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Effects of Complement Depletion in Experimental Chagas' Disease: Immune Lysis of Virulent Blood Forms of *Trypanosoma cruzi*

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In mice infected with virulent blood (trypomastigote) forms of *Trypanosoma cruzi*, complement depletion with cobra venom factor caused a marked exacerbation of the disease evidenced by significantly increased parasitemia levels and early mortality as compared with those of untreated infected animals. The effect was greater in mice receiving cobra venom factor on day 7 postinfection, i.e., at the time when the parasites had had time to localize and multiply in the tissues and appeared in the circulation in appreciable numbers. The possibility that complement participates in host defense against *T. cruzi* infection through a mechanism involving immune lysis was explored in vitro. *T. cruzi* trypomastigotes were found to undergo immune lysis in sera of patients with chronic Chagas' disease, in sera of immunized mice, and in solutions containing both immune mouse gamma globulin and a source of active complement. This phenomenon failed to take place either in the absence of complement or after complement inactivation by heat or utilizing complement inactivators. The lytic capacity of heated sera was restored by the addition of active complement to the system. During the immune lysis of *T. cruzi* blood forms, complement was activated in human sera via both the classical and the alternate pathways. In mouse sera, activation followed at least the alternate pathway.

Knowledge of the different immunological aspects of host resistance against infection with *Trypanosoma cruzi*, a monocellular flagellate causating Chagas' disease or American trypanosomiasis, is very limited (6). Thus, important questions such as whether antibody and complement play a role in this defense still lack definite answers (1). Some in vitro studies along these lines have been carried out in the past (1, 4, 10, 15), but they have dealt mainly with culture (mostly epimastigote) forms of *T. cruzi*. These forms have been reported to be susceptible to antibody-mediated lysis caused by complement that has been found to be activated via the classical pathway (1). The virulent trypomastigote form, instead, is so far only known to undergo lysis when incubated with the sera of normal animals—birds and frogs which are naturally resistant to infection with *T. cruzi* (15). By using sera of susceptible hosts, two attempts have been made to find out whether these forms of the parasite may undergo immune lysis. The first one (10) was based on the observation of the occurrence of morphological changes; it failed to yield definite conclusions since only 2 out of 10 sera caused alterations in the parasites. The second one (16), in which the experimental conditions were essentially similar, yielded negative results. The failure to demonstrate the susceptibility of the parasite to immune lysis and the fact that *T. cruzi* trypomastigotes may be found in the blood of both Chagasic patients (3, 14) and experimental animals (11) with circulating antibodies have unwarrantedly led researchers to think that these forms might be resistant to immune lysis (1).

The present investigation was undertaken to determine the effects of complement depletion on the course of *T. cruzi* infection in mice and to study whether virulent blood forms of this parasite are susceptible to immune lysis, including the mechanisms whereby complement is activated during the process.

MATERIALS AND METHODS

Animals. Five-week-old male Rockland mice, obtained from a colony maintained at the Immunochemistry Section of this Institute, were used in all studies.

Parasite. Tulahuen strain *T. cruzi* were main-

tained by serial passages in 4-week-old Rockland mice. The number of trypanosomes per milliliter present in the blood of infected animals and in other suspensions was determined by a standardized microscopic method described previously (7). The 50% lethal dose of *T. cruzi*, established by the method of Reed and Muench (12), was 1,000 parasites. Inoculation of the organisms was always performed intraperitoneally.

Human sera. Blood samples from chronic Chagasic patients (kindly provided by R. Lelchuk and A. Patrucco of the Institute of Medical Investigations of this University) and from normal individuals were allowed to clot at 37 C for 30 min. These sera will be referred to as immune human serum (IHS) and normal human serum (NHS), respectively.

Mouse sera. Immune mouse sera (IMS) were obtained from mice that had been first immunized with killed culture forms of *T. cruzi* (F. Kierszenbaum and D. B. Budzko, Fed. Proc. **33**:764, 1974) and later challenged with 100 50% lethal doses of *T. cruzi* trypomastigotes. Blood was withdrawn 180 to 360 days after challenge. Normal mouse sera (NMS) were obtained from untreated animals. All samples of IHS and IMS gave positive complement fixation and agglutination tests. They were either fresh or kept at −70 C until used.

