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# Modulation of Sensitivity of Blood Forms of *Trypanosoma cruzi* to Antibody-Mediated, Complement-Dependent Lysis

FELIPE KIERSZENBAUM[1]* AND MIGUEL A. RAMIREZ[2]†

*Departments of Microbiology and Public Health[1] and Pathology,[2] Michigan State University, East Lansing, Michigan 48824-1101*

Received 17 July 1989/Accepted 10 October 1989

**The numerous reports on lysis of blood (trypomastigote) forms of *Trypanosoma cruzi* by specific antibodies plus complement have systematically shown that a certain proportion of parasites survives. However, it is not known whether the insensitive organisms represent a subpopulation (or clones) or a certain developmental phase of otherwise morphologically identical parasites. In this work, we established that partial lysis was not due to the use of insufficient amounts of lytic reagents. Thus, supernatants of lytic reaction mixtures killed the same proportion of *T. cruzi* as previously unused reagents. Moreover, in parallel tests in which the trypomastigote concentration was up to four times greater than that used in standard lysis tests, the percentages of lysis were comparable. Incubation periods as long as 4 h did not increase the extent of lysis beyond the value observed after only 1 h, indicating that the routinely used 1-h incubation was appropriate. The extent of lysis was not increased by additional amounts of antibody, complement, or both. Instead, trypomastigotes surviving immune lysis, washed, and incubated with fresh diluent for 45 to 120 min before being used in new lysis tests did manifest additional sensitivity to immune lysis. Three successive infections in mice with parasites which had survived immune lysis led to the production of trypanosomes that displayed the same level of resistance to immune lysis as the original, untreated parasite population. Of interest, the average parasitemias of these groups of mice did not evidence a tendency to increase, as might have occurred if an immune-lysis-resistant subpopulation had been selected. Since trypomastigotes exhibiting resistance to immune lysis can eventually become sensitive, resistance to immune lysis does not represent an insensitive parasite subpopulation. This resistance appears to be modulated by the presence of the lytic reagents and might involve expression of as yet unidentified surface components playing a role in complement activation.**

In the initial report of the sensitivity of blood (trypomastigote) forms of *Trypanosoma cruzi* to lysis by specific antibody and complement, it was also shown that a certain proportion of the organisms was able to survive (3). This finding was corroborated in subsequent work by the same and other groups of investigators despite their use of different *T. cruzi* isolates, trypomastigotes derived from infected animals or insect vectors, different sources of specific antibodies, and modifications of the lysis test (4, 5, 8, 9, 12, 14, 17, 20). To date, no explanation has been forwarded for the partial resistance of *T. cruzi* trypomastigotes to immune lysis. Because some *T. cruzi* isolates have been shown to consist of a mixture of distinct clones (6), it seemed conceivable that not all clones would be sensitive to immune lysis. Alternatively, this type of resistance could reflect transitional molecular changes occurring on the surface of otherwise morphologically indistinguishable *T. cruzi* trypomastigotes. These possibilities were examined in the present work, in which we used both in vitro and in vivo approaches to establish whether elimination of the immune-lysis-sensitive subpopulation would select a trypomastigote subpopulation displaying increased resistance to lysis by specific antibody plus complement.

## MATERIALS AND METHODS

**Animals.** Crl-CD-1(ICR) Swiss mice (Charles River Breeding Laboratories, Inc., Portage, Mich.) (4 weeks old) were used for maintenance and production of *T. cruzi* trypomastigotes.

**Parasites.** Bloodstream trypomastigotes of *T. cruzi* (Tulahuen isolate subline) were purified from the blood of mice infected intraperitoneally (i.p.) 2 weeks previously with $10^5$ trypomastigotes by centrifugation over a mixture of Ficoll-Hypaque of density 1.077 (2), followed by chromatography through a DEAE-cellulose column (16). The eluted organisms were centrifuged (800 × *g*, 20 min, 4°C) and suspended at $10^7$ organisms per ml in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). This medium is referred to as diluent. Parasite concentrations in blood were determined by a standardized microscopic procedure (11), whereas concentrations in blood cell-free suspensions were established microscopically with a hemacytometer.

