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Immunization against *Trypanosoma cruzi* using killed antigens and with saponin as adjuvant

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Summary

The immunization of mice with killed epimastigotes or trypomastigotes with saponin SPL as adjuvant was challenged by inoculation of blood or metacyclic trypomastigotes of *T. cruzi*. Immunogenic preparations were obtained by freeze-thawing or formalin treatment. The protection was effective against homologous and heterologous challenge, and lasted up to 12 weeks after the last immunization. The immunization was also effective against metacyclic trypomastigote challenge. The immunogenicity of the killed *T. cruzi* suspension was retained after freeze-drying.

Introduction

In an earlier paper (Johnson et al., 1963), the protective effect of trypanosome antigen with adjuvants was described. The present paper gives results of further experiments on immunization against *Trypanosoma cruzi* in mice.

Material and methods

Experimental hosts

A single line of outbred albino mice (Evans line) was used, weighing about 20 g (3 weeks old) when first immunized. The mice were fed on a balanced pelleted diet with water ad lib. and kept at about 23° C.

Strains of T. cruzi

Three Brasilian strains were used, *Y*, *Sonia* and *BH* (see Andrade, 1974, for detailed history). All strains were maintained by subcutaneous inoculation of infected blood into clean mice at 7-day intervals.

Cultivation

T. cruzi strains *Y* and *Sonia* were grown in vitro in Boné and Parent's (1963) liquid medium with rabbit or calf serum. Initially 10% serum was used, though this was later reduced to 5%. Rabbit

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serum was preferred, though calf serum was also satisfactory for growth of epimastigotes. The present work on immunization was completed within 5 years of continuous cultivation in vitro.

Cultures were prepared in 5 to 10 ml volumes of medium in 25 ml screw-capped bottles. For stock maintenance these were sub-cultured weekly into fresh medium. For large scale growth, techniques were scaled up to 1 or 2 litres of medium. The initial cell concentration was about 1×10^6/ml and the epimastigotes reached a peak growth of $1–3 \times 10^7$/ml after 4–6 days incubation at 26° C.

Antigen preparation

The antigen used in these studies was a suspension of *T. cruzi* epimastigotes or trypomastigotes killed by different methods. Trypomastigotes from pooled blood from infected mice were prepared following the methods described earlier (Johnson et al., 1963). The epimastigotes of strains *Y* and *Sonia* grown in vitro were harvested by centrifuging at 4° C, and washing three times with cold glucose saline. Suspensions of trypomastigotes were prepared at a concentration of 1×10^8 cells/ml and epimastigotes at 1×10^9/ml. These suspensions were then treated in different ways:

a) Freeze-thawed. The cell suspension was frozen in a solid CO_2/alcohol mixture then allowed to thaw at room temperature. This was repeated three times.

b) Freeze-dried. Suspensions were ampouled in aliquots containing either 10^8 trypomastigotes or 10^9 epimastigotes. The ampoules were then frozen and dried on a Genevac freeze-drier. The powder was resuspended in distilled water before use. A separate ampoule was used for each experiment.

c) Sonicated. The cell suspension was treated in a MSE Ultrasonic Power Unit until all cells were disintegrated, which usually required 3×60 sec at 1.5 amps.

d) Formalin killed. Formalin (AR) was added to give a concentration of 0.05–0.10% v/v, which immobilized the epimastigotes and rendered them non-viable without immediate disintegration.

e) β-Propiolactone killed. An ice cold 10% v/v solution of β-propiolactone was added to the cell suspension giving a final concentration of 1%. It was then incubated at 37°C for 10 min (La Grippo and Hartman, 1955).

After preparation, the suspensions were examined microscopically for the presence of intact motile organisms. If motility was not observed, the treated epimastigote preparations were tested for viability by culturing about 0.1 ml (equivalent to 1×10^8 organisms) in Boné and Parent's medium. No viability tests were routinely carried out on the trypomastigote preparations, but at intervals the tail blood of immunized mice was examined before challenge. No living parasites were detected by culture or in the blood smears.

