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Protective Effects of Disintegrated Culture form of *Trypanosoma cruzi* on the Mortality of Mice after Challenge

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Although some investigators have dealt with the preparation of a vaccine against experimental Chagas' disease, vaccination with *Trypanosoma cruzi* killed by chemicals, *i.e.* thimerosal (Muniz, *et al.*, 1946), formalin (Hauschka *et al.*, 1950), and merthiolate (Kagan and Norman, 1961) resulted in ineffectiveness. Johnson *et al.* (1963) reported successful immunization in mice using *Trypanosoma cruzi* killed by physical means, alternate freeze-thawing. Goble *et al.* (1964) have examined protective activity of immunization with the cultured organisms killed by various physical means, such as pressure, ultrasonication or shaking with glass beads. Subsequently, the organisms disrupted by ultrasonication (Garcia, 1967), by pressure (González Cappa, *et al.*, 1968; Yanovsky *et al.*, 1969), and by lyophilization (Menezes, 1965) have been used by some investigators as vaccine materials. These antigens prepared by physically killed organisms were found to be effective against the following challenge. It appears to be interest to investigate the relationship between the intensity of protective antigenicity of the parasite and the physical condition of the parasites when they were disrupted by physical means. Furthermore, it appears of interest to analyze the protective antigenicity of subcellular components of the parasites.

The present investigation was undertaken in order to determine whether the subcellular components of the living parasites fractionated by differential centrifugation have a protective effect or not.

Materials and Methods

The Tulahuén strain of *Trypanosoma cruzi* was used in the present experiment. The organisms used for the preparation of antigen were cultured at 29°C in 200 ml tissue culture bottle containing 25 ml of modified LIT medium. The composition of LIT medium which was stated in the previous papers (Fernandes and Castellani, 1966; Kaneda, 1971) was slightly modified in the present experiment; crystalline bovine hemoglobin (200 mg/1,000 ml basic mixture) and the solution of ox liver infusion after boiling at 100°C for 15 minutes and filtration by cheese cloth were mixed into the basic mixture. The organisms cultured for one week were collected and washed three times with 10 mM tris-HCl buffered 0.25 M sucrose solution (pH 7.4). Finally, the organisms were washed once with the sucrose solution containing certain ions. After the last washing, the organisms collected were weighed, disrupted by means of alternate freeze-thawing, and resuspended in the sucrose solution at a concentration of 250 mg or 500 mg of the organisms per ml. The later suspension was mixed with same volume of Freund's complete adjuvant. In case of intravenous inoculation, the suspension was adjusted to a concentration of 1,000 mg of the organisms per ml. In order to check presence of the surviving organisms in the inoculum, the suspension was examined by microscope and cultivation in modified NNN medium. The modified NNN medium

was composed of defibrinated rabbit blood-agar slant and a 0.8 ml overlay of glucose saline. The culture was examined for the presence of the living organisms one month after cultivation.

Four kinds of antigenic inocula were prepared as follows: (1) suspension of whole disrupted organisms; (2) sediment and supernatant at 3,500 g; (3) sediment and supernatant at 12,000 g; (4) sediment and supernatant at 105,000 g. The first inoculum was prepared from the whole organisms disrupted by alternate freeze-thawing and then suspended in a 10 mM tris-HCl buffered 0.25 M sucrose solution (pH 7.4) containing 1 mM $MgCl_2$, 3 mM $CaCl_2$ and 25 mM KCl. The second was prepared by centrifugation of the disrupted organisms suspension at 3,500 g for 30 minutes. The third was prepared by centrifugation of the supernatant obtained by the second procedure at 12,000 g for 30 minutes. In this case, the organisms were suspended in a tris-HCl buffered 0.25 M sucrose solution containing 1 mM EDTA. The fourth was prepared by centrifugation of the supernatant of the third procedure at 105,000 g for 90 minutes. All sediments were suspended in adequate volume of the sucrose solution to be a concentration of 250 mg of the organisms per ml. All preparations were made or produced at 4°C.

Concentrations of protein in the sediment and supernatant at 105,000 g were determined by Lowrly's method (Lowrly, *et al.*, 1951).