**Sera.** Blood was drawn from the retro-orbital venous plexus of ether-anesthetized mice which had been either not infected or infected with a sublethal i.p. dose of *T. cruzi* (50 organisms) 2 to 4 months previously. The blood samples were allowed to clot at room temperature for 2 h, and the sera were separated by centrifugation. Sera from each group were pooled, sterilized by filtration through 0.45-μm-pore-size filters, heat inactivated at 56°C for 30 min, aliquoted, and stored at −20°C until used. These pools are referred to as heat-inactivated normal mouse serum (HI-NMS) and heat-inactivated immune mouse serum (HI-IMS). Guinea pig serum separated from blood drawn from ether-anesthetized animals by cardiac puncture was used as the source of complement. Aliquots of this material were stored at −70°C until used.

* Corresponding author.
† Present address: Estación Experimental Guanapa, El Tigre, Estado Anzoategui, Venezuela.

**Lysis tests.** What we refer to as the standard lysis test consisted of mixing 100 μl of either HI-NMS or HI-IMS (or diluent), 25 μl of a solution containing 0.15 M sodium chloride, 4 mM magnesium chloride, and 1.2 mM calcium chloride, 25 μl of either complement or diluent, and 25 μl of parasite suspension and incubating the mixture at 37°C for 1 h. Parasite concentrations were then determined microscopically with a hemacytometer, and the percentage of lysis was calculated with respect to the parasite count obtained with HI-NMS or diluent (parasite concentrations before and after incubation were comparable when HI-NMS or diluent was used). When larger volumes of reaction mixtures were needed, all volumes were increased proportionately so that the final concentration of each reagent would not vary. In some experiments, parasite concentrations were determined in the same mixtures after various incubation times. In other cases, the standard lysis test was set up and, at the end of predetermined incubation periods (see Results), fresh portions of HI-IMS, complement, or HI-NMS were added and the percentage of immune lysis was determined after an additional 1-h incubation. In some experiments designed to establish whether sufficient antibody and complement had been initially added, the parasites were removed by centrifugation at the end of the standard lysis test and the supernatants were tested for residual lytic activity with a fresh portion of parasite suspension. Experiments were also performed in which the number of parasites was varied (see Results) without changing either the amounts of other reagents or the final volume of the reaction mixture. Another protocol consisted of recovering by centrifugation the parasites that survived the standard lysis test, washing them twice with diluent, and using them in a second lysis test with fresh portions of the original reagents. All determinations were made in duplicate or triplicate. The significance of differences between means was established by Student's *t* test.

**Successive infections of mice with immune-lysis-resistant *T. cruzi*.** A combination in vivo-in vitro protocol was used to establish whether trypomastigotes surviving immune lysis would cause infections in which the newly developing blood forms were more resistant to lysis than the original population. Initially, we infected mice i.p. with $10^5$ untreated blood trypomastigotes and recorded their parasitemias at various times after infection (see Results). Parasites were collected from these mice on day 15 postinfection (p.i.) and purified as described above. A sample (10 ml at $2 \times 10^6$ organisms per ml of diluent) was incubated with 3 ml of HI-IMS and 2 ml of complement diluted 1/3 in diluent at 37°C for 1 h in the presence of calcium and magnesium ions at the same concentrations as used in the standard lysis test. A separate aliquot of the parasite suspension was mock treated with diluent. The parasites were then washed twice with diluent by centrifugation, counted, and adjusted to the desired concentration, and samples were used in standard lysis tests. The rest of the two preparations was used to inject new groups of animals ($10^5$ parasites per mouse, i.p.). The parasitemias of these groups were monitored, and on day 15 p.i., organisms from the mice which had been infected with immune-lysis-resistant organisms were purified to repeat the treatments, lysis tests, and infections. This protocol was repeated twice more, using in every instance parasites collected on day 15 p.i. The same batches of HI-IMS, HI-NMS, and complement were used throughout the entire experiment.

TABLE 1. Percentages of *T. cruzi* lysed by HI-IMS plus complement after various incubation times[a]

| Expt no. | % of parasites lysed after incubation for: | | | |
|---|---|---|---|---|
| | 1 h | 2 h | 3 h | 4 h |
| 1 | 29.9 ± 8.0 | 32.3 ± 8.3 | ND[b] | 32.3 ± 3.6 |
| 2 | 24.9 ± 1.7 | 23.0 ± 2.5 | ND | 20.4 ± 2.0 |
| 3 | 32.3 ± 3.8 | 30.4 ± 4.3 | 25.8 ± 3.0 | ND |

[a] In this and all subsequent tables, the results are expressed as the mean ± standard deviation. A standard lysis test was set up, and samples were taken at the indicated times for microscopic counting of surviving organisms. The percentage of lysis was calculated with respect to the parasite concentration in control reaction mixtures containing HI-NMS instead of HI-IMS.
[b] ND, Not determined.

