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Protective immunity and pathology induced by inoculation of mice with different subcellular fractions of *Trypanosoma cruzi*

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Summary

Mice were immunized with subcellular fractions obtained by differential centrifugation from epimastigotes of *Trypanosoma cruzi* (Tulahuén strain). In a chronic model of Chagas' disease, they were challenged each with 25 bloodstream trypomastigotes. Non-immunized, non-challenged and non-immunized challenged animals were kept as controls. Among the challenged mice, those immunized with 105,000 *g* pellet (Mc) and 105,000 *g* supernatant (Cs) fractions presented positive xenodiagnosis, myocarditis and myositis similar to those shown by non-immunized challenged controls. The fractions enriched in flagella, the 5000 *g* pellet (P5) and the flagellar fraction (F) resulted in fewer animals with positive xenodiagnosis and in hosts partially protected from the development of myocarditis. In the absence of infection, Mc and Cs induced an intense myocarditis while F induced mild lesions similar to those found in the controls. P5 caused a myocarditis intermediate between that elicited by Mc and that in the controls. 50% of the animals immunized with Cs presented pathological electrocardiograms in the absence of challenge. The animals immunized with F and P5 and challenged were protected against the development of pathological electrocardiograms, whereas those immunized with Mc and Cs behaved like the non-immunized controls. The immunized, non-challenged animals presented anti-*T. cruzi* IgG antibodies, with titres which were lower than those shown by the immunized and challenged mice. – The results show the possibility of obtaining tissue lesions with antigenic preparations of *T. cruzi* in the absence of

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infection, and suggest that the mechanisms involved in the generation of myocarditis and electrocardiographic alterations are probably different, since these pathologies can be elicited by different subcellular fractions. Among the antigenic components of the parasite, the flagellar fraction gave the best immunoprotective properties, with little or no immunoaggressive effects.

Key words: *Trypanosoma cruzi;* antigens of *T. cruzi;* Chagas' disease; immunopathology; immunoprotection and immunoaggression.

Introduction

Human infection by *Trypanosoma cruzi*, the causative agent of Chagas' disease, is widely spread in Central and South America (Barreto, 1979). The possibility of using parasite constituents as vaccines to prevent infection and/or disease has been hampered by the finding that subcellular fractions of *T. cruzi* may induce tissue lesions when they are inoculated into rabbits (Teixeira et al., 1975). Recent reports (Laguens et al., 1980; Bijovsky et al., 1983) indicate that mice chronically infected with *T. cruzi* have tissue lesions and electrocardiographic alterations which resemble those found in human chronic Chagas' disease. Therefore, mice were used as experimental host throughout these studies. Microsomal (Mc) and flagellar (F) fractions from *T. cruzi* epimastigotes were effective in protecting mice against lethal acute *T. cruzi* infection (Segura et al., 1976, 1977). However, no information is available on whether these subcellular fractions of epimastigotes of *T. cruzi* are able to: a) induce per se chronic lesions in non-infected animals, and b) prevent chronic lesions in infected or non-infected hosts. Such a study is reported here.

Material and Methods

Antigens

Subcellular fractions were obtained from *T. cruzi* epimastigotes, Tulahuén strain (Pizzi, 1945). Parasites were cultured in biphasic medium (Gerez de Burgos et al., 1976) and harvested, washed and broken as previously described (Segura et al., 1974). Disruption was performed by compression-decompression in a Sorvall-Ribi Cell Fractionator, Model RF-1 (Ivan Sorvall Inc., Norwalk, CT, USA) (Segura et al., 1974). The resultant whole homogenate was centrifuged at 1000 *g* for 15 min, at 4° C. The flagellar (F) fraction was obtained by centrifugation of the pellet on a discontinuous sucrose gradient (Segura et al., 1977). Microsomal (Mc) and cell sap (Cs) fractions from the epimastigotes were obtained by differential centrifugation as previously reported (Segura et al., 1974). Mice were inoculated with the 5000 *g* pellet (P5) or with the Mc or Cs fraction (pellet and supernatant of the 105,000 *g* centrifugation, respectively), all suspended in 0.25 M sucrose, 5 mM KCl (SKS), or with the F fraction, adjusted by dialysis to the same sucrose concentration. Biochemical and electron microscopic characterization of these fractions has already been reported (Segura et al., 1974, 1977).

