Experimental Chagas' Disease in Complement-Deficient Mice and Guinea Pigs

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The course of infection with trypomastigotes of *Trypanosoma cruzi* (House 510 strain) in mice and guinea pigs with genetic complement deficiencies was compared with that in normocomplementemic animals. Parasitemias in a mouse strain (B10.D2/old) genetically deficient in C5 and therefore unable to sustain lysis were similar to or lower than in a congenic normocomplementemic strain (B10.D2/new). The levels of C3 measured immunochemically were generally unaffected. There were no significant differences in mortality rates. These results indicate that, in mice, complement-mediated lysis does not play a significant role in the control of *T. cruzi* (House 510) infections. Studies were also performed in normocomplementemic guinea pigs and in guinea pigs genetically deficient in the fourth component of complement and thus unable to support functions mediated by the classical pathway of complement activation. No significant differences were noted between the two strains in the course of infection, persistence of subpatent infection, or rate of mortality, indicating that if the classical complement pathway plays a role in resistance to *T. cruzi* (House 510) in guinea pigs, this role must be a small one.

The mechanisms of immunity responsible for host defense against *Trypanosoma cruzi*, the causative agent of Chagas' disease in humans, are not fully understood. A role for antibodies in resistance has been demonstrated (9, 11, 16, 19), but their mode of action has not been clearly determined. There is evidence that the antibody-dependent resistance could be mediated by the complement system through such mechanisms as phagocytosis and lysis. Depletion of C3 and late acting components by treatment with cobra venom factor resulted in the exacerbation of *T. cruzi* (Tulahuen strain) infections in mice (3, 15). Lysis of the epimastigote form of *T. cruzi* by complement occurs readily in both immune and nonimmune sera (1, 26, 28), and complement-mediated immune lysis of the trypomastigote (bloodstream) stage has been described in vitro (3, 15). We have investigated whether complement-mediated lysis plays a role in vivo by comparing the course of *T. cruzi* infection in normocomplementemic animals with that in animals with genetic complement deficiencies.

MATERIALS AND METHODS

Experimental animals. Male mice of the congenic normocomplementemic B10.D2/new and C5-deficient B10.D2/old strains (25) were used. The presence or absence of C5 was verified by testing individual plasma samples by double diffusion in agar against anti-mouse C5 prepared in C5-deficient mice (25). C3H/Bi strain

mice (University of Minnesota Mouse Colony, Minneapolis, Minn.) were used to test for the persistence of *T. cruzi* in guinea pigs. Outbred albino mice used for the routine passage of *T. cruzi* were raised from stock obtained from the Department of Zoology, University of Minnesota. C4-deficient (6) and normocomplementemic (NIH) strains of guinea pigs were assayed for total hemolytic complement activity to confirm C4 deficiency or normocomplementemia (6).

Maintenance of parasite. *T. cruzi* (H 510), a myotropic strain (22, 23), was provided by Franklin Neva, Laboratory of Parasitic Diseases, National Institutes of Health. The parasite was isolated initially from a domiciliary *Triatoma dimidiata* in Costa Rica. When we obtained this strain, it had been transferred from a vector (*Rhodnius prolixus*) into NIH Swiss mice and subsequently passaged in mice 23 times. In our laboratory, the trypanosomes were maintained in outbred albino mice by syringe passage of infected blood every 14 to 21 days. There was no apparent change in survival time of infected mice over a period of 2 years or 34 passages. The mean survival time of mice infected at passage 2 was 27 days compared with a mean of 28 days for mice infected at passage 32.

Infection of experimental animals. Trypanosomes were collected from outbred mice on day 10 to 13 of infection by bleeding from the retro-orbital plexus with a heparinized Pasteur pipette. The parasites were counted in a hemocytometer and diluted to the desired concentration with sterile, nonpyrogenic 0.9% NaCl-5% dextrose. Twenty mice of each B10.D2 strain (Jackson Laboratories, Bar Harbor, Maine) were infected intraperitoneally at 4 months of age with a dose of 10^3 trypanosomes. In another experiment, 10 mice of each B10.D2 strain (University of Minnesota

Mouse Colony) were injected intraperitoneally at 2 months of age with a dose of 2.25×10^6 *T. cruzi*.