Immune mouse gamma globulins. Gamma globulins (IMGG) were prepared from IMS by precipitation with ammonium sulfate (2). The concentration of the solution was 6.4 mg/ml in 0.15 M NaCl.

Cobra venom factor. Cobra venom factor (CVF) was prepared from the venom of the cobra snake, *Naja naja* (Ross Allen Reptile Institute, Silver Springs, Fla.), as described by Müller-Eberhard and Fjellstrom (9).

Endotoxin. Endotoxin was extracted from *Escherichia coli* O125:B15 by the phenol-water method of Westphal et al. (17). A 10-mg/ml solution was used.

Phytohemagglutinin. Phytohemagglutinin was prepared in its mucopolysaccharide form from the red kidney bean, *Phaseolus vulgaris*, by the method of Rigas and Osgood (13).

Isolation of T. cruzi trypomastigotes. The parasites used in our studies were obtained from the blood of infected mice in three different ways. Yaeger's technique (18), utilizing mucopolysaccharide phytohemagglutinin to agglutinate the erythrocytes, yielded a suspension of trypanosomes. These were washed three times with a phosphate-buffered saline solution, pH 7.0 (PBS), before use. Plain decantation of heparinized (10 U of heparin per ml) infected blood allowed the recovery of 30 to 40% of the parasites in the supernatant. The third method, developed in our laboratory, consisted in layering a mixture of equal volumes of heparinized blood and 0.15 M NaCl over 1.5 volumes of a solution containing 9.6% of sodium *N*-methyl-3,5,-diacetamido-2-4-6-triiodobenzoate and 5.6% Ficoll (Lymphoprep, Nyegaard & Co. A/S, Oslo) in a centrifuge tube. After centrifugation at 400 × *g* for 40 min at room temperature in a horizontal rotor, four distinct layers are formed. The bottom layer contains the erythrocytes. The next layer above is separation fluid. The third one is a sharp interface containing nearly all the parasites present in the sample plus leukocytes and platelets. The upper layer is the plasma. We utilized the interface suspension collected with a small portion of the plasma. Parasite suspensions were counted and adjusted to 8 to 20 million *T. cruzi* per ml by appropriate dilution with PBS containing 4% bovine serum albumin (PBS-alb).

Determination of the extent of lysis of T. cruzi trypomastigotes. Samples (0.2 ml) of the materials to be tested were mixed with 0.03-ml portions of a solution containing 0.005 M magnesium chloride and 0.0015 M calcium chloride. Complement inactivators were added in a volume of 0.02 ml. Volume adjustments, when required, were made with PBS-alb. After addition of 0.05 ml of the suspension of parasites, the mixtures were incubated at 37 C for 1 h. In the tubes in which the effect of complement restoration was tested, the amounts used were the following: 0.2 ml of heat-inactivated IHS, 0.2 ml of either untreated or heat-inactivated NHS, 0.05 ml of the solution containing calcium and magnesium, and 0.05 ml of the suspension of parasites. After incubation, the number of live trypanosomes was established in all tubes and the percentage of lysis was calculated according to the equation:

$$\text{percentage of lysis} = \frac{N - n}{N} \times 100 \qquad (1)$$

where n and N are the numbers of *T. cruzi* per milliliter counted in the mixtures containing the material tested and PBS-alb, respectively. Alternatively, the counts can be made in equal portions of the same mixture before and after incubation, and the percentage of lysis is calculated as follows:

$$\text{percentage of lysis} = \frac{Ci - Cf}{Ci} \times 100 \qquad (2)$$

where Ci and Cf are the numbers of parasites per milliliter counted before and after incubation respectively.

Complement depletion. In mice, complement was depleted by intraperitoneal administration of 120 U of CVF per kg of body weight. This amount was given in 4 equally spaced doses distributed within 24 h. None of four mice given CVF died within 45 days, a period after which observation was suspended. In normal animals, the treatment resulted in a marked depletion of complement for at least 5 days, evidenced by both the conversion of C3 observed by immunoelectrophoresis and the decreased or nondetectable hemolytic activity of their sera. Mice were given CVF either on the day of infection or on day 7 postinfection to observe the effects of complement depletion at different stages of the disease. To deplete complement in vitro, a solution containing 60 U of CVF per ml was utilized.

