INFECTION AND IMMUNITY, Nov. 1972, p. 860-864
Vol. 6, No. 5
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Role of Complement in Immune Lysis of *Trypanosoma cruzi*

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Received for publication 5 July 1972

Studies were performed on the mechanism of immune lysis of culture forms of *Trypanosoma* (*Schizotrypanum*) *cruzi*. Antibody-mediated lysis is caused by complement, which is activated via the classical pathway. The properdin system is not required. The kinetics of the reaction is similar to that followed by immune lysis of sensitized sheep erythrocytes, as is the concentration of divalent cations required for optimal lysis (0.15 mM Ca^{2+} and 0.5 mM Mg^{2+}), the occurrence of cell membrane uptake of the complement components C3 and C4, and the development of characteristic ultrastructural modifications on the cell membrane.

The susceptibility to immune lysis of the causative agent of Chagas disease, *Trypanosoma* (*Schizotrypanum*) *cruzi*, varies considerably with the developmental stage of the parasite. Whereas the metacyclic and the blood forms are resistant, the crithidial form undergoes immune lysis readily (16, 19). Fresh sera of various mammals, chicken, and frogs produced lysis of parasites in the crithidial stage, but only chicken and frog sera lysed the metacyclic and blood forms (19). Studies using fowl sera suggested that antibody and complement are the serum factors responsible for the lytic reaction (23). Although the exact role of immune lysis in host defense mechanisms against *T. cruzi* has not been delineated, it is important to establish the mechanism responsible for cytotoxic destruction of the forms of the parasite which are susceptible to immune lysis and also to ascertain the reasons why certain evolutive forms are resistant. This report represents an exploration of the first problem. A portion of this paper was published previously (D. F. Anziano, R. Lelchuk, and A. P. Dalmasso, Medicina **30**:51, 1970).

MATERIALS AND METHODS

Trypanosomes. All studies were performed with the Tulahuen strain of *T. cruzi* cultured in biphasic medium (6). The parasites had been subcultured from 55 to 90 times and consisted largely of crithidial forms with a small proportion (1–2%) of metacyclic forms. To carry out the various experiments, we harvested the parasites on the fifth day of culture, after which they were recovered by centrifugation at 700 × *g* and 24 C for 10 min and washed three to four times at 24 C with Veronal buffer (*p*H 7.3) containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (9).

Antisera against T. cruzi. Three sources of antitrypanosomal lytic antibodies were employed. One was an antiserum raised in a rabbit by two injections of 1.2 ml of washed, sedimented trypanosomes subjected to disruption in a Ribbi pressure machine (6) and emulsified with 1.2 ml of complete Freund adjuvant. A first injection was given in the footpads and in the subcutaneous tissue of the back, and a second injection was given subcutaneously and intramuscularly 30 days later. Serum was obtained 15 to 20 days after the last injection. Another source of trypanosomal antibodies consisted of human sera obtained from patients with chronic Chagas' disease, who were diagnosed according to previously defined criteria (11). Finally, in one experiment, advantage was taken of the lytic antibody activity present in relatively high concentrations of normal human and guinea pig sera (19).

Antisera against complement components. Anti-C3 and anti-C4 were obtained by immunization of rabbits with the respective purified complement proteins (14, 17). To eliminate agglutinins to *T. cruzi* present in rabbit serum, the antisera were absorbed as follows. A 125-μliter amount of sedimented parasites was suspended in 0.8 ml of antiserum diluted 1:5 with Veronal buffer and incubated at 24 C for 30 min. The parasites were removed by centrifugation, and the absorption was repeated two more times, which resulted in complete removal of anti-*T. cruzi* agglutinating activity.

Kinetics of immune lysis and role of divalent cations. Reaction mixtures consisted of 0.6 ml of Veronal buffer containing 1.7 × 10^7 parasites to which was added 5 μliters of rabbit anti-*T. cruzi* antiserum (which had been subjected previously to 56 C for 30 min), 10 μliters of fresh, pooled guinea pig serum, and 385 μliters of Veronal buffer. Incubation was

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carried out at 37 C for appropriate times and then interrupted by centrifugation at 1,200 × *g* for 3 min. The pellets were incorporated in a drop of 15% bovine serum albumin, smears were made and stained with Giemsa, and the degree of lysis was calculated after examination of 500 cells. Each determination was performed in duplicate. Lysed trypanosomes were easily recognized by the development of spherical appearance, loss of the flagellum, and gross deterioration of the nucleus and kinetoplast. The same technique was used to study the role of divalent cations in immune lysis, except that a Ca- and Mg-free Veronal buffer and various amounts of stock solutions of $CaCl_2$ and $MgCl_2$ were used. Incubation at 37 C was carried out for 60 min.

