (cumulative)		0%	20%	20%	20%	
1	control	4,375	2,380	140	0	
2	"	3,920	1,990			Died
3	"	17,500				Died
4	"	10,500	2,870			Died
5	"	17,500	2,940	0	0	
6	"	14,000	3,570	315	0	
7	"	4,234	2,730			Died
8	"	4,320				Died
9	"	5,670	3,780			Died
10	"	4,025				Died
MEDIAN		5,022	2,870	0	0	
Mortality (cumulative)		0%	30%	70%	70%	

* Each animal received 6 x 10^3 *PF* parasites by subcutaneous route 30 days before challenge.

TABLE III — Vaccinated and normal mice maintained as controls for mortality rate, body, heart and spleen weight and for the morphology of the hollow internal viscera.

Number of mice	Initial body weight ± s.d.	Duration of		Final body weight (g) mean ± s. d.	Heart weight (mg) mean ± s. d.	Slpeen weight (mg) mean ± s. d.
		Control (days)	Vaccin. (days)			
10	10	120		28.72 ± 0.56	107.80 ± 10.30	218.30 ± 111.35
7	10		120	27.98 ± 2.65	112.28 ± 11.73	378.43 ± 112.21
7	10	270		32.43 ± 4.12	126.57 ± 23.83	154.28 ± 42.39
9	10		270	30.32 ± 3.38	124.89 ± 22.26	226.11 ± 61.83
7	10	360		36.57 ± 2.88	145.28 ± 8.75	145.71 ± 31.75
7	10		360	30.71 ± 3.40	114.86 ± 12.39	159.71 ± 31.49

TABLE IV — Parasitemia and mortality rate from vaccinated * and control animals infected, intraperitonealy, with 5,000 parasites/g, *Y* virulent strain of *Trypanosoma cruzi*.

Mice		Parasites in 5mm³ of blood — Days after infection				Observations
		8	15	30	60	
1	vaccinated	5,215				Died
2	"					Died 360 days after vaccination
3	"	3,640	0	0	0	
4	"	350	0	0	0	
5	"	1,225	0	0	0	
6	"					Died 47 days after vaccination
7	"	665	0	0	0	
8	"	0	0	0	0	
9	"					Died 330 days after vaccination
10	"	0	0	0	0	
MEDIAN		665	0	0	0	
Mortality after infect. (cumulative)		0%	14%	14%	14%	
Mortality before infect.						30%
1	control	38,500				Died
2	"					Died 1 day before infection
3	"	8,400				Died
4	"					Died 60 days before infection
5	"					Died 330 days before infection
6	"					Died 60 days before infection
7	"	52,500				Died
8	"					Died 330 days before infection
9	"					Died 60 days before infection
10	"					Died 60 days before infection
MEDIAN		38,500				
Mortality after infect. (cumulative)		0%	100%	100%	100%	
Mortality before infect.						

* Each vaccinated animal received 6 x 10^3 *PF* parasites, 45 days later was boosted with 5 x 10^5 flagellates of the same strain and challenged, after 360 days.