ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2002, p. 3790–3796 Vol. 46, No. 12
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.12.3790–3796.2002

Benznidazole Treatment following Acute *Trypanosoma cruzi* Infection Triggers CD8$^+$ T-Cell Expansion and Promotes Resistance to Reinfection

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Received 25 March 2002/Returned for modification 2 July 2002/Accepted 27 August 2002

Many studies have shed light on the mechanisms underlying both immunoprotection and immune dysregulation arising after *Trypanosoma cruzi* infection. However, little is known about the impact of benznidazole (*N*-benzyl-2-nitroimidazole acetamide), the drug available for clinical treatment of the infection, on the immune system in the infected host. In the present study we investigated the effect of benznidazole therapy on the lymphoid compartment during the course of experimental *T. cruzi* infection. Although amelioration of a variety of clinical and parasitological signs was observed in treated mice, amelioration of splenocyte expansion was not detected. Interestingly, this sustained splenomegaly observed in benznidazole-treated mice showed a preferential expansion of CD8$^+$ T lymphocytes. Moreover, although benznidazole treatment blocked the expansion of recently activated CD4$^+$ and CD8$^+$ T cells seen in infected hosts, benznidazole treatment led to a selective expansion of effector and memory CD8$^+$ T lymphocytes in association with a lower rate of apoptosis. In addition, the surviving treated animals were protected from reinfection. Together, these data suggest that, in addition to its well-known direct role in blocking parasite replication in vivo, benznidazole appears to directly affect immune regulation in *T. cruzi*-infected hosts.

Chagas' disease, caused by the intracellular protozoan parasite *Trypanosoma cruzi*, is a human parasitosis affecting 16 million to 18 million people in Latin America (42). This disease is characterized by an acute phase with detectable parasitemia and acute myocarditis in 8% of cases (20) and a long-lasting chronic phase in which most infected people remain asymptomatic. However, approximately 36% of chronically infected individuals develop digestive problems and/or cardiomyopathy (42) in association with parasitemia and some parasitism in tissues.

Although the mechanisms involved in the pathogenesis of the target organs have not been completely elucidated, both parasite persistence and autoimmune events have been regarded as important contributors to the tissue lesions (for a review, see reference 43). In this regard, the lymphoid compartment has been seen concomitantly as a target and as an active component of the resistance to *T. cruzi* infection.

Splenomegaly and lymphadenopathy related to polyclonal B- and T-cell activation are hallmarks of the acute phase of infection (24, 25). In parallel, immunosuppression follows as result of the polyclonal nature of lymphocyte activation (26) and of the substantial T-cell death that occurs in the secondary lymphoid organs of the infected mice (22). A huge thymic atrophy due to the depletion of immature T cells is also observed (32).

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Despite these abnormalities, specific immune responses that control parasitemia in the course of infection can proceed (1, 29, 30, 39). The induction of protective immunity seems to be related to CD4$^+$ T cells, as depletion of this subset leads to decreased inflammation and concomitant high levels of parasitemia and tissue parasitism (17, 38). In humans and mice, CD4$^+$ lymphocytes produce substantial quantities of gamma interferon (IFN-γ) (7, 35), which is important to both the humoral and cellular immune responses (1, 29, 30). Recent studies pointed to the prevalence of the Th1 response at the beginning of infection, followed by concurrent Th1 and Th2 responses in later phases of the disease (for a review, see reference 43).

In vivo parasite replication can also be controlled by CD8$^+$ T lymphocytes, another important producer of IFN-γ. In fact, mice lacking CD8$^+$ lymphocytes are more susceptible to infection, showing higher levels of parasitemia and tissue parasitism yet displaying less inflammation in cardiac tissue (39).

Despite the existence of potent controllers of parasite replication, the complete elimination of the parasite and spontaneous cure of *T. cruzi* infection have never been observed in a consistent way (for a review, see reference 11). Additionally, despite the high rates of morbidity and mortality from Chagas' disease, no immunotherapy or chemotherapy is provided as prophylaxis. Moreover, only one trypanocidal drug is available for clinical use: *N*-benzyl-2-nitroimidazole acetamide, named benznidazole. This nitro derivative is known to reduce the level of parasitism and eliminate the acute-phase symptoms, thus abbreviating the course of infection (for a review, see reference 10). However, the efficiency of anti-*T. cruzi* therapy in the chronic phase is still controversial (4, 10, 16, 34).