Lytic reactions with reagents lacking complement components. Inactivation of complement components in guinea pig and human sera was accomplished by treatment with zymosan at 37 C and with hydrazine (9). Selective inactivation of components of the properdin system was achieved by treatment of human serum with zymosan at 17 C (22). As indicated by the acid hemolysis test, serum treated in the latter manner failed to lyse erythrocytes from a patient with paroxysmal nocturnal hemoglobinuria (18), but it retained most of the hemolytic activity against sheep erythrocytes sensitized with rabbit hemolysin. Thus, it was considered deficient in components of the alternate pathway (properdin system) but normal with respect to the classical pathway of complement activation. Reaction mixtures consisted of 0.6 ml of Veronal buffer containing 1.5×10^7 parasites, 40 μliters of pooled, guinea pig serum, or 100 μliters of pooled, human serum from normal donors, and sufficient Veronal buffer to yield a total volume of 1 ml. Incubation was carried out for 30 min at 37 C with intermittent, manual mixing. Then a sample was withdrawn with a platinum wire loop, placed on a microscope slide, and covered with a cover slip, and a semiquantitative evaluation of the degree of lysis was performed immediately without staining.

Uptake of complement components C3 and C4 by *T. cruzi* treated with antibody and complement. A 125-μliter amount of washed, sedimented culture forms was suspended in 375 μliters of Veronal buffer and incubated with 0.5 ml of serum (previously subjected to 56 C for 30 min and diluted 1:20 with Veronal buffer) from a patient with chronic Chagas' disease. The mixture was incubated at 37 C during 30 min. Then 0.5 ml of fresh, normal human serum diluted 1:10 with Veronal buffer was added, and the suspension was incubated at 37 C for 30 min, with manual mixing. In a control tube, 0.5 ml of Veronal buffer was substituted for fresh normal serum. The parasites were washed three times with Veronal buffer at 4 C and suspended in 0.5 ml of Veronal buffer. Agglutination reactions were carried out in well-type plates and evaluated after 10 min at 24 C.

Electron microscopy. A 1-ml amount of washed and sedimented culture forms was suspended in 15 ml of Veronal buffer and divided into 5-ml samples. One sample was mixed with 5 ml of fresh serum from a patient with Chagas' disease. A second sample was mixed with 5 ml of the serum which had previously been subjected to 56 C for 30 min to inactivate complement. Both mixtures were incubated for 60 min at 37 C, with intermittent mixing. Then they were centrifuged, and the pellets were suspended in 10 ml of 5 mM phosphate buffer (*p*H 7.4) and incubated for 30 min at 37 C. A third sample of trypanosomes was not exposed to serum but was centrifuged, suspended in 10 ml of the 5 mM phosphate buffer, and incubated at 37 C for 60 min. The three samples were then centrifuged at 12,000 × *g* and 4 C for 15 min, and the sediments were washed twice with 5 mM phosphate buffer and suspended in 20 ml of this buffer to obtain a concentration of trypanosomal membrane suitable for electron microscopy. A drop of the suspension was placed on carbon-coated grids and negative-stained with 2% aqueous uranyl acetate (3). Electron micrographs were taken at a magnification of 25,000 with an Elmiskop IA apparatus.

RESULTS

The kinetics of immune lysis of *T. cruzi* yielded a sigmoidal curve (Fig. 1). After proceeding slowly during the first 5 min, lysis increased rapidly and reached a plateau in about 30 min, which was maintained for at least 2 hr.