As soon as the all antigenic suspensions were prepared, male DDY mice weighing 16–18 g were injected intraperitoneally with one ml of the suspensions alone or mixture of 0.5 ml antigenic suspension and 0.5 ml Freund's complete adjuvant. Mice were injected with 0.2 ml of the supernatant at 3,500 g for 30 minutes into the tail vein. To make sure of the absence of the infection caused by the surviving trypanosomes, the peripheral blood was drawn from the eye of the experimental mice 14 days after the injection of antigen and inoculated in modified NNN medium.

The mice were challenged by injecting intraperitoneally with 10^5–10^6 trypanosomes at various intervals after the immunization. The organisms which were maintained in mice by serial blood passage every 10 to 14 days were isolated from the blood of the heavily infected mice by Yaeger's method (Yaeger, 1960) and used for challenge. After the challenge, survival time of the mice was observed. Blood of dead mice was examined microscopically for the detection of the trypanosomes.

Results

Whole disrupted Trypanosoma cruzi suspension.

When the suspension of *Trypanosoma cruzi* disrupted by means of freeze-thawing was used as antigenic materials, it was recognized that some of the mice survived after the challenge infection, as is shown in Table 1. Seven mice of the experimental group survived over three months, although all the control mice died within one month. However, there was no prolongation in the survival time of experimental mice comparing with that of control group. Interval of three weeks between the immunization and the challenge seemed to be more effective in prolongation of the survival time than that of other intervals, as is shown in Table 1.

In order to enhance the protective efficacy of the inoculum, Freund's complete adjuvant was added to the suspension. As is shown in Table 1, the addition of the adjuvant did not result in increase of the survival time of the experimental mice.

And then, effect of the repetition of the immunization on the enhancement of protective activity was examined by using disrupted organisms as an antigen. Experimental mice were inoculated intraperitoneally three times on every five days with the disrupted organisms, whereas the control mice were immunized only one time. The challenge infection with the blood forms trypanosomes was performed four weeks after the first immunization in experimental and control mice. As is shown in Table 2, six mice in the experimental group and three mice in

Table 1 Effect of vaccination with killed *Trypanosoma cruzi* on survival of mice received subsequent challenge infection

Series of experiment	No. of mice used	Adjuvant	Interval between inoculation and challenge (weeks)	Challenge dose ($\times 10^4$)	Deaths by one month after challenge	
					No. of mice died	Mean survival time & standard deviation (days)
1	8	+	1	18	8	15.1±1.3
	8	−	1	18	8	13.1±1.2
2	9	+	2	70	9	12.8±2.7
	9	−	2	70	9	10.4±0.7
3	6	−	3	23	3	18.3±6.1
	6	−	3	23	6	15.0±2.9
4	10	+	3	17	10	8.9±1.1
	10	+	3	17	10	9.3±1.7
5	8	−	3	12	4	19.5±4.3
	8	−	3	12	8	19.5±4.8
6	8	+	4	17	8	17.0±3.3
	8	+	4	17	8	16.8±4.0
7	5	−	4	29	5	9.2±1.6
	5	−	4	29	5	6.4±1.9

For each experiment, data on inoculated mice appear on the first line; data for control mice on the second.

Table 2 Effect of repeated vaccination with killed *Trypanosoma cruzi* on survival of mice received subsequent challenge infection

	No. of mice used	Challenge dose ($\times 10^4$)	Deaths by one month after challenge	
			No. of mice died	Mean survival time & standard deviation (days)
Experiment	11	18	5	20.4±2.5
Control	11	18	8	17.6±4.2

Vaccination was made three times every five days.
All mice were challenged four weeks after the first inoculation.
The control mice were inoculated with killed organisms once at the first inoculation.

the control group survived more than one month after the challenge. All the survivors in the experimental group survived more than three months after the challenge. In the control group, two mice died on the 40th and 43rd day after the challenge, and another mouse survived more than three months. The result of this experiment seemed to indicate that the protective effect of the disrupted organisms was increased by the repetition of the immunization. The blood of the survivors was examined three months after the challenge, and all survivors showed parasitemia in low level.

Sediment and supernatant at 3,500 g.