Groups of forty 21-day-old outbred male Swiss mice were given 4 doses of F, P5, Mc or Cs fractions by intraperitoneal (ip) route every 21 days. Each animal received a total of 250, 1000, 3100

and 3000 μg protein (Lowry et al., 1951), of the F, P5, Mc or Cs fractions, respectively. Forty mice which received only SKS served as controls.

Thirty days after the last dose of antigen or SKS, 20 Swiss mice inoculated with each fraction or saline were challenged by ip injection with 25 bloodstream trypomastigotes of the Tulahuén strain maintained by serial passage in mice (Segura et al., 1976). The other 20 mice in each group were not challenged.

In addition, in order to study whether the culture medium used to grow the epimastigotes had any influence on the development of tissue lesions, 20 Swiss mice were inoculated with culture medium (Gerez de Burgos et al., 1976) (1.4 mg protein) by ip injection using the same schedule as above.

Parasitemia

Parasitemia was determined by xenodiagnosis (Cerisola et al., 1971), performed before challenge with trypomastigotes, and 120 days after challenge (sacrifice day). No xenodiagnosis were performed in the meantime, since even under the best conditions, some animals succumb to the loss of blood.

Histopathological studies

All mice were sacrificed 150 days after receiving the first dose of antigen or SKS. Samples of heart and skeletal muscle were fixed in Bouin's and embedded in Paraplast. Sections were stained with hematoxylin and eosin. The lesions found were classified as myocarditis and myositis in a semiquantitative double-blind study. The histopathological study showed inflammatory lesions consisting of an interstitial mononuclear infiltrate in the heart and skeletal muscle. Due to the variability of intensity of the lesions, they were classified in an arbitrary scale as follows: a) absent: normal myocardium (Fig. 1a) or skeletal muscle (Fig. 1b), b) mild: a small number of scattered focal mononuclear infiltrates in the atria (Fig. 1c) or perivascular spaces of skeletal muscle (Fig. 1d), c) moderate: confluent inflammatory infiltrates in the same areas as in b (Fig. 1e, f), d) severe: diffuse inflammatory infiltration in the myocardium and skeletal muscle (Fig. 1g, h). About 10% of the 6–7-month-old normal male Swiss mice from our biotherium showed a spontaneous mild myocarditis, of the type shown in Fig. 1c.

Toxicity assay

To assess a possible toxic effect of the Mc fraction (the one that per se induced more tissue lesions), 2 groups of mice were inoculated with 1 or 3 doses (2000 μg protein each) of Mc. The mice receiving only one dose were killed 3, 10 and 120 days after immunization, for histopathology. Those receiving 2 more doses (at 15 and 30 days after the first one) were killed 37 and 120 days after the first immunizing dose.

Antibodies

Specific antibodies were studied by IIF (Bolomo et al., 1980), using formolated epimastigotes as antigen, and mouse anti-IgG (obtained from Biosys, Compiègne, France).

Results

Non-challenged mice

Parasitemia in mice that received only subcellular fractions, was always negative by xenodiagnosis. Histopathology revealed a mild myocarditis in about 10% of the mice given SKS, culture media or F fraction but none of the mice had myositis. This incidence of myocarditis is the same as that spontaneously shown by Swiss mice of the same age from our biotherium. Further-