Statistical analysis. The significance of differences, P, between means was determined by Student's t test. Differences were considered to be significant if $P < 0.05$.

RESULTS

Effects of complement depletion on the course of T. cruzi infection in mice. To determine the effects of a drop in complement levels on the course of experimental Chagas' disease, mice were given CVF either on the day of infection or 7 days later, i.e., at the beginning of the infection or when the parasites had already established themselves in the tissue of the host and trypomastigotes were found in the blood in appreciable numbers. In both cases, a pronounced exacerbation of the disease was observed, though the effects were more prominent in mice given CVF on day 7 (Fig. 1 and 2). In this group, 58% of the mice died before the animals of the control group, given only the parasites, started to die.

Immune lysis of T. cruzi trypomastigotes: immune lysis assay. The method designed to determine the extent of lysis of *T. cruzi* trypomastigotes is based on the measurement of the number of live parasites in the reaction mixtures, including suitable controls, either after or before and after incubation. According

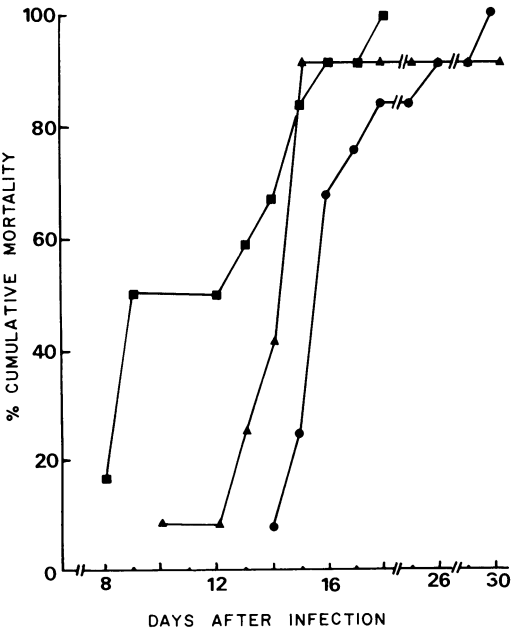

FIG. 1. *Effect of complement depletion with CVF on the mortality of mice infected with blood forms of T. cruzi. Symbols:* ■, *Mice given CVF on day 7 postinfection;* ▲, *mice given CVF on the day of infection;* ●, *control mice injected with PBS on days 0 and 7 postinfection, following the same schedule used to administer CVF. The initial number of animals in each group was 12. All mice were infected with 100,000 parasites.*

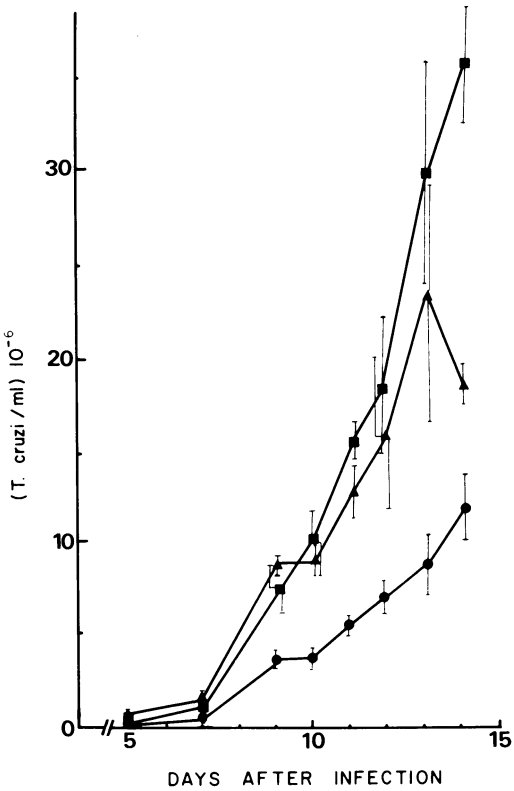

FIG. 2. *Effect of complement depletion with CVF on the course of parasitemia in mice infected with blood forms of T. cruzi. Symbols are as described in the legend to Fig. 1. Each point indicates the average parasitemia of animals surviving at the indicated time. Vertical lines represent the standard error of the mean. Beginning on day 9 postinfection all differences between the experimental points and those of the control group were statistically significant ($P < 0.02$). This set of data corresponds to the same mice whose mortality curves are shown in Fig. 1.*

to both equations 1 and 2, the percentage of lysis is 100% in those tubes in which live parasites can not be detected after incubation. In these mixtures no entire parasites were found either in the suspensions or in the sediments obtained by centrifugation at 2,000 × *g* for 15 min. Under the same conditions, parasites are readily detected if they are present.

