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Experimental Chagas' Disease: Kinetics of Lymphocyte Responses and Immunological Control of the Transition from Acute to Chronic *Trypanosoma cruzi* Infection†

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The responses of spleen cells from mice infected with *Trypanosoma cruzi* to stimulation with T (concanavalin A and phytohemagglutinin) or B (lipopolysaccharide) cell-specific mitogens were monitored during the acute, transition, and chronic stages of the disease. A marked reduction in the responses of infected mouse cells with respect to those of uninfected animals was observed during the acute stage, regardless of whether or not the infective dose was lethal. Reduced or absent responses were recorded with suboptimal, optimal, and supraoptimal concentrations of the mitogens. Normal levels of responsiveness to concanavalin A, phytohemagglutinin, and lipopolysaccharide were observed during the chronic stage of the disease. The trend of return to normal responses was initiated around day 40 after infection with 25 parasites. At this time, a marked decline in parasitemia levels, cessation of mortality, and disappearance of visible signs of disease began to be observed, defining the transition stage that precedes establishment of chronicity. T cell levels of the spleen were markedly reduced during the acute period and returned to normality during the chronic phase. Instead, absolute levels of B cells were significantly increased during the acute period but also normalized in the chronic phase. Immunosuppression of chronically infected mice with cyclophosphamide led to a temporary return to acute infection-type conditions, even in animals with undetectable levels of parasitemia before treatment. These results suggest that reduced T cell responses during acute experimental Chagas' disease might in part be due to depletion of the T cell compartment. Decreased B cell responses in the presence of significant numbers of B lymphocytes implies a suppressive phenomenon, B cell alteration, or a combination of both possibilities. Recrudescence of the disease after immunosuppression with cyclophosphamide suggests that immunological mechanisms play an important role, not only in the gain of control over *T. cruzi* infection by the host but also in the maintenance of the chronic status.

A considerable body of evidence accumulated over the years supports the concept that the immunological status of patients with chronic Chagas' disease, caused by the unicellular flagellate *Trypanosoma cruzi*, is comparable in many ways to that of healthy individuals (3, 7–11, 18, 20). These patients can normally develop humoral (7, 9, 11, 18) as well as cell-mediated (10, 20) immune responses. For this reason, recent reports of suppressed immunological reactivities occurring in experimental animals infected with *T. cruzi* (2, 12–17) have been somewhat puzzling. Aside from genetic differences existing between host species, it is conceivable that discrepancies in the immunological status of the host might also reflect variations in the kinetics of progression of the disease. To date, no chronological studies of lymphocyte reactivity during the course of Chagas' disease have been performed in a single host to eliminate the genetic variability factor. With a dual purpose, we set out to examine first the capacity of lymphoid cells from inbred mice traversing the various stages of *T. cruzi* infection to engage in proliferative responses triggered by T or B cell-specific activators. Second, we attempted to explore the possible role of the immune system in regulation of transition from acute to chronic experimental Chagas' disease. In this paper we show that immunosuppression in mice is a characteristic of the acute, but not the chronic, phase of the disease and that chronicity is likely to be attained and maintained as a consequence of reestablishment of normal immune responsiveness.

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MATERIALS AND METHODS

Animals. Four- to six-week-old CBA/J mice used in this work were purchased from the Jackson Laboratory (Bar Harbor, Maine). General purpose, stock Swiss mice were bred at our own animal facilities.

Parasites. Tulahuén strain *T. cruzi* isolates were maintained by serial syringe passages in mice. Parasite concentrations, whether measured in blood or suspensions, were determined by a standardized microscopic procedure described elsewhere (5) and expressed as numbers of *T. cruzi* per milliliter. Doses of parasites used to infect mice were contained in 0.1 ml of sterile phosphate-buffered saline solution. The administered doses for each experiment are described below.

Mitogens. Phytohemagglutinin P (PHA; Difco Laboratories, Detroit, Mich.), concanavalin A (ConA, Sigma Chemical Co.; St. Louis, Mo.), and the lipopolysaccharide extracted from *Escherichia coli* O55:B5 by the phenol-water procedure (LPS; Difco Laboratories) were used in assays of lymphoid cell responsiveness to T or B cell-specific mitogens. Concentrations of these mitogens for the corresponding experiments are given below.