## RESULTS

Lysis tests were performed by incubating the experimental and control mixtures for 3 or 4 h, and the percentage of lysis was determined at 1-h intervals. The proportions of trypomastigotes lysed by HI-IMS plus complement did not increase significantly over the value obtained after 1 h (Table 1). Several approaches were used to test whether the partial level of immune lysis was due to the presence of limiting amounts of antibody or complement. In one of these approaches, we used a two-step protocol. Initially, we set up a standard lysis test and determined the percentage of lysis after 1 and 2 h of incubation. Then, HI-IMS alone or together with complement was added (equivalent volumes of diluent were added to the controls), and the extent of lysis was measured again after incubating at 37°C for 1 h. At the end of this period, we made a second addition of the same reagents, incubated for another hour, and determined the percentage of lysis once more. The results (Table 2) showed that repeated incorporations of HI-IMS alone or HI-IMS plus complement did not cause significant additional lysis. In a second approach, a standard lysis test was conducted and the supernatants of the experimental and control mixtures were used as the sources of antibody plus complement and HI-NMS, respectively, in a second lysis test with fresh portions of organisms. The supernatants from tests originally performed with antibody plus complement were found to be as lytic as samples of previously unused lytic reagents. Thus,

TABLE 2. Effects of incorporation of additional HI-IMS and complement at the end of standard lysis tests[a]

| Reagent(s) added | Time of addition (h) | % Lysis |
|---|---|---|
| HI-IMS | 2 | 28.0 ± 4.3 |
| Complement | 2 | 28.0 ± 8.3 |
| HI-IMS + complement | 2 | 21.8 ± 6.4 |
| HI-NMS | 2 | 25.3 ± 4.1 |
| HI-IMS | 3 | 30.2 ± 4.8 |
| Complement | 3 | 20.3 ± 4.7 |
| HI-IMS + complement | 3 | 23.4 ± 2.7 |
| HI-NMS | 3 | 29.7 ± 1.6 |

[a] The percentages of lysis obtained in the standard lysis test after 1 and 2 h of incubation (without any new reagent additions) were 29.9 ± 8.0 and 32.3 ± 8.3%, respectively. After the second determination (2-h point), samples of the experimental reaction mixtures received the indicated reagent(s), whereas the control received equal volumes of diluent. The percentage of lysis was determined again 1 h later, at which time new additions of reagent(s) and diluent were made to the experimental and control tubes, respectively. Lysis was determined once more 1 h later. This set of results is representative of three repeat experiments. None of the differences was significant.

TABLE 3. Effects of increasing the concentration of *T. cruzi* in the lysis test on the extent of parasite killing[a]

| Parasite concn ($10^6$ organisms/ml) | % Lysis by HI-IMS + complement | No. of *T. cruzi* lysed |
|---|---|---|
| 1.4 | 35.0 ± 0.9 | $4.9 \times 10^5$ |
| 2.8 | 29.4 ± 3.8 | $8.2 \times 10^5$ |
| 5.6 | 32.0 ± 1.6 | $1.8 \times 10^6$ |

[a] The final parasite concentration in a standard lysis test is $1.4 \times 10^6$ organisms per ml. This set of results is representative of two repeat experiments. None of the differences was significant.

in two repeat experiments, the percentages of lysis produced by supernatants collected at the end of standard lysis tests were 25.4 ± 2.3 and 26.7 ± 1.7%, respectively, and in parallel tests with fresh portions of HI-IMS and complement, the percentages of lysis were 24.6 ± 2.5 and 27.2 ± 1.6%, respectively. Another protocol consisted of increasing the parasite concentration up to four times over that used in standard lysis tests while keeping the final volume and the amounts of HI-IMS and complement unchanged. The findings, summarized in Table 3, revealed that the percentage of lysed organisms did not vary significantly with the increase in parasite concentration despite the presence of enough lytic activity to destroy a number of organisms greater than that present in the standard lysis test.