A total of 5 female and 13 male C4-deficient and 4 female and 16 male normocomplementemic (NIH) guinea pigs were infected. NIH and C4-deficient experimental groups were age matched as closely as possible, as described in Results. Two C4-deficient and two NIH littermates of infected guinea pigs served as uninfected controls and were injected intraperitoneally with the same volume of NaCl-dextrose solution as the infected animals.

Course of infection. The number of parasites in the blood was determined by counts performed at weekly intervals in mice infected with 10^3 parasites and in guinea pigs, or at 1- to 3-day intervals in mice infected with 2.25×10^6 *T. cruzi*. Blood samples for counts were obtained between 0900 and 1100 h from the retro-orbital plexus in mice and by cardiac puncture in guinea pigs. A sample volume of 100 μ l was taken from each mouse with a heparinized capillary tube (Fisher Scientific Co., Pittsburgh, Pa.), and 0.5 to 1.0 ml was obtained from each guinea pig with heparin as anticoagulant. In experiments in mice infected with 10^3 *T. cruzi* and in guinea pigs, the number of parasites per 50 microscopic fields (400 $\times$) was determined from wet mounts of whole blood, or blood samples for hemocytometer counts were diluted 1:10 with 0.87% ammonium chloride which lyses the erythrocytes and permits visualization of the parasites (12). Mice infected with 2.25×10^6 parasites had sufficiently high parasitemias to permit hemocytometer counts of blood diluted >1:50 with 10% Giemsa stain in 1% Formalin-0.9% NaCl.

Measurement of mouse plasma C3. Plasma samples were collected every 5 to 7 days from the mice that were injected with 2.25×10^6 parasites and from uninfected controls on the same schedule. One heparinized capillary tube containing 100 μ l of blood was obtained from each mouse at each bleeding. After centrifugation, the plasma was removed and stored at -70°C . Plasma C3 concentrations were determined by radial immunodiffusion with rabbit anti-mouse C3 as previously described (14). Concentrations were expressed as the percentage of a pooled normal mouse plasma standard obtained from mice of the appropriate strain.

Detection of subpatent infections in guinea pigs. Five to eight months after receiving the infecting dose of *T. cruzi*, all surviving guinea pigs were bled by cardiac puncture with heparin as anticoagulant and then sacrificed with an overdose of ether. The spleen was removed and transferred to a Petri dish containing 2 ml of sterile 0.9% NaCl-5% dextrose. Approximately one-half of the spleen was cut finely with scissors and gently dispersed with a glass tissue homogenizer. Weanling C3H mice were inoculated intraperitoneally with 0.5 ml of either whole blood or spleen homogenate. Two to seven mice were inoculated per guinea pig. Wet mounts of whole blood from the mice were examined once a week for the presence of *T. cruzi* until parasites were found, or a maximum of 10 weeks.

Histology. Tissues from infected and uninfected control animals were obtained at the time of sacrifice or immediately after death. Portions of heart, liver, lungs, spleen, kidneys, adrenals, skeletal muscle, and

ovaries or testes were fixed in 10% buffered Formalin. Specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Statistical analyses. Student's *t* test was used to compare the difference between means. Values of *P* less than 0.05 were considered significant. Survival times of the mice were compared by analysis of variance. Mortality rates and persistence of *T. cruzi* in guinea pigs were compared by the chi square method.

RESULTS

Infections in C5-deficient and normocomplementemic mice. Parasitemias resulting from the inoculation of 10^3 trypanosomes per mouse are illustrated in Fig. 1. The number of parasites in the peripheral blood was similar in both strains through day 21. By day 28, however, there were more circulating parasites in the normocomplementemic B10.D2/n mice (*P* < 0.01). Parasitemias were not followed after day 28 because of the onset of high mortality rates in both strains. Mortality rates were similar in the two strains, as seen in Fig. 2. Two C5-deficient B10.D2/o mice and one normocomplementemic B10.D2/n mouse survived and were in apparent good health when the experiment was terminated on day 70. Mean survival times (excluding the animals surviving on day 70) were 31.5 (range: 24 to 43) days for the B10.D2/n strain and 33.8 (range: 26 to 43) days for the B10.D2/o mice (*P* > 0.05).