Mitogenic stimulation assays. Responses to stimulation with ConA, PHA, or LPS by spleen cells from mice infected with *T. cruzi* were measured at various times postinfection. Parallel experiments were performed with cells from uninfected animals. Mitogenic stimulation assays were performed in triplicate by a procedure described in detail elsewhere (1). Cultures were processed for measurement with a multiple automated cell culture harvester (Microbiological Associates, Walkersville, Md.). Deoxyribonucleic acid synthesis was expressed in terms of incorporation of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.; specific activity 2 Ci/mmol) during the last 24 h of a 3-day culture period. Relative stimulation index (RSI) values were calculated for each mitogen concentration by the equation: RSI = response of infected mouse spleen cells/response of normal mouse spleen cells. In this equation, response values represent the average of triplicate determinations, using pooled spleen cells from 3 to 4 mice, except when tests were performed with cells from randomly selected animals to monitor possible individual deviations. Results applied to this equation were from simultaneously performed experiments conducted under identical conditions. Differences between SI values and the reference value (SI = 1) were considered to be meaningful when the differences between mean values used in the calculation of SI were statistically significant ($P < 0.05$, Student's t test). Individual determinations of [^{3}H]-thymidine uptake usually differed from the mean by less than 8%.

Determination of T lymphocytes. Spleen cell suspensions in cold RPMI 1640 medium were prepared by using a Ten Broeck tissue grinder (two strokes only). After the cells were washed three times with the same medium, they were resuspended, counted, and adjusted to 1×10^7 viable (trypan blue-excluding) nucleated cells per ml. A 1/10-ml amount of these suspensions was mixed with 0.1 ml of a 1:40 dilution of heat-inactivated anti-Thy-1.2 ascites fluid (cytotoxic titer, 1:2,560) prepared in AKR/J mice hyperimmunized with CBA/J mouse thymocytes (1) and 0.1 ml of normal guinea pig serum diluted 1:25 in RPMI 1640 medium. The mixture was incubated at 37°C for 60 min, after which it was cooled in an ice bath. Concentrations of surviving, trypan blue-excluding nucleated cells were established microscopically with a Neubauer hemocytometer. Results were expressed as percentages calculated with reference to control assays in which both anti-Thy-1.2 ascites fluid and diluted guinea pig serum were absent. Results of routinely included control assays, using either anti-Thy-1.2 ascites fluid or diluted guinea pig serum were systematically comparable to those obtained with either heat-inactivated normal mouse ascites fluid or medium alone.

Determination of B lymphocytes. A rosetting technique with latex beads coated with anti-mouse immunoglobulin antibodies (Immunobead; Bio-Rad, Laboratories, Richmond, Calif.) was used to determine B lymphocytes in the spleen. Cell suspension (1/10 ml) at 1×10^7 cells per ml was mixed with 0.1 ml of the Immunobead suspension, incubated at 37°C for 1 h, and further incubated at 4°C for 24 h. A 2/10-ml amount of a 0.2% solution of trypan blue in phosphate-buffered saline was added, and wet mounts were prepared for microscopic examination. Cells with three or more latex beads attached to their surface were considered to be B cell rosettes. Results were expressed as percentages with respect to the total number of nucleated cells. A minimum of 100 cells was counted on each wet mount. Duplicate determinations were made.

CPA treatments. For cyclophosphamide (CPA) treatments, mice received either a single intraperitoneal dose of 3 mg of CPA (Sigma Chemical Co.) or two daily intraperitoneal doses of 3 mg of CPA each.

Presentation of results and statistical analysis. Results presented in Fig. 1 through 6 are representative of a minimum of three separate experiments of each design. Unless otherwise noted, differences between means were analyzed by Student's t test. Differences were considered to be significant if $P < 0.05$.

