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Membrane-bound antibodies to bloodstream *Trypanosoma cruzi* in mice: strain differences in susceptibility to complement-mediated lysis

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SUMMARY

The Y, CL and other strains of *Trypanosoma cruzi* display different morphological and immunological characteristics. Such observations are here extended to the interaction of bloodstream forms of different strains of *T. cruzi* with components of the complement system. We demonstrate that the bloodstream forms of the Y and B strains, but not those of the CL strain, are lysed by normal human serum. Lysis is mediated by combined activities of the alternative and classical complement pathways. These activities are triggered by antibodies on the surface of the parasites as shown by: (a) binding of fluorescein or radiolabelled anti-mouse immunoglobulin to the parasite's membrane and (b) the finding that bloodstream forms from lethally irradiated mice can be sensitized and rendered susceptible to complement-mediated lysis by incubation with sera from acutely infected animals. Bloodstream forms of the CL strain also bear surface immunoglobulin and sensitizing antibodies are present in the sera of mice infected with this strain. However, CL trypomastigotes from acutely infected mice fail to be lysed by human or mouse complement unless the parasites are pre-incubated with sera from chronically infected animals.

The basis of the different interactions between CL and Y trypomastigotes with antibodies and the complement system, and their biological significance are discussed.

INTRODUCTION

Infections in experimental animals by different strains of *Trypanosoma cruzi* have distinct and constant characteristics (Brener, Chiari & Alvarenga, 1974; Brener, 1977). The Y and CL strains exemplify extremes of such strain differences. Although these two strains are 100% lethal for albino mice, they induce very distinct patterns of parasitaemia, including clearance from the circulation at different rates after injection into normal or immune animals. Thus the Y-strain trypanosomes disappear much more rapidly from blood (Brener, 1969) than trypanosomes of the MR strain in which stout forms predominate (Howells & Chiari, 1975; Brener, 1973). Also, relapses during the chronic phase of infection can be induced by immunosuppressive agents in animals infected with the CL-type but not with the Y strain (Brener & Chiari, 1971; Krettli, 1977).

Blood stream forms of the Y and CL strains diversely interact *in vitro* with antisera from chronically infected mice (Krettli & Brener, 1976). Trypomastigotes of the Y strain are agglutinated and lose their infectivity upon incubation with immune serum, whereas CL trypomastigotes are unaffected. In this paper we report studies designed to clarify the basis of these striking strain differences.

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MATERIALS AND METHODS

Animals. Inbred A/J male mice (Jackson Laboratories, Bar Harbor, Maine) or outbred S/W male mice (Taconic Farms, Germantown, New York), 5–8 weeks old were used. Groups of mice were lethally irradiated by exposing them to 650 rads 24 hr before inoculation with trypomastigotes. This irradiation was provided by a 1·2 min exposure to a Gammator (Radiation International Inc., Parsippany, New Jersey). Acidulated water (0·001 N) *ad libitum* and cereal were provided in addition to their usual diet.

Diluents and reagents. Tissue culture medium 199 (TC 199) (Grand Island Biological Co., Grand Island, New York), supplemented with 10% foetal calf serum (FCS), adjusted to a pH of 7·2, was used to wash and dilute the tryomastigotes.

Phosphate-buffered saline (PBS), pH 7·2, served as a diluent for immunofluorescence. Rabbit antibody to mouse 7S immunoglobulin and fluorescein-conjugated goat anti-rabbit immunoglobulin were obtained from Meloy Laboratories (Springfield, Virginia). Disodium ethylenediaminotetra-acetate (EDTA) and sodium ethyleneglycolbis-(β-aminoethylether)-N-N′-tetra-acetate (EGTA) were used at a final dilution of 0·02 M.

Complement reagents. Fresh normal human serum (HuC) was the main source of complement (C). Pooled serum was stored in small aliquots at −70°C until used. C2-deficient human serum was kindly supplied by Dr M. M. Glovsky (Kaiser Medical Group, Los Angeles, California). Human C2 was obtained from Cordis Laboratories, Miami, Florida.