The effect of a larger inoculum (2.25×10^6 *T. cruzi*) on the course of infection is illustrated in

FIG. 1. Comparison of parasitemias in C5-deficient (B10.D2/o) and normocomplementemic (B10.D2/n) mice infected with 10^3 *T. cruzi*. Points represent mean values ($\pm$ standard error) of 20 mice of each strain.

FIG. 2. Cumulative percent mortality of C5-deficient (B10.D2/o) and normocomplementemic (B10.D2/n) mice infected with 10^3 *T. cruzi*. Twenty mice of each strain were infected with 10^3 *T. cruzi*.

Fig. 3. By comparison with Fig. 1, it can be seen that the larger infecting dose resulted in a shortened prepatent period and that the parasitemias reached maximum levels more rapidly. Although there were a greater number of circulating parasites in the B10.D2/n mice on day 11 ($P < 0.01$), the course of infection was similar in both strains. There was no difference in mortality rates, and no mice of either strain survived (Fig. 4). Mean survival time of the B10.D2/n was 21 (range: 18 to 24) days compared with 19.6 (range: 16 to 23) days for the B10.D2/o mice ($P > 0.05$). The larger inoculum was presumably responsible for the earlier mortality observed in this experiment, although the age of the mice may also have been a factor.

Plasma C3 levels were measured in mice that received 2.25×10^6 *T. cruzi*. As seen in Table 1, a slight reduction in C3 levels was observed only in the normocomplementemic mice on day 6 of infection. No decrease in C3 was noted in the course of infection in the C5-deficient mice. A slight increment in C3 levels was noted on day 11 in the normocomplementemic and on day 18 in the C5-deficient infected mice.

Histopathology of infected mice. Abundant nests of *T. cruzi* amastigotes were present in the heart and were found less frequently in smooth and skeletal muscle of the B10.D2 mice that died after infection with 10^3 parasites. Parasites were most numerous in the subepicardial and subendocardial areas of the ventricles, but no region of the heart was regularly free of infection. There was extensive destruction of myocardial fibers with fibrous tissue replacement and mononuclear cell infiltration in these regions. No amastigotes were found in tissues

taken from the survivors of either strain at the time of sacrifice (day 70), and little or no myocardial damage or cellular infiltration was noted. No differences in degree of parasite invasion, tissue damage, or cellular infiltration were apparent between strains.

Infections in C4-deficient and normocomplementemic guinea pigs. The course of infection in normocomplementemic and C4-deficient guinea pigs is illustrated by the experiment shown in Fig. 5. After infection with 1.2×10^5

FIG. 3. Parasitemias in C5-deficient (B10.D2/o) and normocomplementemic (B10.D2/n) mice after infection with 2.25×10^6 *T. cruzi*.

FIG. 4. Cumulative percent mortality of C5-deficient (B10.D2/o) and normocomplementemic (B10.D2/n) mice infected with 2.25×10^6 *T. cruzi*.

TABLE 1. Plasma C3 levels in normocomplementemic (B10.D2/n) and C5-deficient (B10.D2/o) mice infected with *T. cruzi* and in uninfected controls

Day of infection	C3 (% of standard)			
	C5-deficient		Normocomplementemic	
	Infected ^a	Uninfected ^b	Infected ^a	Uninfected ^b
6	107 ± 10	99 ± 8	78 ± 6 ^c	96 ± 6
11	100 ± 10	113 ± 8	109 ± 3 ^c	96 ± 4
18	123 ± 15 ^c	99 ± 4	100 ± 14	105 ± 10

^a Mean ± standard error of five mice per group.

^b Mean ± standard error of 10 mice per group.

^c *P* < 0.05 in comparison to appropriate uninfected controls.

FIG. 5. Comparison of parasitemias in C4-deficient and normocomplementemic (NIH) strain guinea pigs infected with *T. cruzi*. Points represent mean values of 11 C4-deficient and 12 NIH guinea pigs; vertical lines indicate standard error. Age distribution at time of infection was as follows: 4 C4-deficient and 2 NIH, 5 to 10 months old; 7 C4-deficient and 10 NIH, 18 to 22 months old.

parasites, both strains experienced low-grade parasitemias with peak numbers at about 5 weeks of infection. Elimination of parasites was evident by 7 weeks, and by 10 weeks postinfection parasites could no longer be found in the peripheral blood by microscopic examination. Comparable results (not shown) were obtained in another experiment in which seven C4-deficient and eight normocomplementemic animals 2 to 3 months of age were infected with 3×10^6 *T. cruzi*. As seen in Table 2, the mortality rates of each strain during patent parasitemia, after apparent recovery, or during the entire period after infection were not significantly different.