RESULTS

The course of *T. cruzi* infection in mice given different doses. Representative results of the courses of infection caused by 10^4 and 25 parasites are shown in Fig. 1 to serve as reference for the kinetic studies presented below. A relatively faster course resulted from infection with the higher dose of *T. cruzi* as denoted by the greater levels of parasitemia and mortality of the entire group by day 18 postinfection. Results obtained with animals infected with 100 *T. cruzi* (data not shown) indicated production of acute, lethal disease in most cases, with very low percentages of survival observed occasionally. All animals receiving 25 organisms developed a slow-progressing infection with transient parasitemia and a substantial proportion (40 to 60% in different experiments) survived. In these sur-

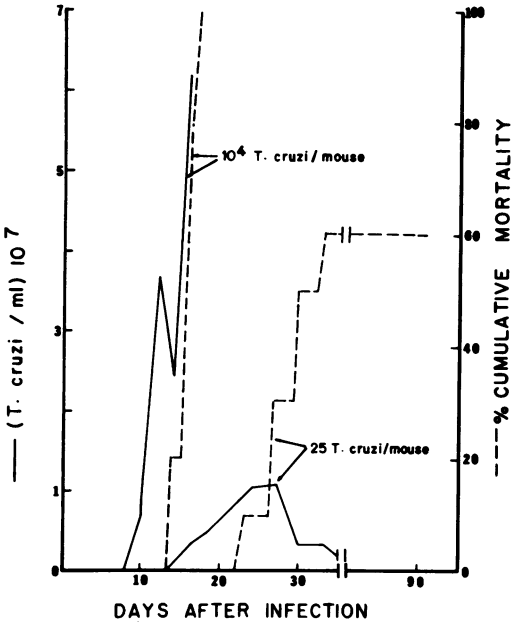

FIG. 1. *Course of infection in mice infected with high and low doses of T. cruzi. Note that intraperitoneal infection with 25 parasites was sublethal for a significant proportion of mice (40% in this experiment). Concentrations of parasite (number of T. cruzi cells per milliliter) represent levels of parasitemia.*

vivors, parasitemias were eventually reduced to minimal, often nondetectable levels, and the animals were considered to have attained the chronic stage of experimental Chagas' disease. The validity of this consideration is supported by the recent work of Laguens et al. (6) on the basis of histological, electrocardiographic, and serological observations.

Kinetics of lymphocyte responsiveness to mitogenic stimulation. Previous reports of immunosuppressed mitogenic responses in mice infected with *T. cruzi* presented results of proliferative responses induced by single doses of mitogens (13, 17). Before embarking on more complex studies, it was important to establish whether or not suppression was the apparent consequence of a shift of the dose-response curves of infected mouse lymphocytes relative to that of normal mouse cells. Significantly reduced ($P < 0.05$) T cell responses to ConA during the acute stage of the disease (days 1 through 20) were not limited to a single concentration of mitogen, and there was no significant shift of dose-response curves (Fig. 2, left panel). Similar observations were made when dose-response curves were obtained for either another T cell-specific mitogen (PHA) or a B cell-specific activator (LPS) (data not shown). It should be noted that background values (spontaneous responses

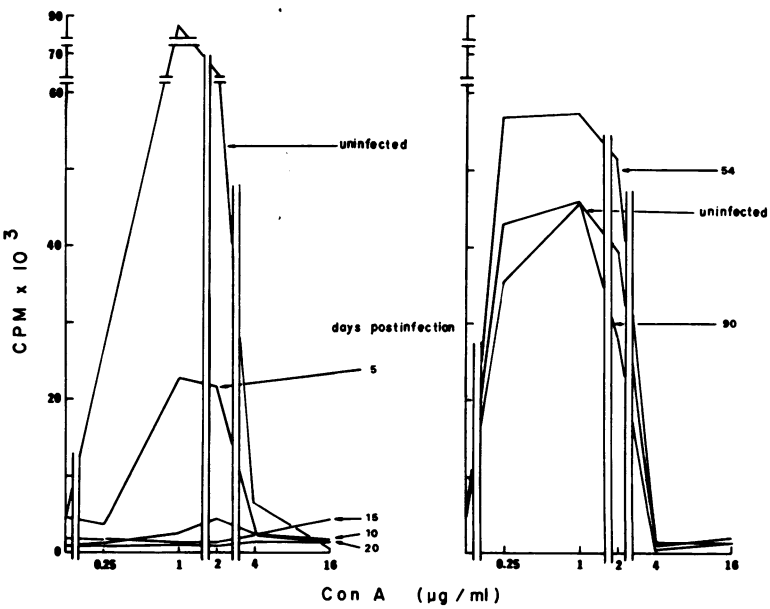

FIG. 2. *Responses to stimulation with ConA by infected and normal mouse spleen cells. Left panel, acute period. Right panel, chronic period. Mice were infected with 25 T. cruzi cells. In the left panel, for all concentrations of ConA, except 16 µg/ml, statistical significance of differences between experimental and control (uninfected) values was demonstrated ($P < 0.05$). In the right panel, none of the observed differences was statistically significant ($P > 0.05$).*