Studies on the lytic activity of serum treated with various substances which alter the complement system preserving the antibody activity are summarized in Table 1. In these experiments, serum from normal guinea pigs at a final dilution of 1:25 (or human serum from normal donors, diluted 1:10) served as a source of both anti-*T. cruzi* antibody and complement. The lytic capacity of guinea pig serum was abolished by treatment with zymosan at 37 C or with hydrazine. Similar results were obtained with human serum (not shown in table). In contrast, no impairment of the lytic capacity occurred when the treatment of human serum with zymo-

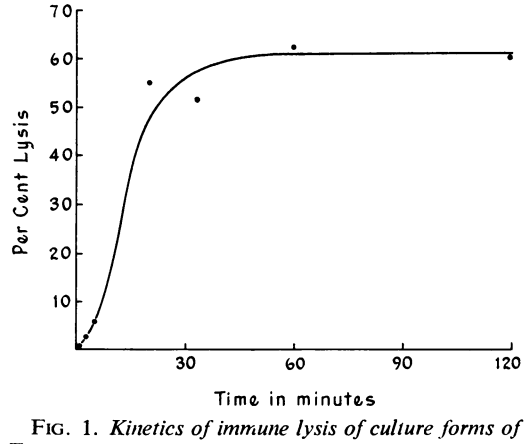

FIG. 1. *Kinetics of immune lysis of culture forms of Trypanosoma cruzi.*

san was carried out at 17 C. Immune lysis was prevented by the presence of 10 mM sodium-ethylenediaminetetraacetate (EDTA) and also by pretreatment of the serum at 56 C for 30 min.

Since the inhibitory effect of EDTA indicated a requirement of divalent cations for immune lysis of *T. cruzi*, a study of the optimal concentrations of Ca^{2+} and Mg^{2+} participating in this reaction was performed. The results indicated that the presence of 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} enhanced immune lysis maximally (Table 2). Higher or lower concentrations affected immune lysis of *T. cruzi* adversely, causing an impairment in the degree of lysis of 38% or more with respect to the degree obtained when an optimal concentration of divalent cations was employed.

TABLE 1. *Role of the complement system in immune lysis of culture forms of Trypanosoma cruzi*

Reagents tested	Degree of lysis[a]
Guinea pig (GP) serum	++++
Heat-inactivated (30 min at 56 C) GP serum	±
GP serum and 10 mM EDTA	0
Zymosan-treated (37 C) GP serum	±
Hydrazine-treated GP serum	±
Zymosan-treated (37 C) GP serum and hydrazine-treated GP serum	++++
Human serum	++++
Zymosan-treated (17 C) human serum	++++

[a] Four plus marks correspond to more than 95% lysis, and ± indicates less than 5% lysis.

TABLE 2. *Optimal concentrations of Ca^{2+} and Mg^{2+} for immune lysis of culture forms of Trypanosoma cruzi*

Immunological reagents	Concn (mM) of		Percent lysis
	Ca^{2+}	Mg^{2+}	
Antiserum and complement	0.015	0.5	46
	0.15	0.5	76
	0.75	0.5	41
	1.5	0.5	47
	7.5	0.5	31
	0.15	0.01	40
	0.15	0.05	46
	0.15	0.1	41
	0.15	5	23
Antiserum	0.15	0.5	1
Complement	0.15	0.5	12
Veronal buffer	0.15	0.5	2

Agglutination studies of complement-treated *T. cruzi* using monospecific anti-C3 and anti-C4 demonstrated that during immune lysis the complement components C3 and C4 become physically bound to the membrane of the trypanosomes (Table 3).

Electron microscope examination of negatively stained trypanosomal membranes isolated from parasites after treatment with antibody and complement revealed ultrastructural changes (Fig. 2). They consisted of irregular, circular

TABLE 3. *Uptake of C3 and C4 by culture forms of Trypanosoma cruzi during immune lysis*

Reagents employed to treat *T. cruzi*	Specificity of antiserum	Degree of agglutination				
		1:10[a]	1:20	1:40	1:80	1:160
Chagas serum + complement	C3	4+	4+	3+	2+	0
Chagas serum	C3	0	0	0	0	0
Chagas serum + complement	C4	2+	1+	0	0	0
Chagas serum	C4	0	0	0	0	0

[a] Antiserum dilution.