Immune lysis of T. cruzi by IHS. Table 1 summarizes the results obtained by measuring the percentages of lysis of virulent blood forms of *T. cruzi* in different mixtures containing human sera. As can be seen, lysis reached large extents only in those tubes where both antibody and active complement were present. The involvement of complement in the reaction was shown in several ways. On the one hand, the

TABLE 1. *Immune lysis of T. cruzi trypomastigotes by utilizing IHS*

Material tested	Lysis (%)				
	Expt. 1[a]	Expt. 2[a]	Expt. 3[b]	Expt. 4[b]	Expt. 5[c]
IHS	94.3	100.0	94.0	98.8	76.2
Heat-inactivated IHS	0	1.5	0	0	0
IHS + CVF	4.5	4.5	—[d]	—	—
IHS + endotoxin	0	—	—	—	—
IHS + EDTA[e, f]	0	—	—	—	—
Heat-inactivated IHS + NHS	86.0	96.7	—	—	—
Heat-inactivated IHS + heat-inactivated NHS	0	0	—	—	—
IHS + EGTA[e]	48.7	42.3	40.4	—	—
NHS	0	0	—	—	0
EDTA[e]	0	—	—	—	—
EGTA[e]	0	0	0	—	—
PBS-alb[g]	0	0	0	0	0

[a] Performed with parasites isolated from blood by the mucopolysaccharide phytohemagglutinin method.
[b] Parasites were obtained by plain decantation of infected blood.
[c] Trypomastigotes were isolated by utilizing Lymphoprep.
[d] The dash means that this particular determination was not done.
[e] Final concentration of the chelating agent was 0.01 M.
[f] EDTA, Disodium ethylenediaminetetraacetate.
[g] Percentage of lysis in these tubes was 0 both by definition and by equation 2.

addition of different complement inactivators to IHS inhibited its capacity to lyse the parasites. Moreover, similar inhibition was observed with heat-inactivated IHS. On the other hand, the addition of NHS restored the lytic activity of heat-inactivated IHS on the parasites. NHS alone did not show lytic activity.

By inhibiting the C142 pathway of complement activation with sodium ethyleneglycol-bis-(β-aminoethylether)-*N*-*N*'-tetraacetate (EGTA), the percentages of immune lysis were found to drop to about one-half of the values obtained with untreated IHS. The results were similar regardless of the method used to obtain the trypanosomes. Since one of the procedures involves plain decantation of infected blood without further treatments, the possibility that the parasites were rendered susceptible to immune lysis by the reagents or techniques utilized in the other two can be ruled out.

Immune lysis of T. cruzi by IMS. The results of the lytic assays utilizing mouse sera are shown in Table 2. IMS showed lytic activity on the trypanosomes which was inhibited both by prior heat inactivation and by incorporation of complement inactivators. Inhibition of the C142 pathway by addition of EGTA to IMS had no major effect on the lytic capacity. Once again, the results were independent from the method used to isolate the parasites from blood.

Immune lysis of T. cruzi by IMGG. Although IMGG alone could not cause the destruction of the parasites, the incorporation of a source of complement to the system in the form of NMS allowed the lytic process to take place. In the tube in which an extra supply of complement (guinea pig serum) was added, parasites could not be detected after incubation (Table 3), suggesting that in this case complement was a limiting factor in reaching 100% lysis in NMS.