in the absence of mitogen) obtained with cells from normal and infected mouse spleen cells were comparable in most cases. Although infection was initiated in these experiments with 25 organisms, suppression was also evident when spleen cells from mice infected with 10^2 or 10^4 *T. cruzi* cells were used (Table 1). Experiments performed with spleen cells from randomly selected, infected animals systematically showed significantly reduced ($P < 0.05$) mitogenic responses to all three polyclonal activators during the initial 3 weeks of infection, regardless of both the dose of parasite and whether or not infection eventually turned out to be lethal for the entire group of animals. By contrast, results of dose-response types of experiments performed with splenocytes from animals that had attained chronicity, in terms of considerably reduced or negative parasitemia, disappearance of visible signs of disease, and cessation of occurrence of mortality, did not differ significantly from those obtained with cells from uninfected control mice of the same age and strain. This point is illustrated in the right panel of Fig. 2 for ConA stimulation, and similar observations were made when PHA and LPS were used (data not shown). Although in some instances, responses higher than normal were recorded with cells from chronically infected animals, this was not the case in the majority of the experiments with identical design, and the general trend was one of return to responses similar to those of uninfected mice.

Additional experiments were performed to more closely establish the correspondence between periods of the disease and variations in lymphocyte responsiveness and to define the time when transition from suppressed responses to normality occurred. Significantly reduced ($P < 0.05$) responses to all three mitogens were first detected 5 days postinfection, with as little as 25 parasites (Fig. 3 through 5), acquired greater proportions as the infection progressed, and reached their highest degree represented by minimal RSI values on days 15 to 20. The initiation of a trend of return to normality was usually noted on or around day 40, although some LPS-induced responses sometimes picked up on day 20. Fluctuations around normal values were observed on day 54 and later.

TABLE 1. *Suppressed responses to T and B cell mitogens by spleen cells from mice infected with T. cruzi.*

Day postinfection	Mitogen[a]	RSI values		
		25[b]	100[b]	10,000[b]
5	ConA	0.3[c]	0.08	0.2
	PHA	0.5	0.3	0.2
	LPS	0.6	ND[d]	0.2
10	ConA	0.03	0.1	0.3
	PHA	0.5	0.5	0.3
	LPS	0.5	ND	0.3
15	ConA	0.02	0.2	0.2
	PHA	0.2	0.5	0.3
	LPS	0.4	ND	0.2

[a] Mitogen concentrations: ConA and LPS, 1 μg/ml; PHA, 1 μl/ml.

[b] Infective dose of parasites per mouse administered intraperitoneally.

[c] Differences between mean values used to calculate all of these RSI values were statistically significant ($P < 0.05$).

[d] ND, Not done.

Variations in T and B cell contents of the spleen during *T. cruzi* infection. A typical set of results is shown in Fig. 6, illustrating the marked drop in both the absolute and relative numbers of spleen T lymphocytes that were observed in the spleens of infected mice. Minimal T cell numbers occurred on day 20, i.e., coinciding with the time when ConA and PHA elicited minimal proliferative responses. B cell contents of infected spleens increased and reached maximal levels on day 20 postinfection. However, variations in the proportion of B cells relative to the total number of nucleated spleen