The above results were consistent with the possibility that *T. cruzi* suspensions contained a subpopulation of organisms resistant to immune lysis. However, when trypomastigotes incubated with HI-IMS plus complement at 37°C for 1 h and washed with and allowed to stand in diluent for 45 to 120 min were used in lysis tests, substantial proportions were killed by HI-IMS and complement (Table 4). Since these results suggested that resistance to immune lysis was not a permanent feature of the trypomastigotes, we designed an independent protocol to probe this concept. Flagellates purified from the blood of infected mice were used to determine the percentage of lysis by HI-IMS plus complement and also to infect groups of mice after treatment with either HI-IMS plus complement or diluent alone. A comparison of the results presented in Fig. 1A and B revealed that the parasitemia levels of animals infected with untreated and diluent-treated organisms measured on alternate days between days 9 and 15 p.i. were similar, whereas those of the group infected with immune-lysis-resistant organisms were lower (Fig. 1B). In the next two steps, trypomastigotes from the mice infected

TABLE 4. Additional lysis of immune-lysis-resistant trypomastigotes after washing with diluent[a]

| Expt no. | Time after washing (min) | % Lysis by HI-IMS + complement | |
|---|---|---|---|
| | | Before washing | After washing |
| 1 | | 34.1 ± 9.2 | |
| | 45 | | 31.1 ± 5.0 |
| | 90 | | 33.1 ± 4.5 |
| 2 | | 47.7 ± 3.3 | |
| | 60 | | 31.7 ± 5.2 |
| | 120 | | 39.7 ± 3.7 |

[a] A standard lysis test was performed. The parasites were then recovered by centrifugation, washed twice with diluent, and allowed to stand at 37°C for up to 90 or 120 min. Samples were removed at the indicated times to perform new lysis tests. The percentages of lysis were calculated with respect to controls containing HI-NMS instead of HI-IMS. This set of results is representative of two repeat experiments.

with immune-lysis-resistant organisms were purified, treated with either HI-IMS plus complement or diluent, and used in lysis tests as well as to infect new groups of mice. A comparison of the results (Fig. 1B, C, and D) showed no tendency of the successive generations of lysis-resistant parasites to cause increased parasitemias. The previously described protective effect of parasite pretreatment with specific antibodies (13) was readily observed in terms of reduced parasitemias with respect to those caused by organisms mock treated with diluent (Fig. 1B, C, and D).

In addition to being used for the successive infections described above, the various batches of parasites were used in lysis tests to establish whether organisms surviving immune lysis represented an immune-lysis-resistant subpopulation or whether they would eventually attain sensitivity to immune lysis. The proportion of trypanosomes killed by HI-IMS plus complement in the initial preparation (untreated trypomastigotes from the experiment depicted in Fig. 1A) was 29.3 ± 6.7%. The percentages of lysis obtained with the successive generations of immune-lysis-resistant organisms were comparable with this value (Table 5). Pretreatment of these parasite generations with diluent, complement, or HI-IMS plus complement and two washings with diluent before their use in lysis test did not alter significantly the proportion of parasites killed by HI-IMS plus complement.

## DISCUSSION

These results showed that the survival of some *T. cruzi* trypomastigotes after exposure to the lytic effects of antibody plus complement observed in our experiments was not the consequence of less than adequate incubation times, as virtually constant levels of parasite destruction were achieved over 3- and 4-h incubation periods (Table 1). Furthermore, and because the incorporation of additional amounts of HI-IMS and complement did not increase the extent of lysis beyond the level achieved in the initial standard tests (Table 2), we were able to rule out the possibility that limiting amounts of the lytic reagents were restricting lysis despite extended incubation. Moreover, supernatants of lysis test mixtures originally containing the standard amounts of HI-IMS and complement were still capable of lysing a similar number of trypanosomes in a second test with fresh samples of parasite suspensions. Further evidence for both the presence of ample amounts of antibody and complement and the selection of appropriate experimental conditions was derived from tests in which the parasite concentration was increased up to fourfold without a significant decrease in the percentage of lysis (Table 3). Clearly, the amounts of lytic reagents were sufficient to kill a number of trypomastigotes greater than that used in a standard lysis test but could not destroy all the flagellates present in the reaction mixture.