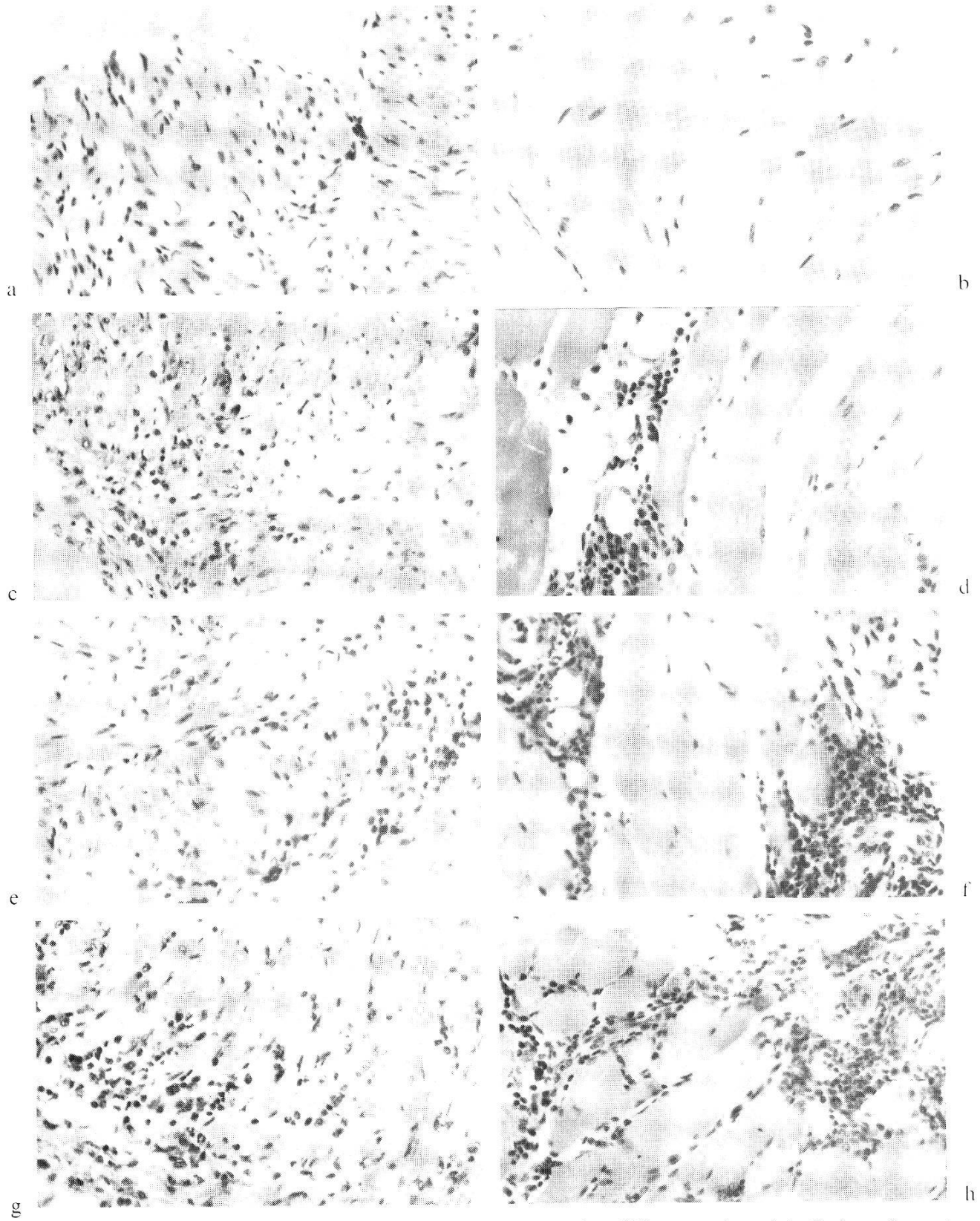

Fig. 1. Myocarditis and myositis (skeletal muscle) intensity differences in adult Swiss mice: a) control mice normal myocardium; b) control mice normal skeletal muscle; c) mild myocarditis, nodular inflammatory infiltrate; d) mild myositis, perivascular and interstitial mononuclear infiltrate can be seen; e) moderate myocarditis; diffuse inflammatory infiltrate in the ventricular wall; f) moderate myositis; diffuse inflammatory infiltrate among the muscle fibres; g) severe myocarditis; a diffuse mononuclear infiltrate can be seen beneath the epicardium and among the ventricular fibres; h) intense myositis; severe muscle atrophy with replacement of adipose tissue; scanty infiltrate remains among the muscle cells.

more, in experiments (not shown) with BALB/c mice, which did not develop spontaneous myocarditis, the animals immunized with the F fraction did not present heart lesions at all. On the other hand, those animals receiving Mc fraction had an intense myocarditis and a mild myositis. These effects are not attributable to the different protein doses used, since in another experiment (not shown) in which 1000 μg of both the F and the Mc fractions were used, similar results were obtained. In those mice given P5 and Cs, myositis and myocarditis were less frequent and of lower intensity (Table 1).

In order to discount the possibility that the myocarditis induced by Mc might be caused by a direct toxic effect, the short-term action of the administration of high doses of this fraction was studied. The animals killed within the first 72 h post-inoculation did not show lesions at the level of heart or skeletal muscle. In the animals injected with only one dose and studied 10 and 120 days post-inoculation, the incidence of myocarditis in terms of number of mice with lesions/total number of animals studied (1/22 and 2/9, respectively) and myositis (0/22 and 0/8) was minimal and the lesion patterns at both times were similar. On the other hand, animals infected with 3 doses and studied 37 days after the first inoculation, showed a high incidence of myocarditis (13/19) and a lower incidence of myositis. Observations at 120 days in these animals showed almost the same degree of myocarditis (13/20) and myositis as those at 37 days.