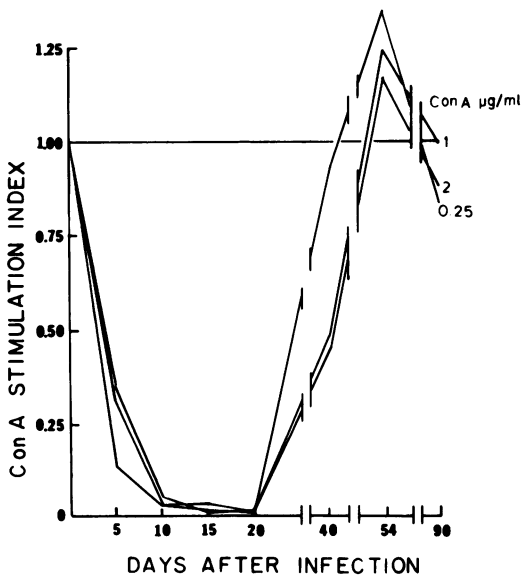

FIG. 3. *Kinetics of lymphocyte responsiveness to ConA during experimental Chagas' disease. The horizontal line (RSI = 1) represents the normal level of responsiveness. Mice were infected with 25 T. cruzi cells. Differences between experimental and control (uninfected) values used to calculate the indices for day 5-40 were statistically significant ($P < 0.05$); other differences were not significant.*

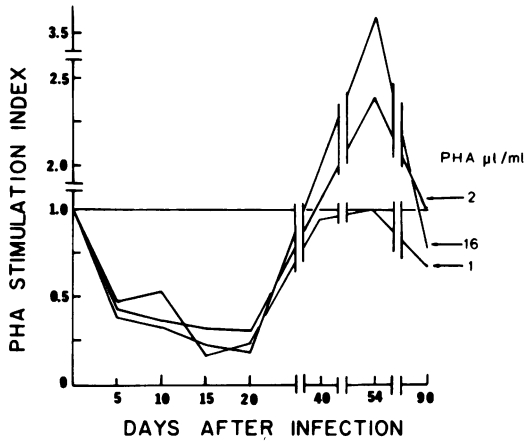

FIG. 4. *Kinetics of lymphocyte responsiveness to PHA during experimental Chagas' disease. RSI = 1, normal level of responsiveness indicated by the horizontal line. Mice were infected with 25 T. cruzi cells. Differences between experimental and control (uninfected) values used to calculate the indices for days 5 through 20 and day 54 (16 µl/ml only) were statistically significant (P < 0.05).*

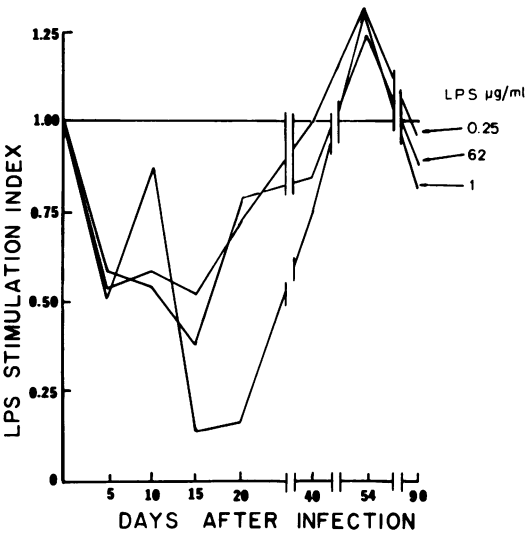

FIG. 5. *Kinetics of lymphocyte responsiveness to LPS during experimental Chagas' disease. RSI = 1, normal level of responsiveness indicated by the horizontal line. Mice were infected with 25 T. cruzi cells. Differences between experimental and control (uninfected) values used to calculate the indices for days 5 through 20 and day 40 (1 µg/ml only) were statistically significant (P < 0.05).*

cells were not as marked, denoting a certain degree of parallelism between increase of B cell levels and the splenomegaly that is typical in acute Chagas' disease. Both T and B cell levels were found to be within normal ranges during the chronic stage of the disease.

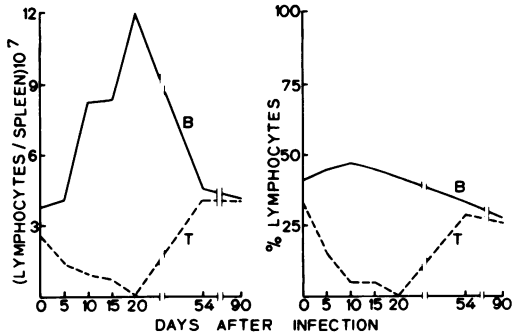

FIG. 6. *Variations in the levels of spleen T and B lymphocytes during experimental Chagas' disease. Left panel, absolute levels of T and B cells per spleen. Right panel, relative levels of T and B cells in the spleen. A horizontal line in the right panel would indicate an increase in cell number at the same rate as that of splenomegaly development. Mice were infected with 25 T. cruzi cells. Determinations were made with pooled spleen cells from 2 to 3 mice.*

