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The Membrane Characters of Different Forms of *Trypanosoma cruzi*

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In culture conditions, the lytic effect of normal human and guinea pig sera on the crithidial form (epimastigote) of *Trypanosoma cruzi* (*T. cruzi*) has been observed by Muniz and Borriello (1945). The same lytic effect was obtained with fresh sera from several species of mammals except mouse (Rubio, M. 1956). In all cases, the metacyclic trypanosomes (trypomastigotes) were not affected.

In this report, the lytic effect of various mammalian sera was examined on the amastigote form *in vitro* which appeared in our modified monophasic medium. The characterization of the surface antigen and electronmicroscopic observation of the amastigotes as compared with other forms were also undertaken.

Materials and Methods

The parasite

Tulahuen strain of *T. cruzi*, obtained from the National Institute of Health, Bethesda, Maryland, U.S.A. through the Keio University, Tokyo, Japan, and maintained in the laboratory by syringe passage in mice and in the NNN diphasic medium was used.

The blood forms of *T. cruzi* from infected mice were inoculated into test tube containing about 5 ml of the modified monophasic medium and cultured at 37°C for collecting the amatigote forms. Subcultures were made every 7 days.

The modified monophasic medium used for this study consists of 1% trypticase, 0.5% glucose, 0.8% NaCl and 5% defibrinated and hemolyzed rabbit blood.

Lytic effect of normal rabbit, guinea pig and human sera

Trypanosomes from the monophasic medium after the 12 th transfer were collected and concentrated to about 10^8 per ml by centrifugation at 2,000 rpm for 5 min at room temperature. To 1 ml of each of the sera was added 0.2 ml of concentrated parasites. The mixture was kept for 1 hr at 37°C and then centrifuged again. The sediments were smeared on slide glass, stained with Giemsa and examined under the microscope. The same strain which had been maintained in the NNN diphasic medium for more than one year and contained epimastigotes in about 98% was used as a control.

Electronmicroscopy

The amastigotes obtained after the 4 th transfer in the monophasic medium which contained amastigote form in about 96%, the epimastigotes from the NNN diphasic medium, and the trypomastigotes from infected mice were separately collected and fixed at 4°C for 1 hr in collidine buffer pH 7.4, containing 4% gluteraldehyde. After washing the specimens at least 3 times in collidine buffer, post-fixation in 1.5% osmic acid for 1 hr was done. The specimens were dehydrated in a series of increasing concentrations of ethanol followed by propylene oxide and then a 1 : 1 mixture of propylene oxide and resin. The whole dehydration process took 2 hours, after which the speci-

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mens were transferred into resin and left overnight in a dessicator. After polymerization in increasing incubator temperature (37°C, 45°C, 60°C) for at least 3 days, sections were cut and stained with uranyl acetate for 30 min at 45°C and in lead citrate for 3 min at room temperature. Washed specimens in grids were dried and micrographs were taken by a Hitachi 11 DS electron microscope.

Analysis of surface antigen(s) by the fluorescent antibody

Trypanosomes in the monophasic medium from the 5th to 10th transfer were pooled, collected and washed at least 4 times in saline by centrifugation at 2,000 rpm for 10 min at room temperature and then stored at -25°C. Equal volumes of trypanosomes and Freund's complete adjuvant were mixed well and used to immunize rabbits by intramuscular inoculation of 1 ml of the mixture. Immunization was done 5 times at seven days interval. Seven days after the last immunization, the rabbit was exsanguinated by cardiac puncture, the blood was collected and the antiserum was conjugated with fluorescein isothiocyanate (FITC).

Conjugation of antiserum with FITC was done according to the method of Riggs *et al.* (1958). The conjugated antiserum was passed through Sephadex G-25 and then fractionated in a stepwise manner through DEAE cellulose column with phosphate buffer at 0.005 M, pH 8.0; 0.03 M, pH 7.2; and 0.1 M, pH 7.0. The fraction eluted by 0.03 M was concentrated by polyethylene glycol 6,000, dialyzed in phosphate buffer saline (PBS), pH 7.2 and used for direct staining of the blood form trypanosomes (trypomastigotes) collected from previously infected mice, and the epimastigotes from the diphasic medium.

For staining of the amastigote, the conjugate was absorbed by the epimastigotes from long established cultures, until the conjugate lost its agglutinating ability for the epimastigotes. Amastigotes obtained after the 8th transfer in the modified monophasic medium was used for staining.

Living parasites were mixed with the conjugate for 10 min at room temperature (Klein 1966). Afterwards, the trypanosomes were washed in PBS until the excess and unreacted conjugate were removed. Observation under the fluorescent microscope was done immediately.