Factor B-depleted serum was obtained by heat inactivation of human serum at 50°C for 30 min (Pillemer *et al.*, 1954). Properdin-depleted serum was obtained by incubating 1 ml of human serum with 2 mg of zymosan at 15°C for 1 hr, after which the zymosan was removed by centrifugation at 1000 ***g*** for 20 min in the cold (Pillemer *et al.*, 1954); the supernatant serum was used immediately. Properdin purified from pooled human serum and factor B were kindly supplied by Dr M. Takahasi and G. Fastaia (Department of Pathology, New York University Medical Center). Fresh serum from S/W and A/J mice was obtained by bleeding from the retro-orbital plexus. The blood was collected in pre-cooled plastic tubes, kept on ice, and centrifuged at 1000 ***g*** for 15 min at 4°C.

Strains of T. cruzi. The Y, Berenice (B), CL and Gilmar (G) strains were used. Some of their biological and immunological characteristics have been described previously (Brener *et al.*, 1974; Brener, 1977; Krettli & Brener, 1976). The strains were kindly given to us by Dr Z. Brener (Centro de Pesquisas René Rachou, Belo Horizonte, where they are maintained in mice by serial blood passage). Acute infections were produced in A/J or S/W mice by i.p. inoculation of bloodstream trypomastigotes, contained in blood samples, which were collected from acutely infected animals in 3·8% sodium citrate.

To standardize inocula, 3 μl of pooled blood were placed between slide and coverslip (18 mm × 18 mm). Parasites were counted in fifty fields as previously determined (Brener, 1962). Dilutions were then made with TC 199-FCS so that each mouse received the parasite inoculum in a volume of 0·2 ml.

Isolation of trypomastigotes. Mice were bled during the acute phase of infection from the retro-orbital plexus. Blood was collected into tubes containing glass beads, defibrinated and then centrifuged at 100 ***g*** for 10 min at room temperature. This separated the serum which contained a large proportion of the trypomastigotes, from the formed elements of the blood which were concentrated in the pellet. For further separation of trypomastigotes from the sediment, the pellets were resuspended in ~3 ml TC 199-FCS and recentrifuged for 10 min at 100 ***g*** at room temperature. To concentrate the trypomastigotes contained in the supernantants resulting from both centrifugations, they were spun in the cold at 1000 ***g*** for 15 min. The trypomastigotes were thereby pelleted; sera of infected mice were stored at −20°C. The pooled trypomastigotes were washed three to four times in cold TC 199-FCS (1000 ***g*** for 15 min) and counted in a haemocytometer. In several instances, washed trypomastigotes were kept overnight on ice before use.

In vitro coating of trypomastigotes. Parasites isolated as described above from irradiated infected mice were diluted with TC 199-FCS to 0·8–2 × 10^7 trypomastigotes/ml. Fifty μl of this parasite suspension were incubated with 200 μl of heat-treated sera (56°C, 30 min), obtained from acutely or chronically infected mice. After incubation for 1 hr at 37°C, these trypomastigotes were washed by repeated centrifugation (at 1000 ***g*** for 15 min), resuspended in 0·2 ml of TC 199-FCS and observed for lysis upon addition of HuC.

Lysis of trypomastigotes with HuC. Trypomastigotes suspensions were diluted to 2–5 × 10^6 ml with TC 199-FCS. Fifty μl aliquots were placed in plastic tubes containing 50 μl of HuC. The mixture was then incubated at 37°C for 1 hr. Control tubes contained equal volumes of the parasite suspension and heat-inactivated human serum (56°C for 30 min = HIS). After incubation the tubes were placed on ice and the trypomastigotes counted. Each count was done in duplicate. Percentage of lysis was determined by the equation:

$$\text{Percentage lysis} = 100 - \frac{\text{number of parasites after incubation with HuC}}{\text{number of parasites after incubation with HIS}} \times 100.$$

Immunofluorescence staining of the parasites. Most of our immunofluorescence observations were done on air-dried acetone-fixed trypomastigotes. A drop of purified and washed parasites, suspended in TC 199-FCS (1 × 10^6 trypomastigotes/ml) was allowed to dry overnight on slides at room temperature and was then refrigerated until used. Direct immunofluorescence was performed with fluorescein-conjugated rabbit antibodies (7S) to mouse immunoglobulins, diluted to 1 mg/ml in PBS. Indirect staining was with rabbit antibodies to mouse immunoglobulins (0·5 mg/ml) overlaid with fluorescein-conjugated goat anti-rabbit (7S) immunoglobulin (1·0 mg/ml).

For direct staining, the slides were washed in PBS for 15 min, fixed in acetone for 30 sec, then washed in PBS for 15 min

and overlaid with the fluorescent antibody for 30 min in a moist chamber. Slides were then washed in PBS for 15 min and mounted in 10% glycerol in PBS.

