Brief Definitive Report

STUDIES ON THE SELECTIVE LYSIS AND PURIFICATION OF *TRYPANOSOMA CRUZI*

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Studies in this laboratory have been examining the interiorization, subcellular localization, and fate of *Trypanosoma cruzi* within mouse peritoneal macrophages. Employing the mixed population of parasites available from culture medium, it had been noted that trypomastigotes survive and multiply within the macrophages whereas epimastigotes are largely destroyed (1). To define the intracellular fate of this organism in more detail, methods were required to separate the forms and establish infection with purified populations. It was noted in the course of these experiments that fresh, normal sera had the ability of selectively lysing epimastigotes whereas trypomastigotes were unaffected. Although this observation had been made previously by others (2, 3), the mechanism remains poorly understood.

In this report we describe that the lysis of epimastigotes by human, rabbit, and guinea pig sera is dependent upon the alternate pathway of complement activation and the use of this phenomenon to purify trypomastigotes in a dense albumin column.

Material and Methods

Parasites. The Y strain of *T. cruzi* was obtained from Dr. S. C. Correa (I. Oswaldo Cruz, Rio de Janeiro, Brazil). The Tulahuén and Racoon Maryland strains were obtained from Dr. B. Bloom (Albert Einstein School of Medicine, Bronx, N. Y.). The parasites were grown in Tobie's medium and passed weekly (4). Parasites grown in the serum-free medium of Boné and Parent (5) were used for certain experiments. Parasites were harvested from 8-day old cultures, washed two times with phosphate-buffered saline (PBS) (Dulbecco's, Grand Island Biological Co., Grand Island, N. Y.) at 750 *g* for 20 min, and counted in a hemacytometer with a 40 × phase objective. The Y strain cultures contained 85% epimastigotes and 15% trypomastigotes and transition forms and the Talahuén and Rc Md strains over 95% epimastigotes. Ca^{++} and Mg^{++} ions were omitted from wash solutions in experiments where the effect of divalent metal ions was studied.

Complement Reagents. Guinea pig serum and cobra venom factor (CVF) were obtained from Cordis Laboratories, Miami, Fla. The C4-deficient guinea pig serum was a gift from Dr. V. Nussensweig, New York University School of Medicine, New York). Human and rabbit sera were obtained from normal donors. All sera were used fresh or stored at −70°C and thawed just before use. Guinea pig serum diluted 1:4 was incubated for 30 min at 37°C with CVF and kept at 4°C until assayed. Zymosan (Sigma Chemical Co., St. Louis, Mo.) was boiled for 30 min in water, washed five times in PBS and incubated with guinea pig serum (1:3) at 17°C for 60 min to remove selectively properdin (6).

Sensitized Erythrocytes. Sensitized sheep erythrocytes (EA) were prepared by incubating 1 vol of 5% washed sheep erythrocytes (Animal Blood Centre, Syracuse, N. Y.) with 1 vol of 1:100 dilution of rabbit antisheep hemolysin (GIBCO) for 15 min at 37°C. The cells were washed and resuspended in PBS to 1%. Ethyleneglycol-bis-(β-aminoethyl ether) *N*, *N'*-tetracetic acid (EGTA) and ethylenediamine tetracetic acid (EDTA) were purchased from Sigma Chemical Co.

Platelet-Rich and Platelet-Poor Rabbit Plasma Sera. These were prepared by the method of Hirsch (7).

Lytic Assay. 50 μl of 1% SRBC or trypanosomes (5×10^5 culture forms) and 50 μl dilutions of normal sera were incubated at 37°C for 30 min and lysis scored by counting red cell ghosts or lysed epimastigotes with a 40 × phase objective. The serum dilutions refer to final dilutions in each tube.

Absorption of Sera. 1 ml of serum diluted 1:2 in PBS was absorbed with 2×10^7 trypanosomes at 4°C for 60 min, centrifuged (4°C) and the process repeated. Heat-inactivated sera (56°C for 30 min) after a single absorption no longer agglutinated *T. cruzi*.

Purification of Trypomastigotes. Parasites were incubated for 30 min at 37°C with 25% fresh normal serum. The suspension was then diluted with 2 vol of PBS, centrifuged at 750 *g* for 20 min and resuspended in 3 vol of PBS and 1 vol of a 1.052 $g \cdot cm^{-3}$ albumin solution, to a concentration of 6×10^7 parasites/ml. 1 ml of the suspension was layered on top of 8 ml bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Chicago, Ill.) solution in PBS, pH 7.2–7.4, with a density of 1.085 $g \cdot cm^{-3}$ and centrifuged at 10,000 *g* for 40 min at 4°C in a Sorvall RC-2B centrifuge (Dupont Instruments, Sorvall Operations, Newton, Conn.). The live trypomastigotes and transition forms were recovered from the interface between the overlay and the albumin column, diluted in 5 vol of PBS, and centrifuged at 750 *g* for 20 min. The resuspended parasites were counted and viability assessed by their motility.