Subpatent infections in guinea pigs. Surviving guinea pigs were tested for the persistence

of subpatent *T. cruzi* infection by the subinoculation of whole blood and spleen cell suspensions into susceptible mice. *T. cruzi* was detected in 7 of the 23 guinea pigs that survived the acute parasitemic stage. Trypanosomes were detected up to 8 months after the initial infection, or approximately 5 months after apparent recovery from acute infection at which time the experiment was terminated. There was no significant difference between strains in the persistence of subpatent infections (Table 3).

Histopathology in guinea pigs. *T. cruzi* amastigotes were found very infrequently in the hearts of representative C4-deficient and normal guinea pigs that died during the acute stage of infection, and the amastigote nests appeared to be undergoing destruction. No parasites were found in sections of heart from two C4-deficient and two normal guinea pigs that had survived the acute stage but had persistent subpatent infections. However, limited myocardial disruption, fibrous tissue replacement, and focal or diffuse mononuclear cell infiltration were noted in all animals examined. There were no apparent differences between C4-deficient and normal strains.

TABLE 2. Comparison of mortality rates between C4-deficient and normocomplementemic guinea pigs infected with *T. cruzi*^a

Experimental group	Acute stage ^b (No. dead/ no. infected)	Chronic stage ^c (No. dead/ no. surviving acute stage)	Total (No. dead/no. infected)
C4-deficient	7/18 (38.9) ^d	3/11 (27.3) ^d	10/18 (55.6) ^d
Normocomplementemic	4/20 (20.0)	1/16 (6.3)	5/20 (25.0)

^a Combined data from the two experiments described in the text.

^b From day 0 of infection through 10 weeks postinfection.

^c From 11 weeks postinfection until sacrifice at 20 or 32 weeks postinfection.

^d *P* > 0.05 in comparison to normocomplementemic group. Figures in parentheses are percents.

TABLE 3. Persistence of *T. cruzi* in C4-deficient and normocomplementemic guinea pigs demonstrated by subinoculation of blood and spleen into mice

Experimental group	No. of guinea pigs positive for <i>T. cruzi</i> /no. of guinea pigs tested by subinoculation of		
	Blood	Spleen	Blood and spleen
C4-deficient	3/8 (37.5) ^a	1/6 (16.7) ^a	4/8 (50.0) ^a
Normocomplementemic	3/15 (20.0)	0/12 (0)	3/15 (20.0)

^a *P* > 0.1 in comparison to normocomplementemic guinea pigs. Figures in parentheses are percents.

DISCUSSION

The results of this study indicate that complement-mediated lysis does not play an essential defensive role during acute *T. cruzi* (H 510) infections in mice. We found no marked differences in either the course of infection or survival time between two congenic strains of C5-deficient and normocomplementemic mice infected with *T. cruzi* (H 510). In spite of the inability of C5-deficient mice to sustain lysis, the numbers of circulating parasites in these animals were similar to, or lower than, those in normocomplementemic mice. A previous study also suggested that lysis may not be an effective defense mechanism against *T. cruzi*: trypomastigotes and amastigotes enclosed in diffusion chambers and implanted in the peritoneal cavity of immune mice were not lysed, but remained intact (11). On the other hand, there is evidence of in vitro complement-dependent immune lysis of the trypomastigotes of both the Y and Tulahuen strains of *T. cruzi* (3, 15). Possibly these apparent contradictions can be explained by differences in the strain of parasite, source of antibody, or other experimental conditions employed.