Despite the large number of studies focusing on the immune response following *T. cruzi* infection, little is known about the

impact of benznidazole treatment on this response. This fact prompted us to investigate the effect of benznidazole therapy on some alterations of the lymphoid compartment of the immune system seen in the course of experimental *T. cruzi* infection.

MATERIALS AND METHODS

Animals. Male albino Swiss mice (age, 6 to 8 weeks; weight, 16 to 20 g) were maintained under controlled conditions in our animal facilities. All procedures carried out in this work were done according to the rules of the Animal Ethics Committee of the Oswaldo Cruz Foundation.

Reagents and antibodies. *N*-Benzyl-2-nitroimidazole acetamide (benznidazole; Rochagan, Roche, Rio de Janeiro, Brazil) was used as a trypanocidal drug in the experimental therapy schedules. The following rat anti-mouse monoclonal antibodies were used for lymphocyte cytofluorometric analysis: anti-CD3–fluorescein isothiocyanate (FITC), anti-CD3–phycoerythrin (PE), anti-CD4–FITC, anti-CD4–PE, and anti-CD8α–PE (all purchased from Sigma Chemical Company, St. Louis, Mo.); anti-CD4–peridin chlorophyll *a* protein (PerCP), anti-CD8α–allophycocyanin (APC), anti-CD62L–FITC, and anti-CD69–FITC (all from BD PharMingen, San Diego, Calif.); and B220 (Pan B cell)–Tri-Color (Caltag Laboratories, San Francisco, Calif.). Blockage of immunoglobulin Fc-portion receptors was done with the monoclonal antibody 2.4G2 (anti-FcγRII/III), kindly provided by Marc Daëron (Institut National de la Santé et de Recherche Médicale, Unité 255, Paris, France). The in vivo cell death detection and quantitation studies were carried out with 7-aminoactinomycin D, purchased from Sigma Chemical Company.

Experimental groups. Mice were separated into the following experimental groups: noninfected, nontreated mice; noninfected, benznidazole-treated mice; *T. cruzi*-infected, nontreated mice; and *T. cruzi*-infected, benznidazole-treated mice.

Experimental infection. Animals from the *T. cruzi*-infected groups were inoculated by the intraperitoneal route with 10^4 trypomastigotes of *T. cruzi* strain Y (36) in 200 μl of saline solution. The parasites were obtained from the blood of infected albino Swiss mice at the time of the peak level of parasitemia by differential centrifugation, as described previously (5).

Reinfection procedures. At day 50 postinfection, the *T. cruzi*-infected, benznidazole-treated mice were reinfected with trypomastigotes of *T. cruzi* strain Y, and the remaining noninfected, nontreated mice were infected as age-matched controls for infection. This reinfection was carried out as described above for the primary infection. In the present approach, no treatment was performed. The survival rates for both groups were monitored for an additional 40 days.

Benznidazole treatment. Noninfected, benznidazole-treated mice and *T. cruzi*-infected, benznidazole-treated mice were submitted to treatment ad libitum by the addition of 0.25 mg of benznidazole per ml to the drinking water from 7 to 21 days postinfection, for a calculated daily dosage of 100 mg/kg of body weight (12). The drug solution was sonicated for 15 min and stored at 4°C in the dark. The daily volume consumed by the animals was monitored for calculation of the amount of drug ingested, which corresponded to 62.5 mg of benznidazole/kg/day, as described previously (37).

Parasitological parameters. At several times after infection, the levels of parasitemia in mice from the *T. cruzi*-infected, treated or *T. cruzi*-infected, nontreated groups were monitored as described previously (8). Briefly, 5 μl of fresh blood taken from the mouse tail was compressed between a glass slide and a coverslip (18 by 18 mm). The number of parasites per milliliter was determined by scoring 50 fields, and then that number was multiplied by a conversion factor which takes into account the number of microscopic fields in the area under specific magnification. The survival time of each animal and the overall survival rate were daily monitored until day 40 of infection. The weight evolution was monitored weekly.