Results

Incubation of fresh normal human, rabbit, and guinea pig sera with trypanosomes lysed all the epimastigote forms, leaving trypomastigotes and transition forms unaffected. A kinetic study had shown that lysis reached completion by 30 min, and further incubation up to 2 h did not increase the number of forms lysed.

The presence of natural agglutinating antibodies in these sera did not seem to influence the lytic process. The agglutination titer for the epimastigote forms of all the sera tested was never higher than 1:20. Absorption with trypanosomes of the same strain being tested abolished the agglutinating activity without affecting the epimastigote lysis. Fig. 1 shows the dose-response curve for the two times absorbed and nonabsorbed sera. There is no significant difference between the two curves.

Factors released by the clotting process or by platelets were not responsible for the epimastigote lysis since plasma, platelet-poor and platelet-rich plasma sera were no different from blood sera in their lytic capacity. The lysis of epimasti-

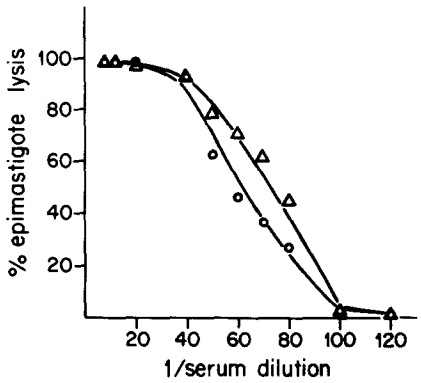

FIG. 1. Guinea pig serum induced lysis of Y strain epimastiogotes. The serum was unabsorbed (Δ) or absorbed twice (O) with trypanosomes.

gotes by fresh, normal human, rabbit, and guinea pig sera was completely abolished by treatment with 10 mM EDTA or by heating to 56°C for 30 min. suggesting that the process involved complement (Table I).

The role of the alternate pathway of complement activation was then studied. Treatment of guinea pig serum with several concentrations of CVF for 30 min at

TABLE I

Effects of Serum Pretreatment on the Lysis of T. cruzi Epimastigotes.

Serum*		Lysis	
Source	Treatment	Trypanosomes‡	Erythrocytes§
		%	%
Guinea pig	EGTA 2 mM $MgCl_2$ 3 mM	>90	<1
	EGTA 2 mM $MgCl_2$ 3 mM $CaCl_2$ 2 mM	>90	>90
	EDTA 10 mM	<1	<1
	50°C/30 min	<1	>90
	Zymosan 17°C/60 min	<1	>90
	CVF 1:10 37°C/30 min 1:90 1:180	<10 <50 <75	>90 >90 >90
	56°C/30 min	<1	<1
C4-deficient guinea pig	None	>90	<1
Human	EDTA 10 mM	<1	<1
	EGTA 2 mM $MgCl_2$ 3 mM	>90	<1
	56°C/30 min	<1	<1
Rabbit	EDTA 10 mM	<1	<1
	EGTA 2 mM $MgCl_2$ 3 mM	>90	<1
	EGTA 2 mM $MgCl_2$ 3 mM $CaCl_2$ 2 mM	>90	<1
	56°C/30 min	<1	<1

* Final serum dilutions 1:4 except in CVF (1:16) and zymosan (1:12).
‡ Y Strain.
§ SRBC coated with rabbit antiserum to SRBC.

37°C reduced the lysis of epimastigotes under conditions in which the lysis of EA was not affected. Treatment of sera with 2 mM EGTA inhibited the lysis of epimastigotes. The lytic activity against epimastigotes was restored by addition of excess Mg^{++}. The same concentration of EGTA and Mg^{++} was sufficient to inhibit lysis of EA. Parasites grown in serum-free medium were lysed in an identical fashion. Heating sera at 50°C for 30 min, condition which inactivates C3 Proactivator abolished the lysis of epimastigotes, without affecting the lysis of

EA. Pretreatment of guinea pig serum with zymosan at 17°C for 60 min to selectively remove properdin completely abolished the lysis of epimastigotes, without interfering with lysis of EA. C4-deficient guinea pig serum was fully active in lysing epimastigotes again supporting the conclusion that the alternate pathway of complement activation generates the lytic process. The extent and mechanism of lysis was similar for both the Tulahuén and Racoon Maryland strains.

The selective lysis of epimastigotes was then exploited to prepare purified populations of trypomastigotes and transition forms. After exposure to fresh serum, the viable trypomastigotes and transition forms were found to have an equilibrium density of less than 1.085 $g \cdot cm^{-3}$. Trypanosomes were then overlayed on an albumin column of this density and centrifuged at 10,000 *g* for 40 min. The lysed epimastigotes which are permeable to albumin form a pellet at the bottom of the tube, while the live trypomastigotes and transition forms are recovered at the overlay-column interface. The initial load volume contained 85% lysed epimastigotes and 15% live trypomastigotes and transition forms whereas the population collected at the interface consists of 85% live trypomastigotes and 15% lysed epimastigotes. The recovery of viable forms when compared to the original population varies from 40–50% (Fig. 2). A second cycle of centrifugation yields populations of trypomastigotes and transition forms of greater than 95% purity which are actively motile.