All killed *T. cruzi* suspensions were stored at $-20°$ C after preparation for not longer than 2 to 3 weeks before use.

Adjuvants

Since previous work (Johnson et al., 1963) had shown that saponin, an extract of *Quillaia* bark, was a successful adjuvant with *T. cruzi*, it was used exclusively. The same batch of saponin SPL (Boake Roberts & Co) was used throughout this work. Solutions were prepared in distilled water and used at a dose of 100 μg in 0.1 ml.

Immunizing procedure and challenge

Experimental immunization was studied in groups of 8 to 10 mice. Each mouse received 0.1 ml of *T. cruzi* suspension (equivalent to 10^8 epimastigotes or 10^7 trypomastigotes) and 0.1 ml (100 μg) of saponin SPL. Saline or water was used if one component was omitted. Antigen and adjuvant were mixed before injection and administered between the scapulae. A second identical injection was given 2 weeks later.

The protection induced by immunization was challenged by the inoculation of blood stream trypomastigotes from infected mice; 10^5 trypomastigotes were inoculated subcutaneously 2 weeks

after the second immunization. In one experiment the immunized mice were challenged with metacyclic trypomastigotes injected subcutaneously. The metacyclic forms were obtained from infected *Rhodnius prolixus*. Adult bugs were fed on infected mice and killed 3 to 4 weeks later. A suspension was prepared in saline of the rectal contents. The proportion of trypomastigotes was determined by microscopical examination of a Giemsa stained smear.

Assessment of result of challenge

The tail blood of each mouse was examined microscopically for trypomastigotes three times per week. The number of trypomastigotes in 20 microscopic fields ($10\times$ ocular, $40\times$ objective) was counted on a thin blood smear. One trypomastigote in 20 microscope fields is equivalent to about 2.5×10^4/ml, while the maximum parasitaemia of 16 parasites in each microscope field represents about 1×10^7/ml. The experiments were generally terminated by the 33rd or 34th day, though mice were sometimes observed for considerably longer, up to 1 year.

The effect of immunization on the parasitaemia was assessed by calculation of a parasitaemia index for each mouse and then the mean mouse determination with its standard error was computed for the group. The mice were checked daily for survivors and the mean survival time and standard error calculated for each group. Since observations were terminated at the 33rd or 34th day the mean survival time is a biased term with a maximum of 33 or 34 days. An occasional death in the absence of trypomastigotes during the period of immunization, was disregarded and omitted from the analysis of results.

Results

1. Comparison of methods of antigen preparation

The protective effect of formalin-killed antigen and comparison with freeze-thawed and freeze-dried antigen is shown in Table 1. The results with homologous (*Y* strain) challenge show that all three trypomastigote preparations (group 2, 3 and 4) were equally effective. Epimastigote antigen prepared by formalin treatment or by freeze-thawing was not significantly different from each other (group 5 and 6) and similar to trypomastigote antigens.

Studies with heterologous challenge (*Sonia* antigen, *Y* strain challenge) (group 7) showed a level of protection equal to that observed in mice protected by the homologous antigen.

Mice challenged with *T. cruzi* strain *Sonia*, again showed that mice immunized with the heterologous *Y* strain antigen were as effectively protected as those immunized with the homologous *Sonia* antigen.

One year later the survivors of groups 9, 10 and 11 were challenged with 10^6 blood stream trypomastigotes of *Y* strain. No mice died and microscopic examination of tail blood (12 examinations of each group over 34 days after rechallenge) detected trypomastigotes in one microscopical examination in group 10 (1/8 mice) and group 11 (1/4 mice), but none in group 9 (0/5 mice).