Collection of peripheral lymphoid organs. The spleens and subcutaneous lymph nodes (axillary, brachial, and inguinal) of three to four animals in each experimental group were collected at days 9 and 14, and their cell numbers and absolute mass were determined. Cell suspensions were gently obtained in a tissue grinder in RPMI medium supplemented with 5% fetal calf serum. The red blood cells in the spleen were lysed through hypotonic shock. Cell numbers and viability were determined by trypan blue exclusion, and the cells were counted in a hemocytometer. Lymphoid organ analysis included the determination of the absolute mass of the spleen, together with the number of viable cells in the spleen and subcutaneous lymph nodes.

Lymphocyte phenotyping analysis. For cell surface phenotyping of the lymphocyte subsets and activation status, 10^6 cells from the suspension of each organ from each experimental group were placed in 96-well round-bottom plates for cytofluorometric analysis. The first step in the blocking of the immunoglobulin Fc receptors was a 20-min incubation, followed by centrifugation and resuspension of the cells with appropriate dilutions of the monoclonal antibodies. After incubation for 20 min at 4°C, the cells were washed and fixed with 1% paraformaldehyde in phosphate-buffered saline and immediately analyzed by flow cytometry. Data acquisition and three- or four-color analysis were performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.) equipped with Cell Quest software (Becton Dickinson). A total of 10,000 to 25,000 events were collected for a viable lymphocyte-enriched region, defined according to the forward- and side-scatter parameters, and further confirmed in some experiments by using propidium iodide staining. Further data analysis was carried out with Cell Quest (Becton Dickinson) or WinMDI2.8 (by Joseph Trotter, Scripps Research Institute, San Diego, Calif.) flow cytometry application software.

Lymphocyte death analysis. For in vivo detection of apoptotic cell death, 10^6 cells were incubated with monoclonal antibodies against CD4 and CD8 molecules as described above. After these cells were washed, they were incubated with 20 μg of 7-aminoactinomycin D per ml for 20 min at 4°C and immediately acquired on the flow cytometer. Gates defining viability and distinguishing early apoptotic cells versus late apoptotic and necrotic cells were prepared as described previously (33).

Statistical analysis. Statistical analyses were performed by the Mann-Whitney test, with values being considered significant when P was <0.05.

RESULTS

A schedule of parasitic load reduction with benznidazole increases rates of survival after acute *T. cruzi* infection in mice. In the present work, Swiss mice infected with the Y strain of *T. cruzi* exhibited the classical pattern of parasitemia and the survival rates described by Silva and Nussenszweig (36). *T. cruzi* infection in nontreated mice led to an evident parasitemia, with no survivors after 25 days postinfection (Fig. 1a and b). We also confirmed previous reports (2, 31) showing that benznidazole treatment is effective in reducing the level of parasitemia (Fig. 1a). With this treatment we also detected a higher percentage of survivors, with the survival rate reaching 78.5% (Fig. 1b). Comparing the parasitemia curves for treated mice and the nontreated groups, we observed similar kinetics of parasitemia. However, maximal parasitemia levels were lower in treated mice, which presented an early decline in the numbers of circulating parasites as well (Fig. 1a).

In addition, we observed marked differences in weight loss in treated versus nontreated mice. Loss of body weight was noticeable in infected, nontreated mice from day 9 postinfection, increasing further with the progression of infection. Conversely, the therapeutic schedule applied was able to partially block the weight loss, as seen by the comparison with infected, nontreated and noninfected, nontreated mice (Fig. 1c).

Benznidazole therapy leads to increased levels of lymphocyte expansion in peripheral lymphoid organs of *T. cruzi*-infected mice. The previously described alterations in the homeostasis of the immune system during *T. cruzi* infection (for a review, see reference 13) prompted us to study the main lymphocyte subsets found in the peripheral lymphoid organs of infected mice under therapy with benznidazole. Analyses were performed immediately after the peak level of parasitemia had been reached, which was at day 9 postinfection, and at day 14 postinfection, when the number of circulating parasites had decreased.