DISCUSSION

The involvement of complement in host resistance to *T. cruzi* infection was clearly denoted by the development of an exacerbated disease in mice after complement depletion

TABLE 2. *Immune lysis of T. cruzi trypomastigotes by utilizing IMS*

Material tested	Lysis (%)					
	Expt. 1[a]	Expt. 2[a]	Expt. 3[a]	Expt. 4[a]	Expt. 5[b]	Expt. 6[c]
IMS	91.1	77.2	76.0	92.0	82.9	97.7
Heat-inactivated IMS	0	0	3.0	6.5	11.5	—[d]
IMS + CVF	—	21.2	15.9	6.5	—	—
IMS + EDTA	4.5	0	—	—	—	0
IMS + EGTA	76.2	85.4	78.5	90.6	—	—
NMS	—	—	0	0	2.8	0
PBS-alb[e]	0	0	0	0[f]	0	0
EDTA	0	0	—	—	—	—
EGTA	0	0	0	0	—	—

[a-d] See footnotes under Table 1.
[e] In these tubes the percentage of lysis was 0 by definition. In all cases but one the percentage of lysis was also 0 by equation 2.
[f] In this tube the percentage of lysis established by equation 2 was 6.0.

TABLE 3. *Lysis of T. cruzi[a] trypomastigotes by mouse antibody and complement*

Material tested[b]	Lysis (%)
IMGG	0
IMGG + NMS	73.9
IMGG + NMS + GPS[c]	100.0
IMGG + heat-inactivated NMS	0
NMS	0
GPS	0
NMS + GPS	0
Saline-alb	0

[a] Parasites used in these experiments were obtained by plain decantation of heparinized infected blood.
[b] Total volume of these reactions was 0.5 ml. Volume adjustments were made with 0.15 M NaCl containing 4% bovine serum albumin (saline-alb).
[c] GPS, 0.1 ml of guinea pig serum.

with CVF. The effect was more dramatic when complement was depleted on day 7 postinfection, i.e., when the parasites had had time to localize and multiply in the host's tissues and were found in the circulation in appreciable numbers in their trypomastigote form. The implication is that maybe the effects of complement are on the circulating parasites and, therefore, complement may cause their destruction by immune lysis and also by facilitating their removal and killing by phagocytic cells through its opsonic capacity. In these studies we have explored the first of these possibilities by utilizing an in vitro system.

Several pieces of evidence are presented in this paper showing that *T. cruzi* trypomastigotes may, at least in vitro, undergo antibody-mediated lysis caused by complement. The capacities of IHS and IMS to lyse the parasites were abolished or reduced to minimal extents after heating at 56 C for 30 min, and the gamma globulin fraction of IMS was lytic only when a source of active complement was included in the system. In addition, the supply of complement in the form of NHS to heat-inactivated IHS restored its lytic effect. In agreement with these observations was the fact that the incorporation of complement inactivators to either IHS or IMS reduced their lytic extents to an either minimal or nondetectable degree. Moreover, the fact that the addition of guinea pig serum increased the lytic capacity of IMGG mixed with NMS suggests that complement was a limiting factor in reaching complete lysis of all the parasites.

The C142 pathway of complement activation requires ionized calcium and is inhibited by EGTA (5). The alternate pathway, instead, requires ionized magnesium but not ionized calcium and takes place in the presence of EGTA. The addition of this chelating agent to IHS decreased its capacity to lyse blood forms of *T. cruzi* by about one-half, indicating that, in these sera, complement may be activated via both the classical and alternate pathways. Instead, no major changes were observed after incorporating EGTA into tubes containing IMS. It is conceivable that the preferential mechanism whereby complement is activated in the sera of different hosts to cause immune lysis of *T. cruzi* trypomastigotes may vary. However, the alternative possibility should also be considered that, in mouse serum, immune lysis reaches its maximal extent by activating complement via the C3 shunt when the classical pathway is inhibited. Under normal conditions, the C142 pathway might provide either a part or all the lytic activity. Also conceivable is the possibility that the class of antibody responsible for the lytic effect is different in mouse and human serum, determining activation of complement through different pathways.

Previous work to establish the susceptibility of *T. cruzi* trypomastigotes to immune lysis yielded either inconclusive (10) or negative (16) results. It is of interest that in these cases the parasites used in the tests were preferentially myotropic, whereas we worked with a highly reticulotropic strain. A different tropism implies dissimilar biological features, of which susceptibility to immune lysis might be one.

Circulating antibody, capable together with complement of causing the destruction of virulent blood forms of *T. cruzi* in vitro, could play its role in host resistance not only through immune lysis but also by facilitating phagocytosis, known to result in vivo in the killing of these forms of the parasite (8). This and the possibility that complement might also contribute to host defense through its opsonic properties deserve further investigations.

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