Antigen prepared from β-propiolactone inactivated *T. cruzi* epimastigotes with saponin was equivalent in activity to formalin prepared antigen with saponin (Fig. 1). Without adjuvant, the antigens were less effective. Saponin alone was not effective as measured by the mean survival time, but the parasitaemia index was reduced to 59 ± 3.2 as compared to the parasitaemia index of the untreated group, 128 ± 34.

Table 1. Comparison of methods of antigen preparation. All vaccines contained 100 μg saponin SPL. Period of observation 33 days.

Group	Antigen	Proportion of survivors (%)	Mean survival time ± SE	Parasitaemia index ± SE
	Challenge with T. cruzi Y trypomastigotes			
1.	Untreated	2/10 (20)	20 ± 2.3	70 ± 9.8
	Y trypomastigotes			
2.	Formalin	8/9 (89)	31 ± 1.9	17 ± 9.7
3.	Freeze-thawed	7/9 (78)	29 ± 2.6	6.2 ± 2.4
4.	Freeze-dried	10/10 100	>33 ± 0	11 ± 6.3
	Y epimastigotes			
5.	Formalin	6/10 (60)	28 ± 2.4	16 ± 7.8
6.	Freeze-thawed	9/10 (90)	32 ± 1.4	7.5 ± 3.0
	Sonia epimastigotes			
7.	Formalin	7/9 (78)	29 ± 3.1	31 ± 9.5
	Challenge with T. cruzi Sonia trypomastigotes			
8.	Untreated	1/9 (11)	26 ± 1.1	79 ± 17
	Sonia epimastigotes			
9.	Formalin	10/10 (100)	> 33 ± 0	2.9 ± 0.79
	Y epimastigotes			
10.	Formalin	10/10 (100)	> 33 ± 0	4.9 ± 1.7
	Y trypomastigotes			
11.	Formalin	5/7 (71)	31 ± 1.4	8.1 ± 3.0

Comparison of freeze-thawed antigen with antigen prepared from *T. cruzi* strain *Y* epimastigotes ruptured by ultrasound showed no difference between the two antigens when given with saponin (Fig. 2). Both antigens increased the survival time and decreased the mean parasitaemia. Vaccine containing the freeze-thawed antigen was significantly better at reducing the parasitaemia, 11 ± 3.5 compared to 24 ± 8.8.

2. Influence of varying antigen dose

Since the significant protection described above was obtained with a standard dose of antigen, attempts were made to obtain a high level of protection using more antigen. Using an 8-fold variation of strain *Y* antigen with a uniform 100 μg dose of saponin, no graded effect was seen with homologous challenge (Fig. 3). However with heterologous challenge (strain *BG*), the highest dose was significantly better than the lower dose of antigen.

3. Duration of protection against challenge

A group of mice was immunized with *T. cruzi* strain *Y* blood stream trypomastigote freeze-thawed antigen with 100 μg saponin as adjuvant. The mice