At day 9 after infection, we observed a clear enlargement of the spleen that was accompanied by a parallel increase in the numbers of cells in both the spleens and lymph nodes from infected, nontreated mice (Fig. 2). However, on day 14 postin-

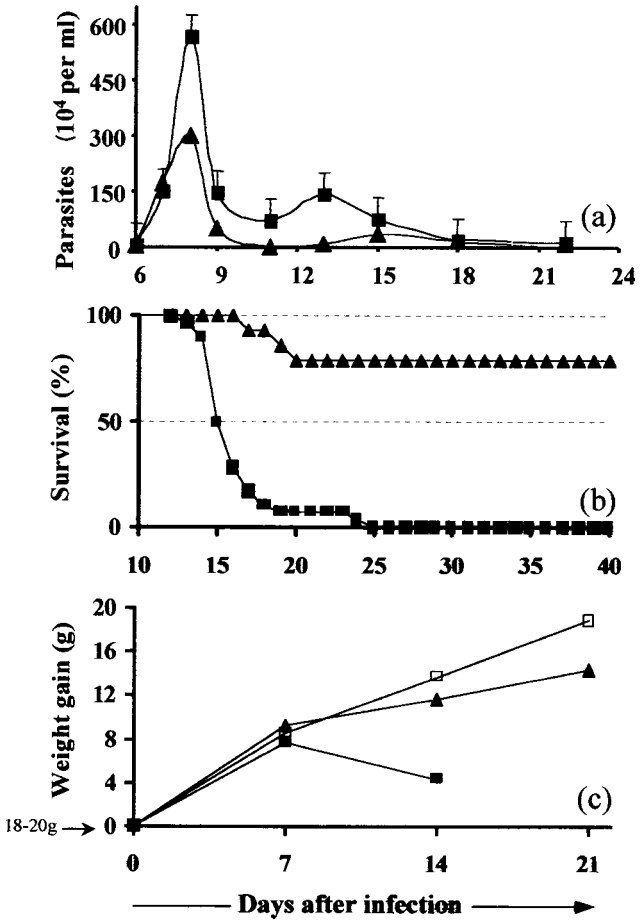

FIG. 1. Benznidazole improves the resistance of mice to *T. cruzi* infection. Each point consists of data for 16 to 22 mice in the noninfected (open squares), infected, nontreated (filled squares), and infected, benznidazole-treated (filled triangles) groups from three independent experiments. (a) Quantitation of parasitemia, with vertical bars representing the means + standard errors for each time point; (b) survival rates; (c) body weight evolution of the experimental groups.

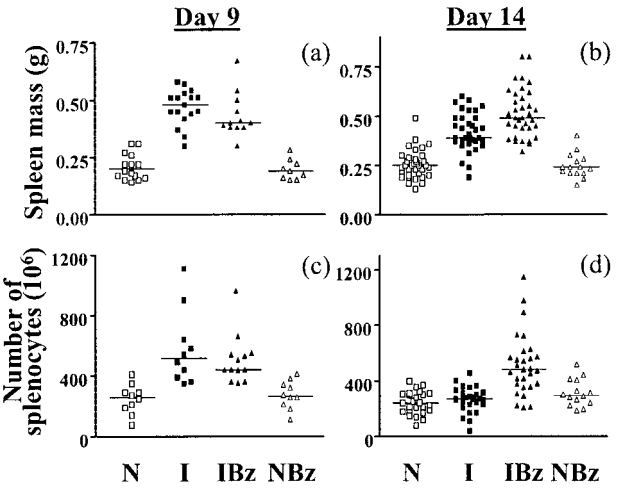

FIG. 2. Sustained absolute spleen mass and cellularity in benznidazole-treated mice following *T. cruzi* infection. Data are for noninfected (open squares), infected, nontreated (filled squares), infected, benznidazole-treated (filled triangles), and noninfected, benznidazole-treated (open triangles) mice. Absolute spleen mass values (a and b) and spleen cell numbers (c and d) on days 9 (a and c) and 14 (b and d) after infection. Horizontal bars represent the median values of three independent experiments for the analysis at day 9 and nine experiments for the analysis at day 14.

fection, these infected mice exhibited splenocyte numbers similar to those in the noninfected, nontreated controls (P = 0.3501), despite the maintenance of an increased spleen mass (Fig. 2b and d).