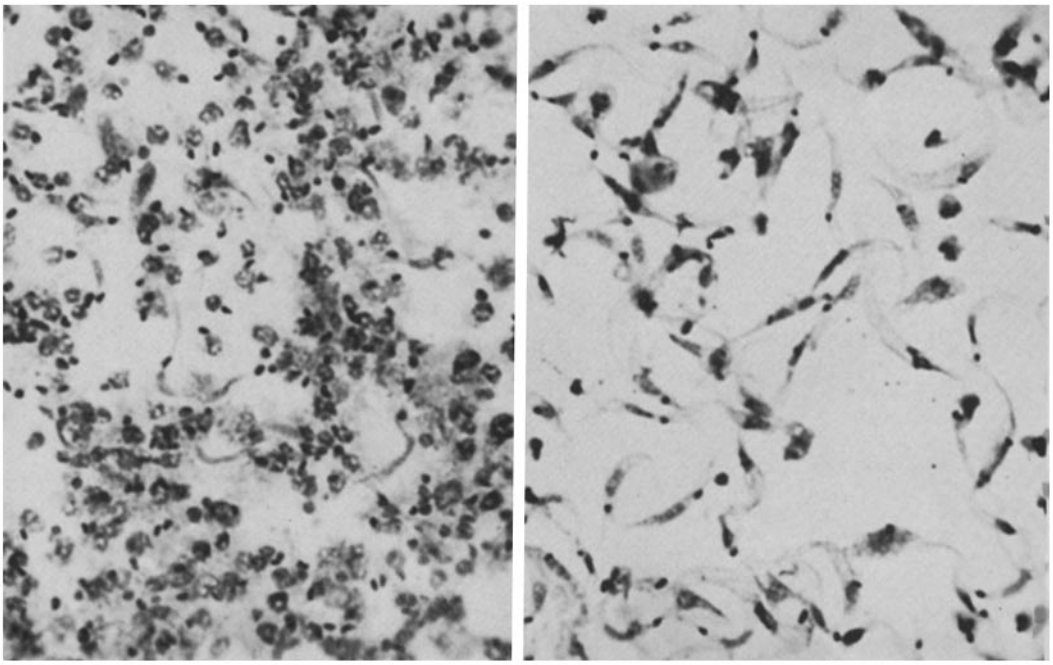

FIG. 2. (A) Trypanosome preparation after serum-induced lysis. The majority of the field is covered with swollen, lysed epimastigotes. Occasional live trypomastigotes are seen. Wright-Giemsa stain. × 1,900. (B) Enriched population of trypomastigotes from the same preparation after separation in a bovine serum albumin column. Wright-Giemsa stain. × 2,560.

Discussion

The fact that a variety of fresh, normal sera are lytic to the epimastigote forms of *T. cruzi* has long been realized (2, 3). It has also been described that the trypomastigote forms can be lysed by normal sera of chicken and frog (3). It has been suggested that the lysis of epimastigotes by immune sera is dependent on the classical pathway of complement activation, and that this mechanism is also involved in the lysis by normal human and guinea pig sera (10).

In this study we re-examined the role of complement in the lysis of epimastigotes by normal sera and the contribution of the alternate and classical pathways of complement activation to the process. The dependence of the process on Mg^{++} ions and not Ca^{++} (8), the heat lability at 50°C for 30 min (9), the inhibition of lysis by the selective removal of properdin with zymosan (6), and the fact that C4-deficient guinea pig serum is fully active in epimastigote lysis all indicate that the alternate pathway of complement activation is sufficient to generate the lytic activity, and that naturally occurring antibody is not essential for lysis. The possibility that the parasites carried absorbed antibodies from the blood-containing culture medium was ruled out by using parasites grown in serum-free medium, with no difference in the experimental results.

Although the alternate pathway was capable by itself of generating lysis under these conditions, lysis will occur in the presence of specific antibodies and consequent activation of the classical pathway. It is possible that surface components of the epimastigote forms trigger the C3 activator system. Those molecules may not be present or may be present in lower amounts on the surface of the trypomastigote and transition forms. This is in keeping with the fact that trypomastigotes can be lysed by specific immune serum and are therefore not insensitive to complement lysis (11).

Summary

The mechanism by which culture forms of *Trypanosoma cruzi* are lysed by normal mammalian sera was examined. Lysis is restricted to the epimastigote form of the organism and is not dependent on the presence of agglutinins. Lysis is a complement-dependent process, the activity being generated by the alternate pathway. The selective lysis by serum was exploited to purify viable trypomastigotes by means of centrifugation in an albumin column. Essentially pure trypomastigote populations are now being employed in cell culture experiments.

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