The findings discussed above did not contradict the notion that only some of the parasites might be sensitive to immune lysis. However, this concept was shaken by the observation that after the lytic reagents were washed off, the seemingly immune-lysis-resistant *T. cruzi* subpopulation could be readily lysed by antibody and complement (Table 4). This suggested that sensitivity to lysis was a transient property of the trypomastigotes whose manifestation might be regulated by the presence of the lytic reagents themselves. This possibility found additional support in the results of our in vivo experiments, showing that three successive generations of parasites originating in inocula consisting of immune-lysis-resistant trypomastigotes steadfastly maintained their

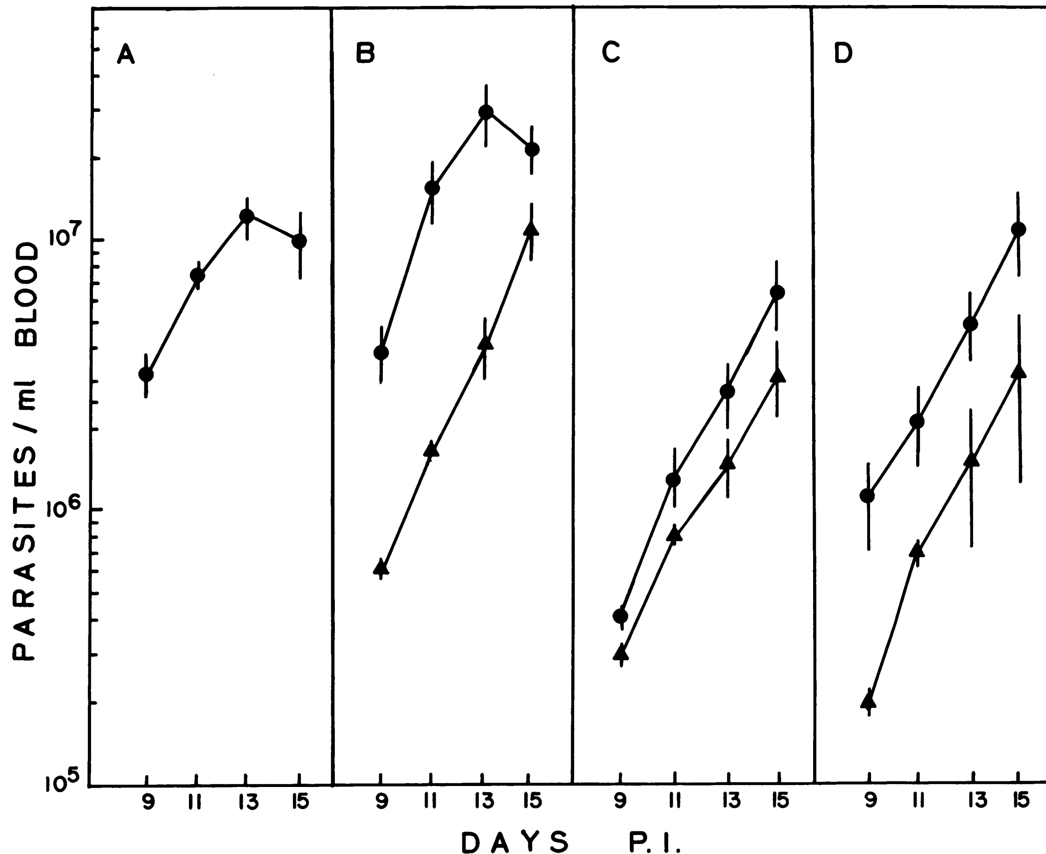


FIG. 1 Parasitemia levels of mice infected with successive generations of immune-lysis-resistant trypomastigotes. In the experimental phases depicted in panels A, B, C, and D, the numbers of mice infected with diluent-treated organisms (●) were 18, 10, 10, and 10, respectively. In panels B, C, and D, the numbers of mice infected with trypomastigotes surviving immune lysis (▲) were 18, 18, and 10, respectively. Points and vertical lines represent mean values and the standard error, respectively. (A) Course of parasitemia in animals infected with the initial, untreated trypomastigotes. (B) Parasitemias of mice infected with trypomastigotes from the previous step after treatment with HI-IMS plus complement or diluent and washing with diluent. (C and D) Parasitemias of animals receiving trypomastigotes from the mice infected in the previous phase with immune-lysis-resistant parasites after incubation with HI-IMS plus complement or diluent and washing with diluent. In all cases, parasites were obtained on day 15 p.i. and the i.p. inoculum consisted of $10^5$ parasites.

level of sensitivity to antibody-mediated, complement-dependent lysis (Table 5). In addition, infections with the immune-lysis-resistant parasites did not lead to infections accompanied by increased parasitemias, as might have been anticipated if the newly developed flagellates had resisted immune lysis in vivo.