Results

Table 1 shows the relative frequency of the 3 types of *T. cruzi* during 10 serial passages at 37°C in the monophasic medium after transfer from infected mice.

The sera obtained from rabbits, guinea pigs and men showed the unanimous lytic action on the epimastigotes while they showed the action on neither the trypomastigotes nor the amastigotes. Table 2 shows the result with one rabbit serum. The epimastigote lysis was observed in Photos. 1-6.

Electronmicroscopic observations show distinct differences among the different forms as regards the cell membrane. Both the amastigote and the trypomastigote have much thicker membrane than the epimastigotes (Photos. 7, 8, 9). Although the former two are provided with thick cell membrane, the trypomastigotes exhibit short, fine, irregular bristle-like projections while the amastigotes show a relatively smooth, compact surface.

Table 1 The relative frequency of the 3 types of *T. cruzi* during 10 serial passages at 37°C in the monophasic medium after transfer from infected mice

Subculture	Trypomastigote (%)	Epimastigote (%)	Amastigote (%)
1			100
2		1	99
3		1	99
4	1	3	96
5	2	7	91
6	2	26	72
7	1	13	86
8	1	20	79
9	1	25	74
10	2	37	61

Table 2 The percentage of the three forms and the destructed cells of *T. cruzi* before and after treatment with a rabbit serum

	Trypomastigote (%)	Epimastigote (%)	Amastigote (%)	Destructed cell (%)
The culture forms in the diphasic medium before treatment	2.0	98.0		
The same above after treatment	1.9			93.1
The culture forms from the 12th passage in the monophasic medium before treatment	12.8	34.0	53.2	
The same above after treatment	10.1	0.9	67.7	41.3

The FITC conjugated antibody, after absorption by the epimastigotes, stained only the amastigotes (Photo. 10) while both epimastigotes and trypomastigotes were left unstained. The unabsorbed conjugate stained the agglomerated epimastigotes (Photo. 12). The blood form trypanosomes (trypomastigotes) were not stained with the unabsorbed conjugate except a few (Photo. 13).

Discussion

The lytic effects of normal sera from various animals to the epimastigotes were observed by Muniz *et al.* (1945) and Rubio (1956). The lytic action is suggested to be an immunological reaction by natural antibody which needs complements because inactivated serum can agglutinate the epimastigotes but not lyse.

The results from the present study consistently show the resistance of the amastigotes to the lytic action of rabbit, guinea pig and human sera as like as the typomastigotes. Electronmicroscopy revealed the presence of thick cell membrane in both the trypomastigote and the amastigote. Although not as distinctive as the outer thick layer (Kubo, 1968) or the "surface coat" in salivarian trypanosomes (Vickerman, 1969), the fine, bristle-like projections on the bloodstream form (trypomastigote) of *T. cruzi* and the smooth, compact surface of the amastigote were observed. By fluorescent antibody technique, the surface substance of the amastigote was shown to be antigenically different from the epimastigote, because the fluorescent antibody absorbed with epimastigote stained it.

The existence of different surface antigen on the trypomastigote (blood form) was indicated from the fact that the used fluorescent antibody didn't stain it.

These findings may explain why the sera are unable to lyse the two forms.

Since the amastigote used in this study is the form produced in the monophasic medium, it is difficult to say whether the same characteristics are true for the intracellular amastigotes. Inoki *et al.* (1973) noted the presence of villi-like structures on the surface of the form released from the destruction of infected cells *in vitro*. It is assumed that such structures may also produce resistance to the lytic action of mammalian sera because of the same reason.

Summary

Amastigote form of *T. cruzi* produced in the monophasic medium was found to resist against the lytic action of rabbit, guinea pig and human sera as like as trypomastigote form.

The surface membrane and antigen of different forms of *T. cruzi* were investigated in order to elucidate the resistant mechanism against the lytic effect. Electron-

microscopic observation showed that both amastigote and trypomastigote forms have surface coats surrounding the whole body. Fluorescent antibody technics revealed that the surface antigen of each form was different one another.