Effects of CPA-induced immunosuppression during chronic *T. cruzi* infection in mice. The results described in preceding paragraphs do not disclose whether establishment of the chronic phase of experimental Chagas' disease follows normalization of immunological function or vice versa. To clarify this question, experiments were designed in which chronically infected mice were immunosuppressed with CPA. A considerable proportion (6:9) of mice, survivors of a 25-*T cruzi* dose challenge, whose parasitemia had become undetectable showed measurable numbers of circulating trypanosomes after treatment with CPA. (Table 2) By contrast, only one of the animals in the control group showed a low-grade, transient parasitemia during the observation period. A similar change was recorded in a second type of experiment in which all of the animals had low-level parasitemias before receiving CPA (Table 3). These mice were considered to have entered the chronic stage on the basis of normal T and B spleen cell responses to ConA and LPS, respectively, lack of occurrence of mortality during the week before, and markedly declining levels of circulating parasites. In these animals, CPA-induced immunosuppression resulted in a marked recrudescence of the disease; parasitemias increased to levels far higher than those of untreated, infected mice, and signs of the disease became evident. One of these mice died. No major variations in parasitemia were observed in the group of mice that did not receive CPA, and there was neither mortality nor evidence of exacerbation of the disease.

TABLE 2. *Effect of CPA-induced immunosuppression of chronically infected mice with undetectable parasitemia on the ulterior course of T. cruzi infection*

Treatment of infected mice	Day of treatment[a]	Mean *T. cruzi* × 10^5 per ml on the following day postinfection:[b]				
		78	80	82	84	87
PBS (0.1 ml intraperitoneally)	79	0 (0:9)	0 (0:9)	0.10 (1:9)	0.03 (1:9)	0 (0:9)
CPA (3 mg intraperitoneally)	79	0 (0:9)	0.10 (3:9)	0.85 (4:9)	0.71 (6:9)	1.05 (4:9)

[a] Day postinfection.

[b] Mean of all individual values of the group whether or not parasitemia was detected. Ratios in parentheses represent the number of mice with detectable parasitemia per the total number of mice.

TABLE 3. *Effects of CPA-induced immunosuppression of chronically infected mice with low-level parasitemia on the ulterior course of T. cruzi infection*

Treatment of infected mice	Days of treatment[a]	Mean *T. cruzi* × 10^5 per ml ± SEM on the following day postinfection[a]:				No. of dead mice per total
		42	52	58	61	
PBS (0.1 ml intraperitoneally)	43 and 44	0.4 ± 0.6	0.2 ± 0.4	0.2 ± 0.1	0.2 ± 0.1	0:10
CPA (3 mg intraperitoneally)	43 and 44	0.5 ± 0.3	2.5 ± 0.9	9.2 ± 3.4	12.0 ± 4.1	1:10
Statistical significance[b]		NS	S	S	S	

[a] SEM, Standard error of the mean.

[b] NS, Not significant; S, $P < 0.05$.

DISCUSSION

These results highlight the contrast between markedly suppressed mouse T and B lymphocyte responses occurring during acute *T. cruzi* infection and normal responsive ability of these cells when the infection becomes chronic. These observations, together with the finding that immunosuppression in chronically infected mice reverts the infection back to its acute form, support the hypothesis that transition from the acute to the chronic phase of Chagas' disease and maintenance of the chronic status are under immunological control.