Challenged mice

In the animals challenged with 25 trypomastigotes, parasitemia could be only demonstrated by xenodiagnosis. Thus, the results obtained by direct observation of blood samples were persistently negative. Parasites were found in 50% of the non-immunized and challenged controls. Immunized and challenged groups of animals presented different numbers of mice showing parasitemia (Table 1).

The fewest number of animals with positive xenodiagnosis were those immunized with F, and the highest number (similar to that found in controls) were those immunized with Mc. As expected, non-immunized, non-challenged controls did not present a parasitemia. Histopathological study revealed that, in the non-immunized but challenged controls, a high incidence of myocarditis and myositis was present: 14/17 and 13/17 animals, respectively. In the animals immunized with Cs or Mc, lesions similar to those observed in the challenged controls were present (Table 1). The lowest incidence of myocarditis was found in the group immunized with F (Table 1). Pre-immunization with P5 and subsequent parasite challenge, produced a more intense and frequent myocarditis than that present in the group immunized with F, but less severe than that in the group given the Mc and Cs fraction (Table 1). As regards myositis, animals immunized with F presented the lowest intensity (Fig. 1) and incidence (Table 1). A more intense myositis was observed as a result of challenge in animals immunized with any of the other fractions (Table 1).

Table 1. Heart and striated muscle lesions in mice given subcellular fractions from *T. cruzi* epimastigotes and challenged with *T. cruzi* trypomastigotes

Inoculum challenge	Intensity of myocarditis/myositis				% of animals presenting			
	Number of mice with lesions				myocarditis	myositis	positive xenodiagnosis	pathological ECG
	absent	mild	moderate	severe				
Flagellar (F)								
None	17/20	3/0	0/0	0/0	15	0	0	5
Tryps	12/13	6/3	2/3	0/1	40	35	10	5
5000 g pellet (P5)								
None	12/16	3/0	1/0	0/0	25	0	0	6
Tryps	6/6	4/0	5/4	0/5	60	60	27	7
Microsomal (Mc)								
None	1/17	10/1	5/0	2/0	94	5	0	12
Tryps	3/5	9/5	3/2	1/5	81	70	50	50
Cell sap (Cs)								
None	11/16	4/1	2/0	0/0	35	6	0	50
Tryps	2/2	4/2	2/3	0/1	75	75	40	50
SKS* (Controls)								
None	15/11	2/0	0/0	0/0	11	0	0	0
Tryps	3/4	7/5	7/4	0/4	82	76	38	44