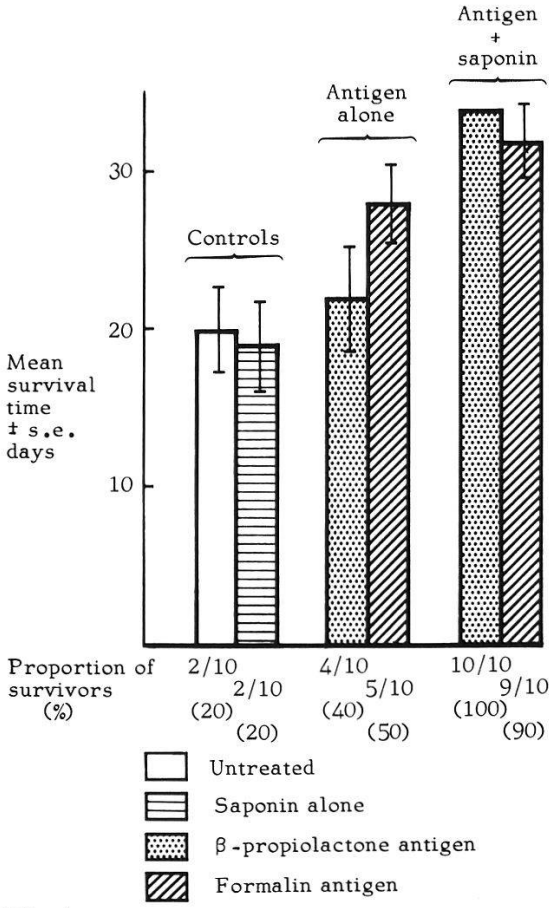

Fig. 1

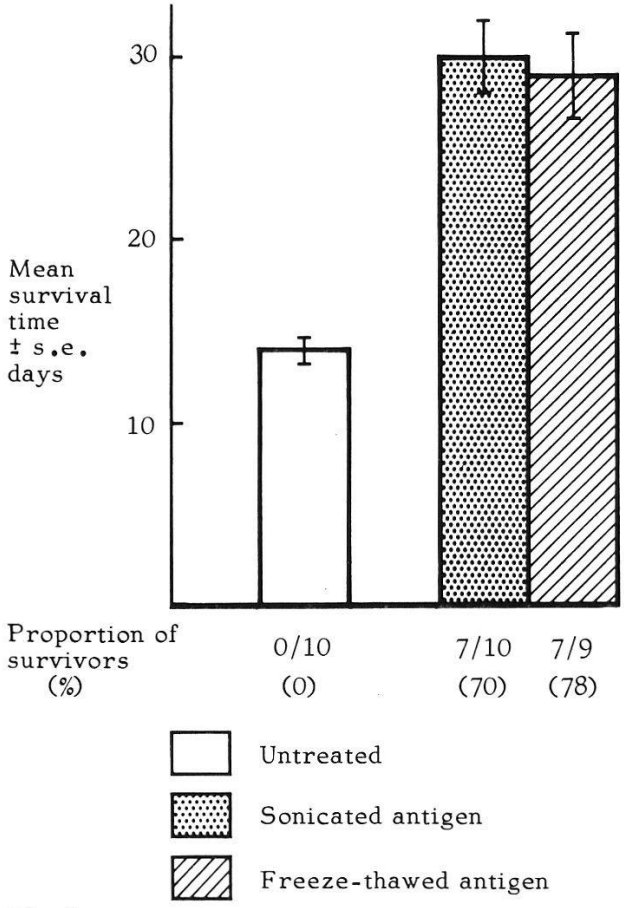

Fig. 2

Fig. 1. Comparison of epimastigote antigen (Sonia strain) inactivated by formalin or β-propiolactone. Saponin used as adjuvant. Mice challenged with blood stream trypomastigote of *T. cruzi* strain *Y*. Period of observation: 34 days.

Fig. 2. Comparison of antigen prepared by freeze-thawing or sonicating a suspension of *T. cruzi* strain *Y* epimastigotes. Saponin used as adjuvant. Challenge with blood stream trypomastigote of *T. cruzi* strain *Y*. Observation period was 33 days.

were challenged at intervals with *T. cruzi* strain *BG* blood stream trypomastigotes, using non-immunized mice from the original batch, for inoculum control at each time interval.

This experiment showed that maximum protection was present up to the 4-week challenge time (Table 2). In fact, the reduction of parasitaemia index was better at the 4-week than the standard 2-week challenge time. At the 8th and 12th week, the parasitaemia index in the immunized mice was increasing though there was still considerable protection of mice against death. However, by the 16th week no significant protection remained.