The susceptibility of *T. cruzi* trypomastigotes to immune lysis by complement may be a variable strain characteristic. The heterogeneity of *T. cruzi* with regard to other parameters such as tissue tropism, drug susceptibility, and virulence is already documented (9). Differences in the antigenic constitution of strains of *T. cruzi* epimastigotes have been previously detected (9, 27). Likewise, the variable susceptibility of different strains of trypomastigotes to immune lysis by complement may result from differences in the composition of the plasma membrane. Kretzli and Brener (19) found that although both Y and CL strain infections elicited antibody production in mice, only Y strain trypomastigotes were affected by the resulting immune sera. Differences in the quantity, distribution, or accessibility of common antigens were proposed to explain this finding (19). Such differences between *T. cruzi* strains might also be reflected in an ability to evade the host's immune response in vivo. For example, the demonstration of a carbohydrate-containing exoantigen of *T. cruzi* origin in the plasma of infected mice (2, 4, 10) suggests that shedding of antigens or antigen-antibody complexes might occur during *T. cruzi* infections. Such a process could facilitate escape from destruction by decreasing surface antigen density below the critical level required for complement-mediated lysis or other defense mechanisms (20, 21). Further studies are needed to determine which factors are involved in trypomastigote susceptibility to lysis and whether this is indeed a variable strain characteristic.

Although some species of trypanosomes are capable of direct complement activation without the apparent participation of antibody (17, 24, 26), there is no evidence that this occurs with *T. cruzi* trypomastigotes in mammalian sera (3). In addition, we found C3 levels in infected mice to be relatively unchanged during the course of infection, suggesting that little complement activation occurred. Thus, antibody appears to be essential for complement-mediated lysis of *T. cruzi* trypomastigotes. The infections of mice in our study generally terminated fatally between 16 and 43 days of infection, and there may have been insufficient production of lytic antibody during this period. In this regard, Hanson (11) reported that serum from mice infected for 5 to 6 weeks was protective in passive transfer experiments, whereas sera from mice infected for 2 weeks was not. Sera used to demonstrate in vitro lysis of trypomastigotes were obtained from chronic human infection or from epimastigote-immunized mice 6 months to 1 year after challenge with trypomastigotes (3, 15). Similarly, sera from patients with chronic Chagas' disease or from chronically infected mice 7 to 22 weeks after infection were effective at agglutinating and reducing the infectivity of Y strain trypomastigotes, whereas sera obtained from mice at 4 weeks of infection or earlier were ineffective (19). In complement fixation tests, all sera from human infections in the acute stage were negative, whereas most sera were positive in the chronic stage (31). Thus, it appears that complement-fixing lytic antibody may be present primarily during the chronic stage of infection or may be produced in response to repeated exposure to parasite antigens. If so, complement-mediated lysis may be a factor in maintaining parasitemia at subpatent levels in chronic infections.

Phagocytosis by activated macrophages has been implicated as a defense mechanism against *T. cruzi* (18, 30, 32). Treatment of mice with cobra venom factor has been used to effect temporary in vivo depletion of C3, the essential component of complement-mediated phagocytosis (5). *T. cruzi* infections in mice were markedly exacerbated when cobra factor was administered 1 week after infection, whereas cobra factor treatment on the day of infection was less effective (3, 15). These results indicate that defense mechanisms depending on C3 may be critical during the early stages of *T. cruzi* infections.

Complement activities mediated by the classical pathway were not found to be critical to the outcome of *T. cruzi* (H 510) infection in guinea pigs. There were no significant differences between C4-deficient and normocomplementemic animals in the course of infection,

mortality rate, or persistence of subpatent infections. However, the C4-deficient animals had a slightly higher mortality rate (Table 2) and persistence of subpatent infections (Table 3), suggesting that experiments with a larger number of animals might demonstrate that the classical pathway plays a minor protective role. In addition, since the alternative pathway remains functional in C4-deficient guinea pigs (7, 8), these experiments do not exclude a role for this pathway. A marked difference in host susceptibility to *T. cruzi* (H 510) is apparent when the infections in mice are compared with those in guinea pigs. Infections in mice were characteristically acute and fatal with high parasitemias. In contrast, guinea pigs infected with comparable doses of *T. cruzi* per gram of body weight experienced low parasitemias, chronic subpatent infections, and lower mortality. Thus, guinea pig infections appear to be more similar to chronic human infections and may be a more suitable model than infections in mice.

We have previously shown that complement-mediated lysis is not essential to the control of *T. musculi* infections in mice, whereas C3-mediated phagocytosis may contribute to parasite elimination (14). Neither lysis nor C3-mediated phagocytosis appears to be involved in control of *T. lewisi* infections in rats, even though extensive complement activation occurs during *T. lewisi* infections (13), and this trypanosome can be lysed by complement and immune serum in vitro (29). The relative involvement of complement in defense mechanisms therefore appears to be a unique feature of the individual host-trypanosome interaction.

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