Similar spleen enlargement and enhanced cellularity were observed in the benznidazole-treated infected group on day 9 postinfection (Fig. 2a and c). Surprisingly, at day 14 after infection, the treatment (benznidazole in infected mice) was unable to reverse significantly the increase in cell numbers, as observed in infected, nontreated mice. Instead, treated mice maintained not only the splenomegaly but also the elevated numbers of cells detected at day 9 of infection (Fig. 2b and d).

Noninfected, treated animals exhibited values statistically similar to those of noninfected, nontreated mice for all parameters analyzed, as described above (Fig. 2). Analysis of the cellularity in lymph nodes showed similar findings (data not shown).

The $CD8^+$ T-cell subset is highly expanded in *T. cruzi*-infected mice receiving benznidazole therapy. Aiming to evaluate if the modifications observed in the lymphoid organs were due to the expansion of a specific cell population, we studied their lymphocyte compositions by determination of the relative frequency of CD4-, CD8-, and B-cell subsets at day 14 postinfection.

Analysis of the lymphocyte subsets in the spleen showed that mice from both the infected and the infected, benznidazole-treated groups had increased relative numbers of T cells (Fig. 3a, b). Interestingly, benznidazole treatment of infected mice promoted a preferential expansion of the $CD8^+$ splenocyte subset compared to the numbers of $CD8^+$ splenocytes in infected, nontreated mice (P = 0.002) (Fig. 3b). This difference was even greater if we take into account the absolute numbers of these T-cell subsets (data not shown), since benznidazole-treated mice had significantly higher numbers of splenocytes compared to the numbers in the nontreated group at day 14 after infection (Fig. 2d).

Analysis of the absolute numbers of cells showed a significant expansion of all subsets studied in the lymph nodes of both the treated and the nontreated groups, with a slight increase in the frequency of B cells in the infected, nontreated group (data not shown). It must be pointed out that the effects described above cannot be ascribed to the drug alone, since similar lymphocyte subset numbers were seen in the spleens (Fig. 3) and lymph nodes (data not shown) of treated and untreated, noninfected mice.

Benznidazole treatment reduces T-cell activation but sustains expansion of effector and memory $CD8^+$ T cells. In order to understand the maintenance of the lymphocyte expansion in the lymphoid organs of mice receiving trypanocidal therapy, we performed phenotypic analysis for the cell surface molecules CD44, CD62L, and CD69 to determine the activation

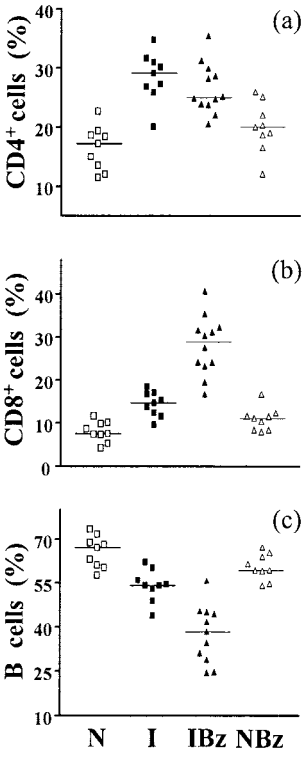

FIG. 3. Prominent CD8$^+$ expansion in the spleens of *T. cruzi*-infected, benznidazole-treated mice. Phenotypically defined CD4$^+$, CD8$^+$, and B lymphocytes in noninfected (N; open squares), infected, nontreated (I; filled squares), infected, benznidazole-treated (IBz; filled triangles), and noninfected, benznidazole-treated (NBz; open triangles) mice were analyzed after 14 days of infection in three to four different experiments. The numbers of CD4$^+$, CD8$^+$, and B lymphocytes in the spleens of individual mice are depicted (a, b, and c, respectively), with the horizontal bars indicating the median value for each experimental group.

status of both the CD4$^+$ and the CD8$^+$ subpopulations. These analyses were carried out with splenocytes at day 14 postinfection.