Indirect staining was performed by incubation of the washed and fixed preparations with non-fluoresceinated rabbit anti-mouse Ig antibody for 30 min, followed by several washes in PBS and incubation with fluorescein-conjugated goat anti-rabbit antibody for 30 min. The slides were then washed in PBS and mounted in PSB-glycerol and subsequently examined with a Leitz Orthoplan microscope illuminated by a 200-W ultra-high-pressure Hg lamp equipped with a KP490 and K480 excitation filter plus a K510 suppression filter.

RESULTS

Lytic effect of fresh human serum on T. cruzi

When trypomastigotes isolated from the blood of acutely infected mice were incubated with normal human serum (HuC), many parasites were lysed. This phenomenon was strain-dependent. Trypomastigotes of *T. cruzi* strains in which slender forms predominate, i.e. the Y and B strains, were extremely sensitive to C-mediated lysis. On the other hand, strains which had mostly broad trypomastigotes, i.e. CL and G strains, could not be lysed by HuC.

Complement-mediated lysis required a high concentration of human serum. Incubation of trypomastigotes in an equal volume of undiluted HuC resulted in lysis of 80% or more of the parasites. However, lysis was considerably lower when an eight-fold dilution of HuC was incubated with Y or B trypomastigotes (Table 1).

TABLE 1. Lytic effect of human complement (HuC) on bloodstream forms of *T. cruzi* from acutely infected mice

Strain of *T. cruzi*	Percentage lysis Final dilution of HuC		
	1 : 2	1 : 4	1 : 8
Y	88	63	38
Y*	0	0	0
B	79	71	13
CL	0	0	n.d.
G	0	0	0

* Trypomastigotes obtained from lethally irradiated mice.

Lysis of trypomastigotes is antibody-mediated

The Y trypomastigotes from acutely infected mice varied widely in susceptibility to lysis, according to the day of infection on which parasites were harvested (Table 2). Parasites collected very early during the infection (day 3–6 after inoculation) were not lysed upon incubation with HuC. With parasites collected on day 7 after inoculation, the degree of lysis varied considerably from experiment to experiment. From day 8 onwards, the percentage of lysis of the trypomastigotes with HuC was consistently very high.

The time of occurrence and degree of lysis also depended on the size of the inoculum used to infect the animals. For example, mice infected i.p. with 200,000 trypomastigotes had 'sensitized' bloodstream forms 1 week earlier than animals which had received a ten-fold smaller inoculum.

When Y trypomastigotes were obtained from mice which had been lethally irradiated before inoculation with parasites, lysis failed to occur (Tables 1 & 2). However, these parasites could be lysed when incubation with HuC was preceded by incubation with sera collected from acutely infected non-irradiated animals. Using these uncoated trypanosomes it was possible to determine that (a) sera collected from day 7 onwards from acutely Y strain-infected non-irradiated mice could sensitize these parasites;

TABLE 2. Lysis by HuC of *T. cruzi* from acutely infected mice on different days after inoculation

Source of trypomastigotes		Percentage lysis Day after inoculation								
Mice	Strain of *T. cruzi*	3	5	7	8	10	11	12	16	19
Non-irradiated	Y	—	—	0	97	87	—	—	—	—
		—	—	27	78	90	85	—	—	—
		—	0	48	—	—	—	—	—	—
		0	0	83	91	—	—	85	75	—
Irradiated*	Y	—	0	0	0	0	—	—	—	—
Non-irradiated	CL	—	—	—	0	0	—	0	0	0

* The mice had been lethally irradiated 24 hr prior to their inoculation.

(b) these antibodies also occurred in the sera of mice infected with parasites of the CL strain; and that (c) this property was absent from the sera of irradiated infected animals (Table 3). In contrast, trypomastigotes of the CL strain from acutely infected mice were not lysed upon incubation with HuC even when collected as late as on day 19 after infection (Table 2). However, these parasites were extensively lysed when pre-incubated with sera from chronically infected mice. In fact, dilutions of $\geqq 1 : 16$ of these chronic immune sera produced lysis of $>90\%$ of the CL parasites (Table 4).