In order to determine the protective effect of the fractionated organisms, mice were inoculated intraperitoneally with the sediment or supernatant obtained by centrifugation at 3,500 g for 30 minutes, and survival of the mice against the subsequent challenge was observed. The challenge infection was made by a lethal dose of blood form trypanosomes three weeks after the immunization. The results are shown in Table 3. All the control mice died within 14 days after the challenge,

Table 3 Effect of vaccination with centrifugal fractions of disrupted trypanosomes upon subsequent challenge infection: (1) Sediment and supernatant by centrifugation at 3,500 g for 30 minutes

Series of experiment	Inoculum	No. of mice used	Interval between inoculation and challenge (weeks)	Challenge dose ($\times 10^4$)	Deaths by one month after challenge	
					No. of mice died	Mean survival time & standard deviation (days)
1	None	5	3	6	5	9.2±2.5
	Sediment	5	3	6	5	9.4±1.5
	Supernatant	5	3	6	5	8.2±1.7
2	None	4	3	25	4	12.3±1.3
	Sediment	4	3	25	4	14.5±1.8
	Supernatant	4	3	25	3	14.3±2.6
3	None	6	3	41	6	11.3±2.2
	Sediment	6	3	41	6	12.3±0.7
	Supernatant	6	3	41	6	11.2±1.5

Table 4 Effect of vaccination with centrifugal fractions of disrupted trypanosomes upon subsequent challenge infection: (2) Sediment and supernatant by centrifugation at 12,000 g for 30 minutes

Inoculum	No. of mice used	Challenge dose ($\times 10^4$)	Deaths by one month after challenge	
			No. of mice died	Mean survival time & standard deviation (days)
None	9	20	9	14.0±1.7
Sediment	9	20	8	14.8±5.7
Supernatant	9	20	8	16.5±2.2

The sediment or supernatant was mixed with Freund's complete adjuvant.
The interval between inoculation and challenge was three weeks.

and all experimental mice died within one month excepting one mouse which died on the 43rd day after the challenge. Average survival period of the experimental mice which died by one month after the challenge did not show any signiffcant difference from that of the control mice. Moreover, the trypanosomes were detected in the blood of all dead mice by microscopic examination.

Sediment and supernatant at 12,000 g.

Subsequent to the above experiment, 3,500 g supernatant was centrifuged at 12,000 g for 30 minutes. Mice were inoculated intraperitoneally with the sediment or supernatant at 12,000 g and their survival was observed after a subsequent challenge. Non-immunized mice were used as control. The experimental and control mice were challenged by inoculating 20×10^4 blood form trypanosomes intraperitoneally three weeks after the immunization. The results are shown in Table 4. All the control mice died within 16 days after the challenge. One of the mice inoculated with the sediment survived more than four months after the challenge. It showed parasitemia of low level when the blood was examined two months after the challenge. This mouse was

Table 5 Effect of vaccination with centrifugal fractions of disrupted trypanosomes upon subsequent challenge infection: (3) Sediment and supernatant by centrifugation at 105,000 g for 90 minutes

Inoculum	No. of mice used	Challenge dose ($\times 10^4$)	Deaths by one month after challenge		Protein concentration (mg/100 ml)
			No. of mice died	Mean survival time & standard deviation (days)	
None	9	15	9	16.6±4.5	—
Supernatant	9	15	8	15.6±4.4	18.75
Sediment	9	15	7	15.1±2.8	21.88

The interval between inoculation and challenge was three weeks.
The sediment or supernatant was mixed with Freund's complete adjuvant.

killed for detection of the trypanosomes four months after the challenge, however, the trypanosomes were not detected by cultural examination of the blood. One of the mice inoculated with the supernatant died four months after the challenge. It showed parasitemia by microscopic examination. Relatively marked prolongation of the survival time was recognized in supernatant-immunized mice comparing with those of control and sediment-immunized mice.

Sediment at 105,000 g and final supernatant.