* SKS = 5 M KCl – 0.25 M sucrose

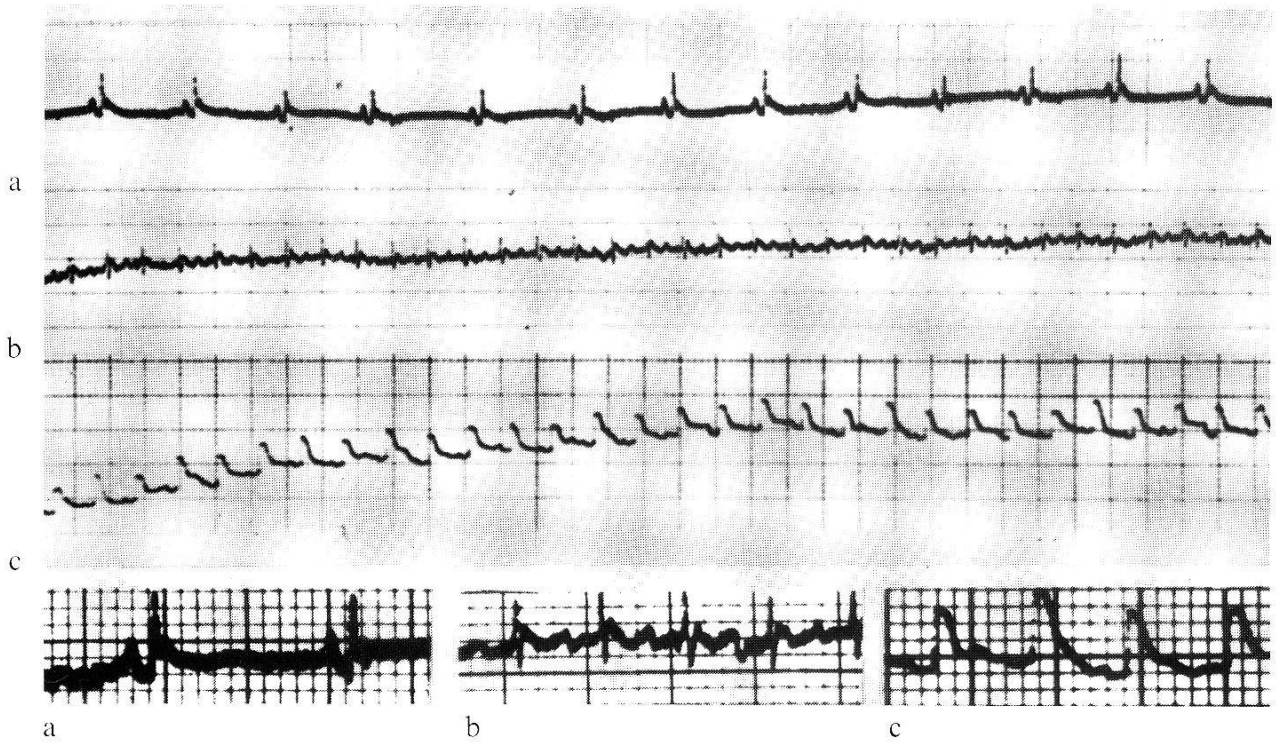

Fig. 2. Electrocardiographic pattern from a) normal mouse (PR = 0.02 sec; QRS = 0.01 sec); b) animal immunized with the Cs fraction; atrioventricular block of the first degree (PR = 0.04 sec); c) animal immunized with the Mc fraction; increase in the length of the QRS complex (QRS = 0.03–0.04 sec).

Pathological ECGs (1st and 2nd degree atrio-ventricular block and QRS complex higher than 0.03 sec, Fig. 2) were observed in 50% of the animals immunized with CS, whereas those animals immunized with F, P5 and Mc presented pathological ECGs in only 10% or less of the animals studied (Table 1). No abnormal ECGs were observed in the controls injected with SKS. When challenge was performed, the mice immunized with F or P5 fractions did not modify the incidence shown by the non-challenged animals. The mice immunized with Mc or Cs, on the other hand, behaved like the challenged controls.

Specific anti-*T. cruzi* antibodies were demonstrated by IIF in the sera of mice immunized with the different subcellular fractions, before and after challenge. The mice showed antibody titres between 1:4 and 1:64 before challenge and higher titres, from 1:16 to 1:128, after challenge, irrespective of the fraction used as immunogen (Table 2).

Discussion

Our results indicate that Mc and Cs fractions contained components able to induce a myocarditis similar to that found in chronic experimental Chagas' disease in mice (Laguens et al., 1980).

The induction of myocarditis in mice after immunization with *T. cruzi*-antigens is in agreement with the results of Teixeira et al. (Teixeira and Santos-

Table 2. Titres of anti-*T. cruzi* antibodies detected by indirect immunofluorescence against formolated epimastigotes, in sera of mice immunized with different subcellular fractions from *T. cruzi* epimastigotes, without or with challenge with virulent trypomastigotes

	Number of mice									
Subcellular fraction:	F		P5		Mc		Cs		Control	
Challenge:	none	tryps	none	tryps	none	tryps	none	tryps	none	tryps
Inverse of titre:										
128	0	0	0	1	0	2	0	2	0	2
64	0	8	0	6	0	3	1	3	0	5
32	3	10	1	6	6	7	2	1	0	3
16	14	2	8	2	8	5	8	2	0	5
8	2	0	6	0	4	0	5	0	0	2
4	1	0	1	0	0	0	1	0	4	0
<4	0	0	0	0	0	0	0	0	7	0

Buch, 1974; Teixeira et al., 1975), who described a myocarditis in rabbits immunized with a 100,000 *g* pellet obtained from a mixture of amastigotes plus trypomastigotes. This effect was not specific for *T. cruzi*, since the microsomal fraction of the non-pathogenic Trypanosomatid *Crithidia fasciculata* was also capable of inducing similar tissue lesions (Cabeza Meckert et al., 1984).