Suppressed T and B cell responses to mitogenic stimulation were readily demonstrable over a full-titration range of mitogen concentrations. Therefore, it was clear that suppression was not the apparent consequence of a displacement of the titration curves of infected mouse spleen cells with respect to those of cells from uninfected animals. These results confirm and extend those of Rowland and Kuhn (17) and Ramos et al. (13), who used single concentrations of mitogens in their work. Suppressed responses to ConA, PHA, or LPS were detectable as early as 5 days postinfection, even when only 25 parasites were injected. Since this relatively low dose was insufficient to cause a lethal infection in all of the animals and suppression was not observed among the chronically infected, surviving mice, it follows that suppression is a characteristic of the acute stage of the infection, independent of both the rate of progression of the disease and its final outcome. This notion is supported by several observations. First, suppression was demonstrable with cells from mice given doses of *T. cruzi* varying by as much as 400-fold and ranging from the lethal (10^4 organisms) to the partially lethal (25 organisms) ones. Second, randomly selected mice infected with 25 parasites systematically showed suppressed T and B cell responses, even when this dose was lethal to 40 to 60% of the animals. We assume that the probability of survival of these randomly chosen mice, had they not been sacrificed, would have been the same as that of the rest of the animals that received the same dose of *T. cruzi* and were spared to determine the rate of survival. Relatively large numbers of mice, often between 100 to 150, were infected for both experimental purposes and determination of the proportion of survivors, and these proportions were readily reproducible within the 40 to 60% range.

A comparison of the course of infection produced with 25 *T. cruzi* (Fig. 1) with the results of lymphocyte stimulation kinetics (Fig. 3 to 5) revealed a coincidence between the times of initiation of the transition period of the disease and the trend of return of B and T cells to a normal responsive status. The term transition period as used in this paper refers to the time postinfection, when parasitemia levels start to decline to eventually become either minimal or undetectable and occurrence of mortality ceases. Normality of lymphocyte responses was clearly attained during the chronic period with fluctu-

ations around control levels being observed occasionally. Normal responses to ConA, PHA, or LPS persisted until day 90 postinfection, when the experiments were routinely terminated. These results, derived from the use of a single type of host, rule out genetic considerations as a necessary basis for the apparent discrepancies in lymphocyte and immunological status reported by other investigators (13, 17). Instead, suppressed and normal lymphocyte responsiveness represent, at least in the mouse, two distinct periods of Chagas' disease. The vast majority of human cases with this disease reach the chronic phase, and these have been the individuals commonly used in studies of evaluation of immune status. It is tempting to speculate, in the light of these findings, that similar studies conducted with patients with acute symptoms could expose a temporary immunodeficient status. Of interest in this context is the recent report by Teixeira et al. (19) in which it is shown that acute chagasic patients may exhibit reduced cutaneous reactivity to *T. cruzi* antigens.

Suppressor T cells have been implicated in the impaired responsiveness of lymphocytes from *T. cruzi*-infected mice to mitogenic stimulations (14). Although these results do not contradict this notion, they also raise the possibility that the markedly depleted T cell compartment of the spleens of infected animals may be responsible, in part, for the observed deficient responses. Reduced levels of B cell responsiveness in the presence of significant numbers of B cells would point to a suppressive mechanism, an intrinsic B cell defect, as postulated by Ramos et al. (13), or to a combination of both possibilities.

The exacerbation of the infection produced in chronically infected, recovered mice by immunosuppression with CPA strongly suggests that immunological mechanisms are involved, not only in leading to the recovery from acute infection but also in maintaining the chronic status when it is attained. The reversal effect (from chronic to acute conditions) caused by CPA was, however, temporary since most of the treated mice eventually recovered again and became chronic again. The capacity of most of these immunosuppressed animals to survive the exacerbated infection and to prevent the increase of parasitemia to levels comparable to those of mice initially traversing the acute period of the infection is probably attributable to the specific humoral immune response that is known to develop in mice that spontaneously recover from *T. cruzi* infection and will not be immediately removed by treatment with cyclophosphamide. In support of this notion is the well-recognized protective action of passively transferred serum from chronically infected mice (reviewed in reference 4), even when the recipient animals are hypersusceptible to *T. cruzi* infection (3a, 4). Of interest in this context, titration of pools of sera from mice surviving low-dose infection with *T. cruzi* yielded titers of around 40,000, when measured by direct agglutination.

Recent experiments in which infected mouse lymphocyte responses to *T. cruzi* antigens were monitored have revealed the occurrence of a state of unresponsiveness kinetically similar to those described in this paper for polyclonal activators (Kierszenbaum, unpublished data).

In closing, we stress that these results emphasize that immunological events are important contributors to the defense mechanisms of the host against *T. cruzi* infection. Efforts to develop an effective vaccine against this parasite appear to be justified and deserve encouragement.

ACKNOWLEDGMENT

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