Acknowledgement

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Trypanosoma cruzi の膜表面構造に関する研究

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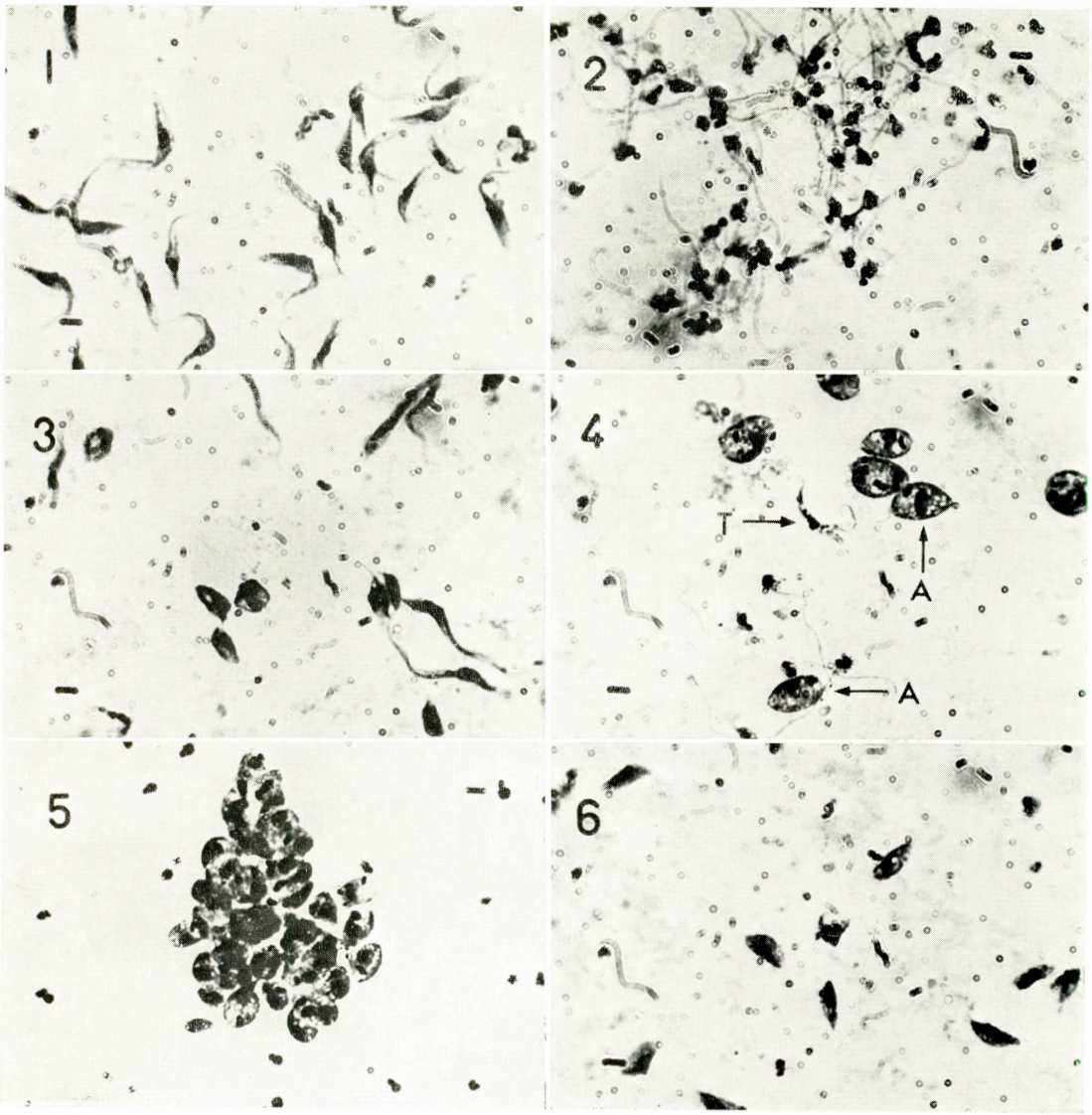
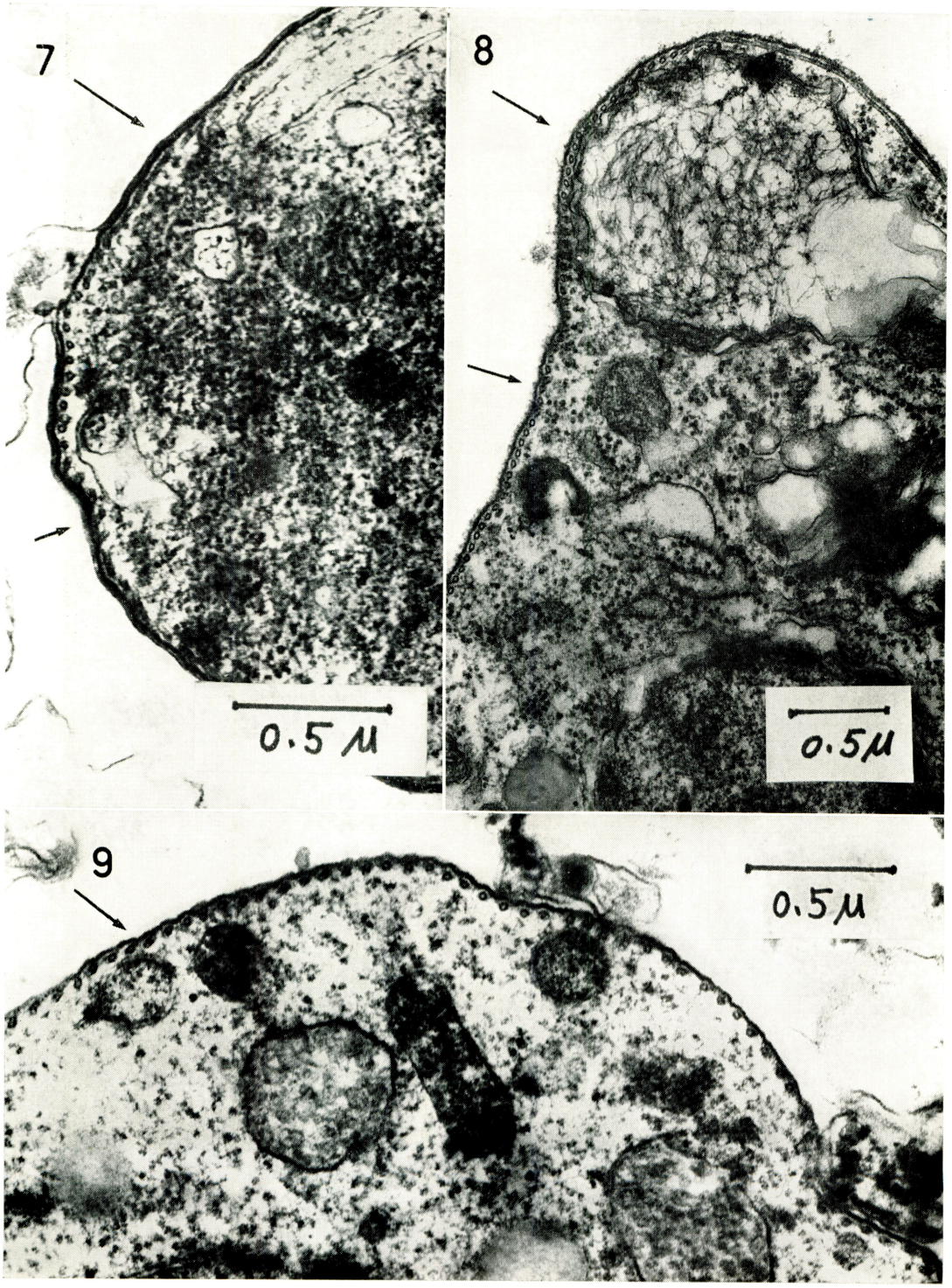
Trypanosoma cruzi の Epimastigote form はマウスを除くいろいろの動物の正常血清により溶解されるが Trypomastigote はこの作用に抵抗する．われわれは感染マウスより新しく液体培地中に移された原虫が多くの Amastigote form をふくむことを利用し，家兎，モルモット，人血清の Amastigote に対する作用を検討するとともにそれぞれの型の膜の性質を電顕および螢光抗体法を用いて調べてみた．その結果 Amastigote は Trypomastigote と同様被験動物血清の溶解作用に対して抵抗性であつた．