4. Stability of freeze-dried antigen

The success described above of small scale trials of freeze-dried trypomastigote antigen prompted a further study of stability. Freeze-dried epimastigote antigen was stored in sealed ampoules at different temperatures for 4 months,

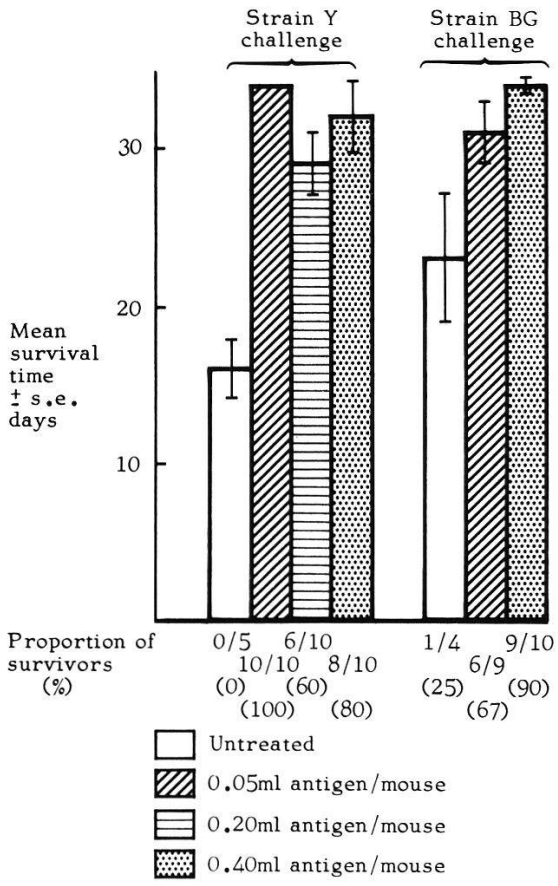

Fig. 3. Effect of 8 fold variation in antigen dose. Antigen prepared by formalin treatment of *T. cruzi* strain *Y* trypomastigotes. Immunity challenged with homologous and heterologous parasites. Saponin used as adjuvant. Observation period was 33 days.

Table 2. Duration of protection after immunization with bloodstream trypomastigotes *T. cruzi* strain *Y* antigen prepared by freeze-thawing and challenged with BG bloodstream trypomastigote. Saponin was used as adjuvant and the period of observation after challenge was 34 days.

No. of weeks after immunization	Treatment	Proportion of survivors	Survival time days ± SE	Parasitaemia index ± SE
2	Immunized	10/10 (100)	>34±0	6.2±2.5
	Control	3/9 (33)	27±2.5	25±10.8
4	Immunized	8/8 (100)	>34±0	2.7±0.6
	Control	–	–	–
8	Immunized	6/8 (75)	30±2.9	22±8.6
	Control	0/9 (0)	20±2.1	46±10.0
12	Immunized	6/9 (67)	28±3.1	35±16.7
	Control	2/10 (20)	22±2.9	92±24.9
16	Immunized	4/7 (52)	26±3.5	40±13.9
	Control	5/9 (56)	28±2.5	70±46.1