As the supernatant at 12,000 g showed a slight protective effect against a challenge, the supernatant at 12,000 g was furthermore centrifuged at 105,000 g for 90 minutes. Experimental mice were immunized intraperitoneally with the sediment or the final supernatant and were challenged three weeks after the immunization. The survival period of the experimental and control mice are shown in Table 5. All the control mice died by one month after the challenge. However, one of the mice inoculated with the final supernatant and two mice inoculated with the sediment survived for four months after the challenge. These mice were killed four months later and their bloods were proved to have no trypanosomes by both microscopic and cultural examinations. These results may indicate that the inoculation of the sediment and supernatant at 105,000 g have incomplete protective effect on the challenge infection.

Intravenous inoculation with antigenic materials.

In order to increase an efficacy of the antigenic materials, mice were inoculated intravenously with 0.2 ml of the supernatant which was prepared by the concentration of disrupted organisms at 3,500 g for 30 minutes. Intact mice were used as control. The experimental and control mice were challenged by inoculating intraperitoneally with the blood form of the parasites. As is shown in Table 6, both experimental and control mice died within 18 days after the challenge showing ineffectiveness of the immunization on the subsequent challenge.

Discussion

Goble and co-workers (Goble *et al.*, 1964; Goble, 1970) have investigated antigenicity of *Trypanosoma cruzi* killed by various physical means such as ultrasonication, pressure, or shaking with glass beads. They showed that the antigens prepared were protective against a lethal challenge in mice. Johnson *et al.* (1963) used freeze-thawing method for disrupture of the blood form trypanosomes suspended in saline. The vaccines thus prepared were effective on experimental Chagas' disease only when given in combination with some sorts of adjuvants among which saponin was the most effective. Judging from the results of the previous experiments,

Table 6 Effect of intravenous inoculation with the supernatant at 3,500 g of disrupted trypanosomes on survival of mice received subsequent challenge infection

Series of experiment	No. of mice used	Interval between inoculation and challenge (weeks)	Challenge dose ($\times 10^4$)	Deaths by one month after challenge	
				No. of mice died	Mean survival time & standard deviation (days)
1	5	1	50	5	11.2±1.1
	5	1	50	5	12.6±1.0
2	4	2	50	4	11.5±1.1
	4	2	50	4	11.0±1.2
3	6	3	71	6	11.0±0.6
	6	3	71	6	11.0±0

For each experiment, data on inoculated mice appear on the first line; data for control mice on the second.

physical methods in disrupting the cells are almost universally more effective than chemical means as a procedure to prepare vaccine. The present experiment also indicated that the inoculation of the culture organisms killed by alternate freeze-thawing had a protective effect against a lethal challenge infection. However, it was not shown that the addition of Freund's complete adjuvant increased a resistance to challenge in mice. In order to keep activity of the components originated from the disrupted cells in normal, the preparations for antigen were suspended in tris-HCl buffered 0.25 M sucrose solution. The mice inoculated intraperitoneally with these suspensions showed some degree of protection against a challenge. However, use of the sucrose solution failed in increase of protective activity.

The repetition of the immunization was effective to enhance the protection against a lethal challenge. It was assumed, from these results, that the second or third immunization had a booster effect on the manifestation of the protection. In the present experiment, intravenous immunization with antigenic materials showed no protective effect at all. The intravenous inoculation seems to be ineffective on production of protective antibody in mice.

By inoculation of the fractionated organisms, it was indicated that the sediment at 12,000 g and at 105,000 g and the final supernatant had a slight protective effect against a lethal infection. Above all, the inoculation of the sediment at 105,000 g showed a most remarkable protection so far as the present experiments concern. The sediment at 12,000 g in the present experiments corresponds to a heavy mitochondria and related fragments, and the sediment at 105,000 g corresponds to a microsome and related particles, as was stated by Boiso and Stoppani (1971). Therefore, it seems to be reasonable to suppose that the microsomal fraction has a protective antigenicity. This result agrees with that of Oka and Osaki (1963) who investigated on the protective antigenicity of fractions of *Trichomonas foetus*.