The presence of immunoglobulin on the surface membrane of *T. cruzi* was directly demonstrated by immunofluorescence. Fluorescein-labelled anti-mouse Ig revealed positive staining of trypomastigotes of both Y and CL strains, isolated from the bloodstream of acutely infected mice (Table 5). In many experiments, the reactions were consistently positive with Y trypomastigotes isolated on or after day 8 of the infection. The Y trypomastigotes obtained earlier during the infection gave consistently negative or $\pm$ readings on day 5. The reactions were positive only in a certain number of the samples collected on day 7 after infection (Table 5).

TABLE 3. Presence of antibodies in the sera of mice during acute phase of *T. cruzi* infection

Source of serum from acute phase of infection			Mean percentage lysis of Y trypomastigotes* incubated with serum and HuC
Mice	*T. cruzi* strain	Serum obtained on day	
Non-irradiated	Y	5	10 (1)†
		7	83 (3)
		8	85 (3)
		9	80 (3)
		16	82 (2)
Irradiated	Y	8	0
		7	0
Non-irradiated	CL	10	87
		15	59
		19	97

* Trypomastigotes obtained from lethally irradiated mice.

† Number of experiments in parentheses.

TABLE 4. Lysis by HuC of bloodstream forms of Y and CL strains upon incubation with sera from chronically infected mice

Strain	Immune serum	Reciprocal of dilution of immune serum						
		2	4	8	16	32	64	128
Y*	Anti-Y	95	94	84	89	73	54	37
Y*	Anti-CL	91	77	72	59	48	0	—
CL	Anti-CL	92	—	98	94	—	—	—

* Trypomastigotes obtained from mice which had been irradiated before inoculation with *T. cruzi*.

Pooled sera obtained from chronically and repeatedly infected mice.

TABLE 5. Immunofluorescence of bloodstream forms of the Y and CL strains from mice during acute phase of infection*

Day of Infection	Y-trypomastigotes		CL trypomastigotes from
	Non-irradiated mice	Irradiated mice	non-irradiated mice
5	± (12)*	± (8)	n.d.
7	± (2)	± (8)	n.d.
7	Pos (12)	Neg. (7)	n.d.
8	Pos (9)	± (4)	n.d.
10	Pos (6)	± (1)	Pos. (3)
12	Pos. (9)	n.d.	Pos. (1)
14	Pos. (1)	n.d.	Pos. (4)
19	n.d.	n.d.	Pos. (2)

* Number of experiments in parentheses.

The CL trypomastigotes obtained from day 10 to day 19 after infection consistently gave positive immunofluorescent antibody (IFA) reactions. Positive reactions were also obtained with a fluorescein-labelled anti-IgM reagent, but only in trypomastigotes isolated on or after day 10 of infection.

The occurrence of surface membrane fluorescence was corroborated by staining live trypomastigotes of both the CL and Y strains in suspension. The staining pattern was different than that seen in acetone-fixed preparations. Detailed observations were difficult due to the fast motility of living parasites.

The Y trypomastigotes from irradiated mice consistently gave negative or ± reactions with anti-mouse Ig, regardless of the day of infection. These mice had very rapidly rising, high parasitaemias; most did not survive beyond day 8.

Mechanisms of lysis of Y trypomastigotes by HuC

The involvement of complement (C) in the lysis of bloodstream forms of *T. cruzi* was initially suggested by the finding that parasites were lysed upon incubation at 37°C with HuC but not with heat-inactivated normal human serum. Lysis failed to occur when this incubation was carried out at 4°C or in the presence of chelating agents.

Direct evidence for participation of the various complement components was provided by experimental findings (Fig. 1). Factor B-depleted human serum failed to lyse these parasites. Lytic activity was totally restored with the addition of physiological concentrations of purified factor B (Fig. 1a). Trypomastigotes also failed to be lysed by properdin-depleted serum. The addition of properdin to this depleted serum restored a considerable proportion of the original lytic activity (Fig. 1b).

Participation of the classical pathway of C activation in the lysis of Y trypomastigotes was investigated by adding purified C2 to C2-deficient human serum (Fig. 1c). Although a 35% lysis of the trypanosomes was obtained with the C2-deficient serum, the addition of increasing amounts of C2 enhanced lytic activity. When Ca^{2+} ions were chelated by 10 mM of EGTA in the presence of 5 mM of Mg^{2+}, lysis of Y trypomastigotes fell from 95% to 65%.

Taken together, these experiments indicate that the lysis of bloodstream trypomastigotes requires an intact alternative complement pathway. The classical pathway appears to enhance lysis.