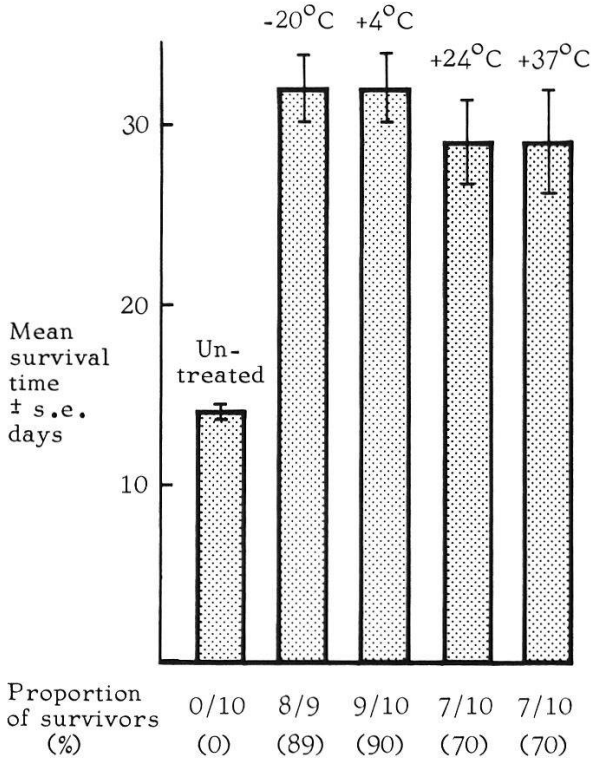

Fig. 4

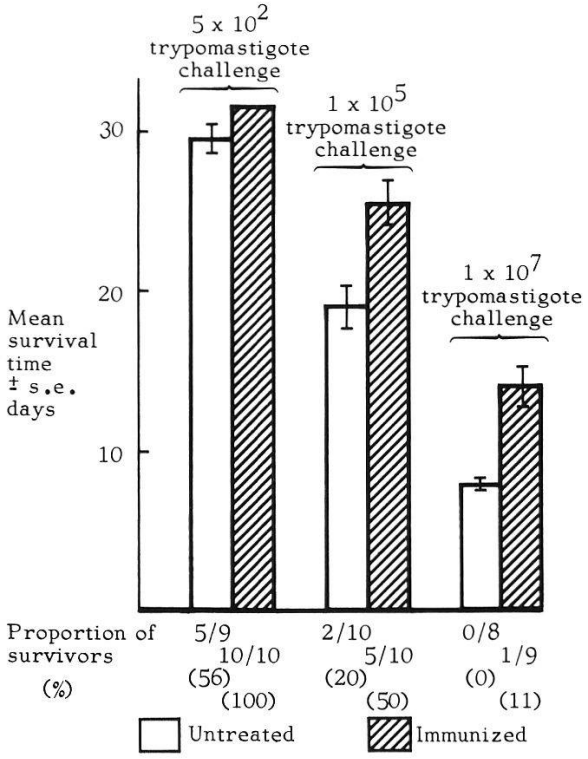

Fig. 5

Fig. 4. Protective effect of freeze-dried antigen of *T. cruzi* after storage at different temperatures for 4 months. Antigen prepared from strain *Y* epimastigote cultures with saponin used as adjuvant. Challenge was blood stream trypomastigotes of strain *Y*.

Fig. 5. Effect of increasing challenge on protection of immunized mice. Formalin-killed antigen prepared from *T. cruzi* strain *Y* epimastigote cultures with saponin as adjuvant. Observation time of 33 days. Protection challenged with blood stream trypomastigote of *T. cruzi* strain *Y*.

then reconstituted by resuspending in distilled water. Saponin at the standard dosage of 100 μg in 0.1 ml per mouse was added to the reconstituted antigen at the time of immunization. The results in Fig. 4 show that protection was not lost after prolonged storage at high temperatures. Neither the survival time nor parasitaemia index showed any correlation with temperature of storage.

5. The effect of increasing challenge

The protection observed so far was determined after challenge with 10^5 blood stream trypomastigotes. The increase of challenge in untreated mice showed a progressively shortening of the survival time (Fig. 5). The protection afforded by immunization with *T. cruzi* strain *Y* epimastigote formalin-killed antigen plus saponin was observed at all levels of challenge, though the proportion of mice surviving decreased with increasing challenge. At the lowest challenge rate of 5×10^2 trypomastigotes/mouse, although the survival time was not markedly different between untreated and immunized groups, the parasitaemia index was reduced in the immunized group, being 33 ± 9.2 and 5.0 ± 1.8 respectively.