The parasitemias exhibited by the mice infected with organisms surviving incubation with antibody and complement were systematically lower than those resulting from infection with organisms mock treated with diluent (Fig. 1B, C, and D). These results are in keeping with the well-established protective role of the humoral immune response in host defense against experimental Chagas' disease (reviewed in references 1 and 15) and are consistent with an earlier report that the virulence of *T. cruzi* is reduced after incubation with specific antibodies (13). The noted protection presumably resulted, at least in part, from phagocytosis of the opsonized flagellates (9, 10), lysis by complement in vivo (3, 14), or both.

Parasite passage through DEAE-cellulose columns during purification might have, conceivably, altered their sensitivity to immune lysis or selected for a subpopulation(s). However, any such alteration could not be the cause for partial sensitivity to immune lysis because this type of effect is readily demonstrable with *T. cruzi* trypomastigotes isolated by other means, including recovery from decanted plasma from infected blood (3). In addition, and because in the present work we used mouse anti-*T.cruzi* antibody in conjunction with guinea pig complement, it is noteworthy that partial lysis occurs also when antibody and complement from homologous sources are used (3).

Although *T. cruzi* trypomastigotes can be destroyed by humoral immunologic mechanisms, they survive in the host plasma and body fluids, where both specific antibodies and complement are present. It is possible that, as in our experiments, a certain proportion of organisms is eliminated, whereas another survives and accounts for the persistence of the parasite in the host. The results of our experiments (Tables 4 and 5) suggested that this scenario is plausible and raised the possibility that *T. cruzi*, in fact, modulates its sensitivity to immune lysis in the presence of specific antibodies, securing the survival of at least some organisms. Such an ability would offer the parasite better chances to reach its safest environment, the cytoplasm of host cells.

*T. cruzi* epimastigotes are lysed by mammalian complement via the alternative pathway without antibody requirement (18, 19), whereas trypomastigote lysis requires both specific antibody and complement (3). Kipnis et al. (12)

TABLE 5. Lytic effect of HI-IMS plus complement on *T. cruzi* from successive groups of mice infected with trypomastigotes surviving immune lysis[a]

| Expt | % of *T. cruzi* lysed by HI-IMS plus complement after pretreatment with: | | |
|---|---|---|---|
| | Diluent | Complement | HI-IMS + complement |
| B | 34.1 ± 9.3 | 24.6 ± 3.9 | 31.8 ± 11.2 |
| C | 27.8 ± 7.3 | 29.1 ± 4.9 | 28.2 ± 3.8 |
| D | 33.6 ± 4.6 | 34.8 ± 4.3 | 33.1 ± 5.9 |

[a] These tests were performed with parasites isolated from the groups of mice infected with organisms resistant to lysis by HI-IMS plus complement. The various batches of trypanosomes were first incubated with diluent alone or diluent containing either HI-IMS plus complement or complement alone at 37°C for 60 min, washed twice by centrifugation, adjusted to $10^7$ parasites per ml, and used in lysis tests with HI-IMS plus complement. In the original, unselected parasite population (Fig. 1A), the percentage lysed by HI-IMS plus complement was 29.3 ± 6.7%. All percentages of lysis were calculated with reference to the parasite concentration in control reaction mixtures containing diluent instead of HI-NMS and complement. No significant lysis was observed in additional controls in which HI-NMS was used instead of HI-IMS (data not shown). None of the differences in this table was significant. This set of results is representative of two experiments with the same protocol.

reported that $F(ab')_2$ from anti-*T. cruzi* immunoglobulin G is as effective in mediating complement-dependent lysis of trypomastigotes as whole antibody. These investigators suggested that immune lysis results from the blocking of parasite surface components capable of inhibiting complement activation. Viewed in this context, partial lysis of trypomastigotes might be interpreted in terms of antibody-modulated expression of a complement inhibitory surface component by some of the parasites. Antibody removal would eliminate the modulatory influence and allow some of the initially resistant organisms to become sensitive.

A trypomastigote surface glycoprotein(s) has been shown to play a role in the resistance of *T. cruzi* trypomastigotes to antibody-independent lysis via the alternative complement pathway (7). Because the epimastigote form (which does not occur in mammalian hosts) is sensitive to this type of lysis, the difference reflects a developmental molecular change with important implications to parasite survival in mammals. The survival of some trypomastigotes after immune lysis might conceivably result from either the expression of surface components that inhibit complement activation or the lack of expression of components required for such activation. The validity of these concepts remains to be assessed.

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