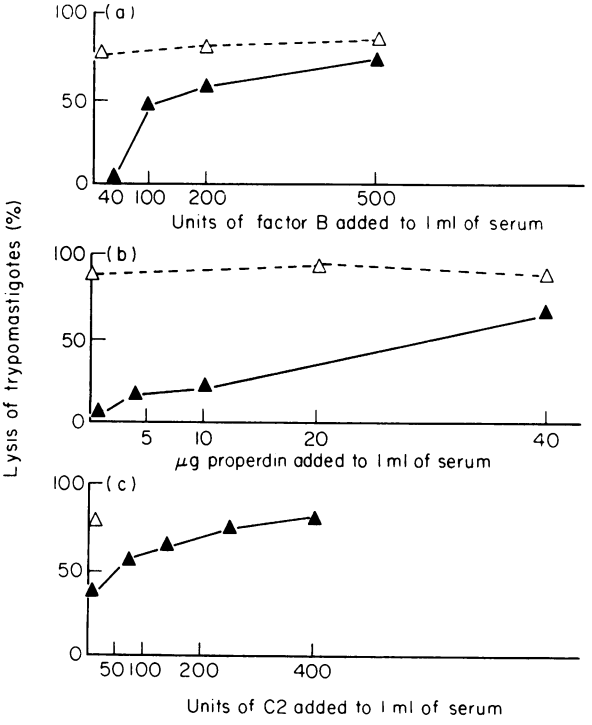

FIG. 1. (a) Inhibition of complement-mediated lysis of *T. cruzi* by depletion of factor B from human serum. Depletion produced by heat-inactivation of HuC for 30 min at 50°C. Lytic activity was restored by addition of purified factor B. Results are the means of triplicate experiments. (△) Non-depleted Hu serum; (▲) factor B-depleted Hu serum. (b) Role of properdin in complement-mediated lysis of *T. cruzi*. Properdin-depletion was produced by incubation of 1 ml of HuC with 2 mg of zymosan at 15°C for 1 hr. Lytic activity was restored upon addition of properdin to the zymosan-treated human serum. Results are the means of triplicate experiments. (△) Untreated Hu serum; (▲) zymosan-treated Hu serum. (c) Reduced lytic activity of C2-deficient serum for *T. cruzi*. Lytic activity restored by addition of purified human C2. (△) Normal Hu serum, (▲) C2-deficient Hu serum.

Lysis of trypomastigotes by mouse complement

Lysis of Y bloodstream parasites was less pronounced upon their incubation with fresh mouse serum. The percentage of lysis on incubation with normal serum from S/W mice varied from 20 to 45% in different experiments. This represented ~50% of the lysis produced by incubation of the same parasite preparation with HuC. Fresh serum from A/J mice, which are C5-deficient, did not lyse the parasites.

DISCUSSION

Ours is the first demonstration that HuC lyses trypomastigotes of *T. cruzi* isolated from the blood of acutely infected mice. This lysis is strain-dependent: trypomastigotes from the Y and B strains were lysed upon incubation at 37°C with HuC, whereas those from strains CL and G failed to do so.

Bloodstream trypomastigotes of *T. cruzi* from infected mice are lysed by fresh sera of chickens, frogs and toads, but not by normal human, mouse or guinea-pig sera (Rubio, 1956; Budzko, Pizzimenti & Kierszenbaum, 1975). The discrepancy with our findings of lytic activity of human complement may reside in the use of CL-like strains in those earlier experiments, and/or the isolation of trypomastigotes from animals with relatively low parasitaemias—a condition which does not favour rapid antibody formation and lysis of the trypomastigotes.

Kierszenbaum, Ivanyi & Budzko (1976) demonstrated that in an avian system the lysis of bloodstream trypomastigotes is mediated via activation of the alternative pathway of complement, and suggested that this lysis occurred in the absence of antibodies—a conclusion based on the finding that lytic activity resided in the sera of agammaglobulinaemic chickens. In view of the present results it is conceivable that the trypomastigotes used in their experiments were already coated with mouse antibodies before their incubation with chicken serum, similar to the Y strain described in this paper.

Our data indicate that the alternative pathway of complement activation is most important in the immune lysis of bloodstream forms of *T. cruzi*. Lysis was abolished by the depletion of factor B or properdin from HuC and was fully restored by physiological amounts of these serum components. The significantly smaller degree of lysis obtained with a C2-deficient serum, or by performing the reaction in the absence of Ca^{2+}, suggests that the classical pathway amplifies the alternative pathway of complement-mediated lysis, as reported for other systems (Pillemer *et al.*, 1954).