電顕的観察では Amastigote は一見二重膜様にみえる滑らかな Surface coat をもつ．一方血中 Trypomastigote はその表面が不規則な突起様構造物で被われている．しかし Epimastigote にはそれらの構造は認められない．

螢光抗体法を用いた生鮮染色を行うと，培養 Amastigote，血中 Trypomastigote はそれぞれ抗原性の異る表面物質で被われていることがわかつた．



Photos. 1–6 show the lytic action of the sera.
Photo. 1. The culture forms in the diphasic medium consisting of 98% epimastigote.
Photo. 2. The lytic effect of a rabbit serum to the epimastigote. The epimastigotes were lysed completely.
Photo. 3. The culture forms from the 12 passages in the monophasic medium consisting of the three forms.
Photo. 4. The lytic effect of a rabbit serum to the culure forms in the monophasic medium. The amastigotes (A) and trypomastigote (T) were not lysed.
Photo. 5. The same treatment as above was done with a guinea pig serum and the same result was shown.
Photo. 6. The same with a human serum and the same result was seen.
Photos. 7–9 show the electronmicroscopic observation on the three forms.
Photo. 7. The amastigote has the smooth and compact surface coat.
Photo 8. The fine bristle-like surface coat was shown on the trypomastigote.
Photo. 9. No surface coat was seen on the epimastigote.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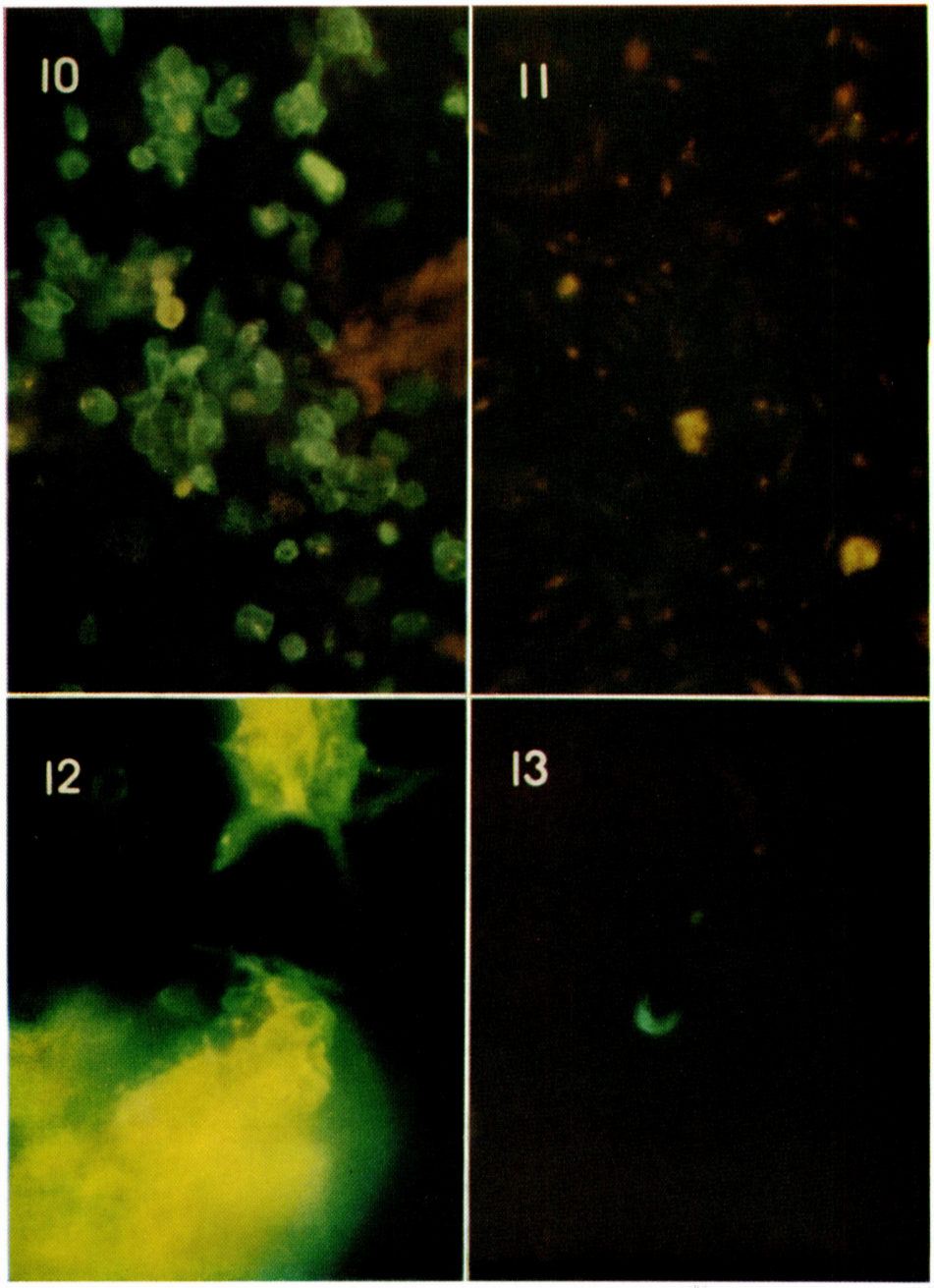

Photos. 10–13 show the observation with fluorescent antibody technics.

Photo. 10. The fluorescent antibody absorbed with the epimastigotes stained the amastigotes but not other forms.

Photo. 11. The fluorescent antibody absorbed with the epimastigotes was confirmed to have lost the staining ability to the epimastigote.

Photo. 12. The non-absorbed fluorescent antibody stained the agglomerated epimastigotes.

Photo. 13. The stained trypomastigote with the non-absorbed fluorescent antibody was shown but almost others were not stained.