Immunization with these fractions seems to have little effect on skeletal muscle, which might suggest the existence of different pathogenic mechanisms for the production of damage to heart and skeletal muscle.

The incidence of altered electrocardiograms among the groups of immunized mice without challenge suggests that components of the Cs fraction of *T. cruzi* could participate in the aetiology of this cardiac alteration in Chagas' disease. The mice immunized with the Mc and the Cs fractions and challenged behaved like the non-immunized, challenged mice in this respect. On the other hand, the animals immunized with the F and P5 fractions of *T. cruzi* epimastigotes were completely protected against the development of altered electrocardiograms. The pattern of the electrocardiographic alterations shown by the animals immunized with the Mc or Cs fractions, and challenged, as well as that of the controls, is similar to that found in humans in endemic areas of *T. cruzi* infection, where Chagas' disease is the main cause of atrioventricular block in the inhabitants younger than 50 years old (Amorim et al., 1979).

These results might be interpreted as a dissociation of the pathogenic effects on the heart, obtained with two different *T. cruzi* fractions. This suggests that different components of the parasite are able, by themselves, to induce the complex of effects configuring chronic chagasic cardiopathy (Andrade and Andrade, 1979). Moreover Mc, at doses used in this study does not induce acute damage to heart muscle and, thus, does not exist a direct toxic effect. The fact

that animals sensitized with only one dose do not develop significant lesions even after 120 days, indicates the need of repeated doses of antigens to attain heart damage. This was confirmed when repeated doses, over the same period, were able to induce lesions.

These results indicate that the Mc fraction from *T. cruzi* contains components able to induce myocarditis in mice, after repeated immunization, thus giving support to the opinion that immunopathological mechanisms are important for the production of damage in chronic Chagas' disease (Andrade and Andrade, 1979).

In the animals immunized with Mc or Cs, no decrease in parasitemia, as shown by the number of animals with positive xenodiagnosis, was observed as a result of immunization.

Among the subcellular fractions of *T. cruzi* assayed in this work for immunoprotection, the flagellar fraction was the most effective. This activity was demonstrated in the chronic model of Chagas' disease in Swiss mice obtained by challenge with few parasites; this model is much nearer the real situation in nature than the acute model previously employed (Segura et al., 1977). Parasitemia was demonstrated only by xenodiagnosis, as in the case of human Chagas' disease. Although all the groups presented parasitemia after challenge, the incidence in parasitemia differed between groups (Table 1).

A lower number of mice with parasitemia was observed among animals immunized with F and P5 fractions, whereas mice immunized with the other fractions behaved similarly to the controls given SKS. Mice preimmunized with F also presented a lower number and intensity of heart and striated muscle lesions.

A decrease in parasitemia, in animals protected by immunization with F and P5, is in agreement with the protection obtained when an acute challenge is performed on animals immunized with F (Segura et al., 1977) or with a glycoprotein from epimastigote membranes (Scott and Snary, 1979). In acute infection, immunization with these antigens produces significant decrease in parasitemia, and high survival (Segura et al., 1977; Scott and Snary, 1979).

The specific anti-*T. cruzi* IgG antibodies, detected by IIF using formolated whole epimastigotes as antigen, remain for at least 120 days after the last immunizing dose (sacrifice day). The higher antibody titres observed in the challenged mice confirm that the immunoprotection obtained with the F fraction was not complete, as detected by the finding of one mouse with positive xenodiagnosis. Another explanation could be that all mice were immunized with only one subcellular fraction, while the challenge includes the whole parasite, containing a greater variety of antigens. Since formolated whole epimastigotes were used as antigen for the detection of the antibodies, the enhancement in the immune response after challenge could be due simply to the introduction of new antigens in addition to those present in the immunizing subcellular fraction.

The appearance and titre of antibodies detected in the mice immunized with the different subcellular fractions was independent from the incidence of myocarditis, altered electrocardiograms and immunoprotection. This finding is similar to the lack of correlation between serologically detected antibodies and pathology in human cases of Chagas' disease (Krettli and Brener, 1982).

The results presented in this paper allow for the first time a direct comparison of protective immunity and pathology induced by different subcellular fractions obtained from epimastigotes of *T. cruzi*. If the antigen or antigens responsible for the protective effects of the F fraction could be isolated, it might be possible to obtain an immunogen capable of achieving complete protection, without inducing any pathology in the host, the main and ultimate goal for the immunoprophylaxis of human Chagas' disease.

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