Our data corroborate previous findings on the complement requirements for the lysis of bloodstream forms of *T. cruzi* sensitized with heat-inactivated sera of chronically infected mice or humans (Budzko *et al.*, 1975). This antibody coating of the bloodstream forms of *T. cruzi* in the course of acute infections in mice has also been documented by Kloetzel & Deane (1977), who demonstrated immunoglobulins on the surface of bloodstream trypomastigotes by immunofluorescence. Our data also reveal striking strain differences in the ability of this antibody coating to induce lysis in the presence of C. Although antibodies have been demonstrated on the surface of Y and CL trypomastigotes from acutely infected mice, and in their sera, only Y trypomastigotes were lysed by human or mouse complement.

How can this paradoxical observation be explained? It is unlikely to be due to a non-complement-fixing class of Ig on the parasite membrane since sera of mice acutely infected with the CL strain mediated lysis of Y trypomastigotes from irradiated mice (Table 3). On the other hand, CL trypomastigotes are not resistant to complement activity, since they were lysed after incubation with immune sera from chronically infected mice. Perhaps, then quantitative and/or qualitative differences influence the interaction of Y and CL trypomastigotes with antibody and complement. This is suggested by preliminary observations in which we used ^{125}I-labelled anti-mouse γ globulin to measure surface-bound IgG. As expected, this reagent bound to the surface membrane of both the Y and the CL bloodstream forms, and could be eluted from their surface after incubation at 37°C in the absence of immune serum. However, kinetic data on this 'release' reaction suggest that CL trypomastigotes lose the bound ^{125}I-labelled antibodies much more rapidly than trypomastigotes of the Y strain (Krettli & Nussenzweig, unpublished observations).

Loss or redistribution of surface immunoglobulin could also be shown by immune lysis: the Y trypomastigotes, which are lysed efficiently with HuC, gradually lose their susceptibility to lysis when pre-incubated at 37°C in the absence of immune serum. After 1 hr pre-incubation, lysis was abolished. If these parasites were re-incubated with immune serum and HuC, lysis occurred again, which suggests that was no significant loss of surface antigens during the 37°C pre-incubation.

Does shedding and/or interiorization of membrane-bound antibodies occur? This will be investigated with the aid of labelled trypomastigotes and radiolabelled antibodies. The present observations appear to be somewhat analogous to the phenomenon of modulation of surface antigens extensively investigated in leukaemic cells (De Vaux Saint Cyr, 1974; Genovesi, Marx & Wheelock, 1977) and recently reported for *Leishmania donovani* (Dwyer, 1976), also a trypanosomatid parasite. This phenomenon is likely to represent a mechanism of evasion by the *T. cruzi* parasite of the host's immune response, which might be responsible in part for strain-linked differences in the course of infection. Our working hypothesis is that trypomastigotes of the CL strain of *T. cruzi* remove the immune complexes from their membrane

more efficiently than those of the Y strain and for this reason CL trypomastigotes are not lysed by complement.

How this *in vitro* lysis relates to the defence mechanisms *in vivo* remains obscure. The relative importance of phagocytosis and complement-mediated lysis in *T. cruzi* infection has not been established and may vary in infections induced by different strains. Since fresh mouse serum was not very effective in mediating lysis in the *in vitro* system, it might appear that immune lysis plays only a minor role in modulating the course of infection in these animals. Furthermore, C5-deficient mice develop a similar course of infection to mice with normal C5 levels (Krettli & Nussenzweig, unpublished observations). In contrast, C3 plays an important role in resistance to this infection, as shown by the increased intensity of parasitaemia and mortality of mice treated with cobra venom factor (Budzko *et al.*, 1975).

Antibody coating and activation of the earlier components of the complement system, followed by deposition of C components on the membrane of the parasites, may modulate the uptake and handling of bloodstream forms of *T. cruzi* by phagocytic cells. A study of the *in vitro* uptake of bloodstream forms of *T. cruzi* by peritoneal macrophages has revealed appreciable strain differences and significantly increased rates of parasite uptake as a result of opsonization (Alcantara & Brener, 1978). Antibody coating and deposition of complement components on bloodstream forms might also affect the capacity of these parasites to penetrate and multiply within various tissue cells and account for some of the differences in the tissue tropism of various *T. cruzi* strains (Melo & Brener, 1977).

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