Seneca and Peer (1964) indicated that *Trypanosoma cruzi* had 9 antigens; A, B, C, D, E, F, G, H, and X; sonic lysates of cultured organisms contained antigens A, B, C, D, E, F, G, and H, saline supernatant had antigens A, B, and F, and phenolized organisms contained A, B, G, and X, while the phenolized cells preserved for long period contained only B. Seneca and Peer (1966) and Seneca *et al.* (1966) reported that im-

munization with "Chagastoxin" (lipopolysaccharide) extracted with phenol from the parasites showed a prolongation of survival time against a challenge when the chagastoxin was administrated twice a week. Furthermore, they pointed out that the chagastoxin contained antigen B and the antigen B had an unstable component which had protective effect. The present result in which fractions containing microsome and related particles showed a protective antigenicity is suggestive that antigen B was derived from microsome.

Summary

The present investigation dealt with the protective antigenicity of the culture forms of *Trypanosoma cruzi* disrupted by freeze-thawing. Furthermore, comparison of antigenicity was made on some fractions obtained by differential centrifugation of the cells. The parasites which were disrupted and suspended in tris-HCl buffered 0.25 M sucrose solution were centrifuged at 3,500 g for 30 minutes, at 12,000 g for 30 minutes, and at 105,000 g for 90 minutes. The disrupted organisms, sediments and supernatants were inoculated intraperitoneally into mice. At various intervals after the inoculation, mice were challenged by inoculating intraperitoneally with lethal doses of the blood form trypanosomes. Survival period of the mice and number of survivors after the challenge were indicative of protective effect of the inocula. From these results, it was concluded that whole disrupted organisms, the sediment at 12,000 g, and 105,000 g, and the final supernatant had a protective effect against a challenge infection in some degrees. The sediment 105,000 g which may correspond to the fraction of microsome and related particles showed the highest effect. Enhancement of the protective antigenicity of the inocula was recognized by the repetition of the immunization but not by addition of Freund's complete adjuvant. Intraperitoneal inoculation of the antigenic materials resulted in no effect on increasing the protective antigenicity.

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Trypanosoma cruzi の防御抗原の研究

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Trypanosoma cruzi の感染死を防御するワクチンの作製はいままでに種々試みられてきた．それによると物理的に虫体を破砕する方法が化学的に破砕する方法より優れているように思われる．今回の実験では，培養した Tulahuén 株を用い，細胞構成物の生理的活性を維持させるような破砕方法および破砕条件を用いて防御抗原を作製し，それによる防御力を検討した．また，破砕虫体を遠心分画法によつて分画し，各々の細胞構成物の防御力についても検討された．すなわち次の種類の抗原について防御力の有無の検討が試みられた．(1) 破砕虫体浮遊液，(2) 3,500 g×30 分の上清と沈渣，(3) 12,000 g ×30 分の上清と沈渣，(4) 105,000 g×90 分の上清と沈渣．この4種の抗原は所定のイオンを含むトリス—塩酸緩衝庶糖液 (pH 7.4) に浮遊した．この浮遊液単独または Freund's complete adjuvant との混合物をマウス腹腔へ接種した．3,500 g の上清はマウス尾静脈より接種する方法も試みられた．接種後1，2，3，4週後に致死量の血液型虫体で攻撃を行ない1ヵ月以内のマウスの死亡率，生存日数を観察した．対照群のマウスは1ヵ月以内に死亡したが，破砕虫体浮遊液を接種したマウス19匹中7匹が1ヵ月以上生存していた．また，adjuvant と混合して接種した場合には防御力の増加は認められなかつた．破砕虫体浮遊液を5日間隔で3回接種した場合には1回接種のマウスより防御力が増加したと考えられる．これは2回または3回目の接種による booster 効果と考えられる．3,500 g の上清または沈渣を接種したマウスでは上清を接種した群に少数の生存マウスを認めた．また，この上清を静脈より接種した場合には感染死を防御する効果はなかつた．12,000 g の上清または沈渣を接種したマウスのうち攻撃後生存していたマウスは少数であつた．105,000 g の上清と沈渣を接種したマウスでは，沈渣を接種した群の方が上清接種群より生存マウスが多かつた．以上のいずれの抗原を接種した群でも1ヵ月以内の生存日数を対照群と比較して延命は認められなかつた．

以上の結果，破砕虫体の生理的活性を維持することによつて防御力の増大の試みは期待できなかつた．また，105,000 g の沈渣すなわち microsome 分画の接種により感染死防御力をマウスに与えることが可能と思われた．