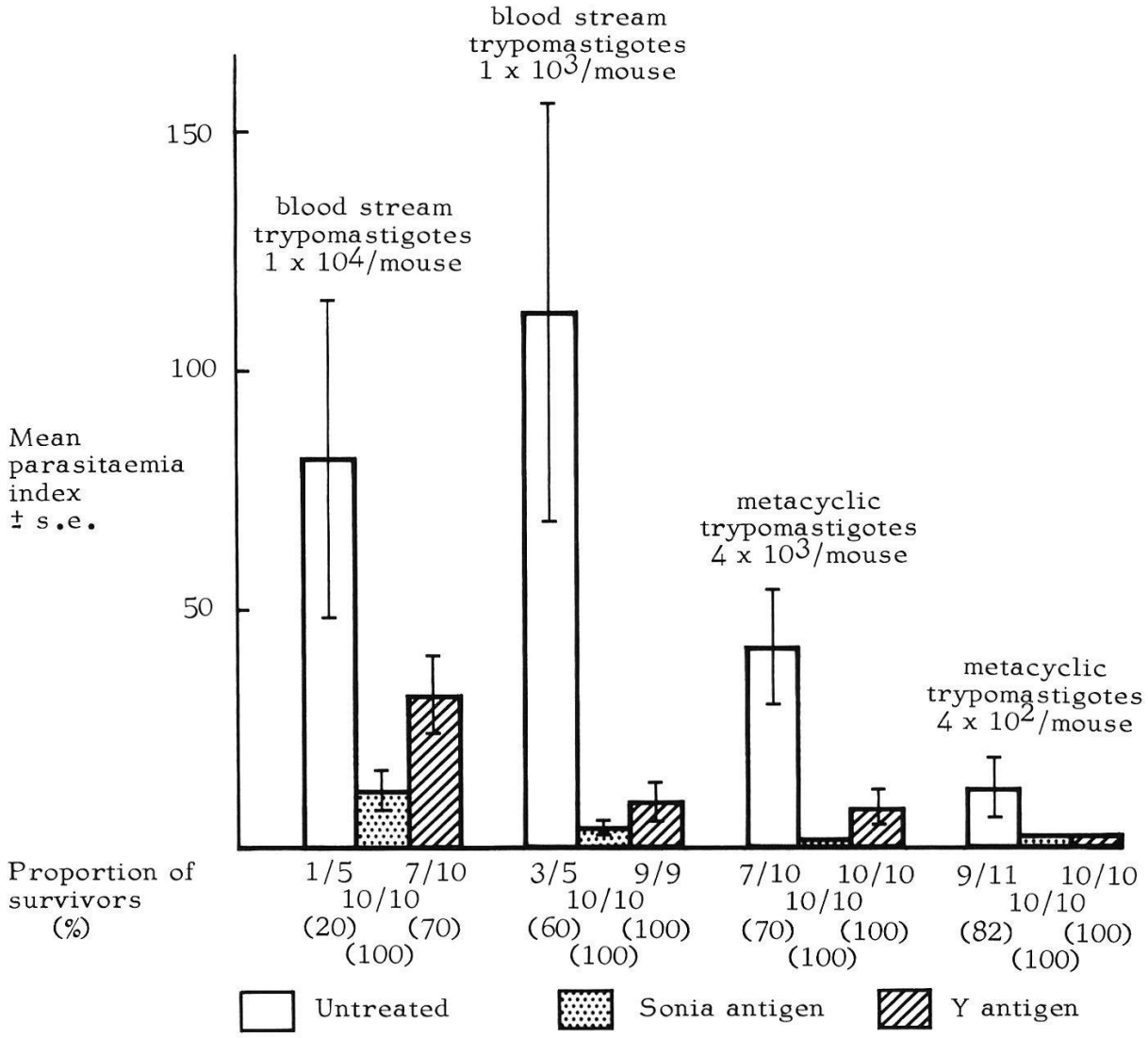

Fig. 6. Comparison of protection again blood stream trypomastigote *Sonia* and metacyclic trypomastigote *Sonia* challenge. Antigen prepared from formalin killed *T. cruzi* strains *Sonia* and *Y* epimastigote culture with saponin adjuvant. Experiment observed for 34 days.