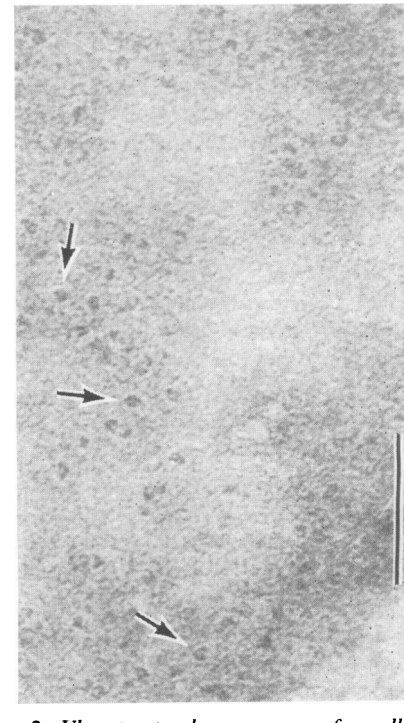

FIG. 2. *Ultrastructural appearance of a cell membrane fragment from complement-treated Trypanosoma cruzi. Arrows indicate typical membrane modifications. Bar represents 100 nm. ×200,000.*

areas that appeared to be filled with the negative stain, had a diameter of 8 to 10 nm, and were surrounded by an irregular, clear rim. These membrane changes were distributed randomly on the membrane surface of complement-treated trypanosomes but failed to appear in control membranes isolated from parasites that were left untreated or were exposed to heat-inactivated (56 C, 30 min) serum.

DISCUSSION

These studies indicate that immune lysis of culture forms of *T. cruzi* follows the general principles which govern complement-mediated lysis of antibody-sensitized sheep red blood cells (9, 13), as demonstrated by the kinetics of the reaction, requirement of divalent cations, uptake of the complement components C3 and C4, and appearance of characteristic ultrastructural changes on the cell membrane.

Some of these results are of interest regarding which of the two main mechanisms of complement activation is responsible for immune lysis of *T. cruzi*. One of the possible mechanisms bypasses C1, C2, and C4 (20) and requires an activator system (8) which appears to be identical with the properdin system (7, 18, 22). Whereas this mechanism is important in bacteriolysis and in lysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria, antibody-mediated lysis of sheep erythrocytes utilizes a mechanism involving activation of C1 (8). Immune destruction of the protozoan *Toxoplasma gondii* depends primarily on the activity of the properdin system (21). In the present study, the following observations indicate that immune lysis of *T. cruzi* proceeds through activation of complement via the classical pathway. C4 was taken up by the trypanosomal membrane; immune lysis was facilitated maximally by concentrations of Ca^{2+} and Mg^{2+} that are optimal for activation of complement via C1 (9, 12); the lytic capacity of serum was abrogated by procedures which inactivate the classical pathway but not by inactivation of the properdin system (18); and finally, the complement-mediated ultrastructural membrane "lesions" had the characteristics previously described in association with activation of the classical pathway and not those associated with the alternate pathway (M. J. Polley, Fed. Proc. **31:** 788, 1972). It is possible, however, that under conditions different from those used in this study the alternate pathway may play a role in lysis of *T. cruzi*.

The ultrastructural changes developing on the membrane of complement-treated *T. cruzi* are similar to those previously described on the membranes of other cell types, e.g., red blood cells (2), bacterial lipopolysaccharides (1), and even on complement-treated artificial membranes (10). Therefore, it appears safe to conclude that the development of typical ultrastructural membrane changes associated with the complement reaction is a phenomenon of general occurrence.

The understanding of the immunological factors controlling the interaction of *T. cruzi* with its host is incomplete (5, 11, 15), including the question of the degree of participation of immune lysis and the possible role of complement in noncytotoxic reactions. The objective of the present study was to establish the main features of immune cytolysis of the crithidial stage, a developmental form readily obtainable in vitro which appears regularly in the insect vector, but not in the mammalian host. The latter may indeed be due to the prompt destruction of crithidial forms by immune cytolysis (15). Work is needed to establish the reaction mechanism of antibody and complement with the other developmental forms, some of which can be obtained in vitro (4), as well as the mechanism of resistance to immune lysis exhibited by certain forms of *T. cruzi*.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-07470 from the National Institute of Allergy and Infectious Diseases and by Comisión para el estudio de la enfermedad de Chagas, University of Buenos Aires. D.F.A. was Research Fellow of the Secretary of Public Health, City of Buenos Aires. R.L. was Research Fellow of the Institute of Pharmacology, Secretary of Public Health of Argentina. A.P.D. and C.V. are Career Investigators of the National Research Council of Argentina.

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