6. Effect of challenge with metacyclic trypomastigotes

The effect of the natural infective stage was studied with strain *Sonia.* Two challenge levels were used of metacyclics obtained from infected *Rhodnius prolixus* bugs and blood stream trypomastigotes from infected mice. The mice were immunized with epimastigote formalin-killed antigen prepared from *Sonia* or *Y* strains cultures. Saponin was used as adjuvant at the standard dose. The parasitaemia index is used to describe the results since *Sonia* strain was less virulent particularly with metacyclic trypomastigote inoculum.

The experiment showed that the immunization protected against the challenges used in terms of both survival and reduction of parasitaemia index (Fig. 6). In the case of the two bloodstream trypomastigote and the higher metacyclic challenges, the protection was greatest against the homologous immunization.

The parasitaemia indices observed after the lowest metacyclic challenge were not large enough to allow detailed analysis, though immunization reduced the parasitaemia index of the untreated group of 12 ± 6.0, to 2.2 ± 0.59 for the group immunized with *Sonia* antigen and to 1.9 ± 0.50 for mice immunized with *Y* antigen.

Discussion

The level of protection produced by the standard immunizing method of crude *T. cruzi* antigen plus saponin as adjuvant, has shown that a high proportion of mice can be protected against death from a lethal challenge and the parasitaemia is considerably reduced. Such mice show a transient parasitaemia, and even the few microscopically negative animals would probably be shown to have latent infection by more sensitive methods of detection.

There is a marked contrast between the duration of artificial immunization and of protection afforded to mice recovered from infections. It is not known whether the mice at the time of rechallenge were infected, but it is known that mice with sub-patent infections have a high resistance to reinfection (Cohen, 1975).

As regards the source of antigen, blood stream trypomastigotes were not consistently better from the epimastigotes derived from culture. However, epimastigote suspensions were 10 fold more concentrated than trypomastigote suspensions. Since epimastigotes can be grown with relative ease in a variety of diphasic and monophasic liquid media, they are clearly the more practical source of antigen. No technique emerged as being superior to others amongst the various methods of antigen preparation tested. Freeze-thawed and formalin-treated antigens are simple to prepare and will be adopted in further work.

An interesting result was the preservation of immunogenicity after freeze-drying. This is contrary to the reports of Gonzalez-Cappa et al. (1968), though they have recently been more successful with storage of a low pressure-release antigen at 4° C (Gonzalez-Cappa et al., 1976). If the above results are confirmed, the method provides a practical method for preservation of antigenic potency.

The results with β-propiolactone inactivation of *T. cruzi* were similar to those obtained by Soltys (1967) with *T. brucei.* However, the details of the inactivation procedure used in the two investigations are different.

Cross protection between strains was reported by Gonzalez-Cappa et al. (1974) and the present data confirm their conclusions. This aspect clearly needs more detailed study, although it would appear that lack of cross-protection is a much smaller problem with *T. cruzi,* than with the *T. brucei* group of organisms (Gray and Luckins, 1976).

The challenge when given by the intraperitoneal route has been shown by McHardy (in press) to be less severe than when given by the subcutaneous route. Thus immunization procedures may appear more effective.

A comparative study with other published methods of antigen preparation (e.g. Gonzalez-Cappa et al., 1968; Kierszenbaum and Budzko, 1975) would be desirable. Further methods of immunization being currently investigated by other workers involve the use of living *T. cruzi* either non-virulent through prolonged cultivation in vitro or attenuated by various physical and chemical means (see Cohen, 1975, for recent summary). However in the present state of knowledge of the chemotherapy and pathology of Chagas' disease, careful consideration needs to be given to the use of living *T. cruzi* for immunization on the large scale.

The saponin adjuvant used in the present work greatly improves the level of protection produced by the antigen. Further chemical studies are required to define what at present is an unknown mixture of compounds extracted from tree bark.

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