Cytofluorometric analysis showed that *T. cruzi*-infected, nontreated mice had both CD4$^+$CD69$^+$ and CD8$^+$CD69$^+$ T lymphocytes at increased frequencies (Fig. 4a), indicating the presence of recent cell activation. This effect was partially inhibited by drug administration, as T cells from infected, benznidazole-treated mice showed lower levels of expression of the CD69 molecule, although the levels of expression were clearly higher than those for noninfected mice (Fig. 4a).

Concomitantly, we also observed an increased frequency of CD62L$^{-/low}$ cells among CD4 and CD8 T-cell subsets in animals in the infected, nontreated group (Fig. 4b). As seen by the frequency of CD69 expression, benznidazole administration in infected mice led to the partial inhibition of this effect in the CD4$^+$ subset (Fig. 4b). However, this group showed no reversion of the CD62L loss in the CD8$^+$ subset (Fig. 4b). In fact, we could observe their expansion in the spleens of the *T. cruzi*-infected and benznidazole-treated animals if we took into account the absolute cell numbers (Fig. 4c). Parallel upregulation of CD44 expression in the T-cell subsets of *T. cruzi*-infected and benznidazole-treated mice was also detected (data not shown). Together, these data indicate the development of effector and memory CD8$^+$ T cells after benznidazole therapy in *T. cruzi*-infected mice.

Diminished frequency of apoptotic cells was detected after benznidazole treatment of *T. cruzi*-infected mice. As apoptosis has been demonstrated to be an active regulatory process involved in the huge expansion of lymphocytes during *T. cruzi* infection, we analyzed apoptotic cell death by cytofluorometric studies with 7-aminoactinomycin D.

Increased numbers of cells in the initial stage of apoptosis were observed in the lymphoid organs of infected, nontreated mice. The proportions of apoptotic cells were increased in the spleens of infected mice, with the proportions of apoptotic cells being 7.6% ± 0.9% and 14.2% ± 4.7% in the spleens of noninfected and infected mice, respectively. Again, this phenomenon was reversed with benznidazole therapy, with the proportions of apoptotic cells decreasing from 14.2% ± 4.7% before treatment to 9.6% ± 0.4% after treatment. Results for the spleens of noninfected mice treated with benznidazole were similar to those for their nontreated controls: 6.4% ± 1.5% and 8.5% ± 2.3%, respectively. Parallel analysis of the lymph nodes revealed a comparable trend in the results, but the difference was not statistically significant (data not shown). In addition, analysis of 7-aminoactinomycin D incorporation by specific T-cell subpopulations revealed similar effects for both CD4$^+$ and CD8$^+$ T cells (data not shown).

Benznidazole therapy promotes resistance to secondary infection with *T. cruzi*. The generation of an important pool of expanded effector T cells following benznidazole treatment of infected mice prompted us to investigate the resistance of these animals to a second challenge. Interestingly, a second infection of surviving benznidazole-treated mice was associated with a rate of survival of almost 100% (Fig. 5). Although the age-matched (previously noninfected) control mice had lower survival rates following this infection procedure (Fig. 5), they showed higher rates of survival compared to those for the animals used in the primary infection experiments (Fig. 1b), which did not survive. This fact is likely related to the older age of the animals in the former group.

DISCUSSION

In Chagas' disease, analysis of immunity pre- and posttreatment is essential for both an understanding of the mechanisms of benznidazole action and the rational development of new trypanocidal agents (10, 11, 40). For example, it has been demonstrated that interleukin-12 is able to increase the trypanocidal activity of benznidazole against a drug-resistant *T. cruzi* strain (23). However, even though this nitro derivative is essentially the only drug used for clinical treatment of Chagas' disease in Brazil, few other studies have analyzed the impact of this therapy on the host immune system (28). In the present study we addressed this issue by analyzing the effects of benznidazole treatment on the mouse peripheral lymphoid compartment following acute *T. cruzi* infection.

As a first step, we searched for an in vivo standardization of the therapeutic schedules used for experimental *T. cruzi* infection in mice. The majority of attempts at experimental chemotherapy with new trypanocidal drugs have been done with Swiss mice infected with the Y strain of *T. cruzi* (2, 3, 4, 15, 19).

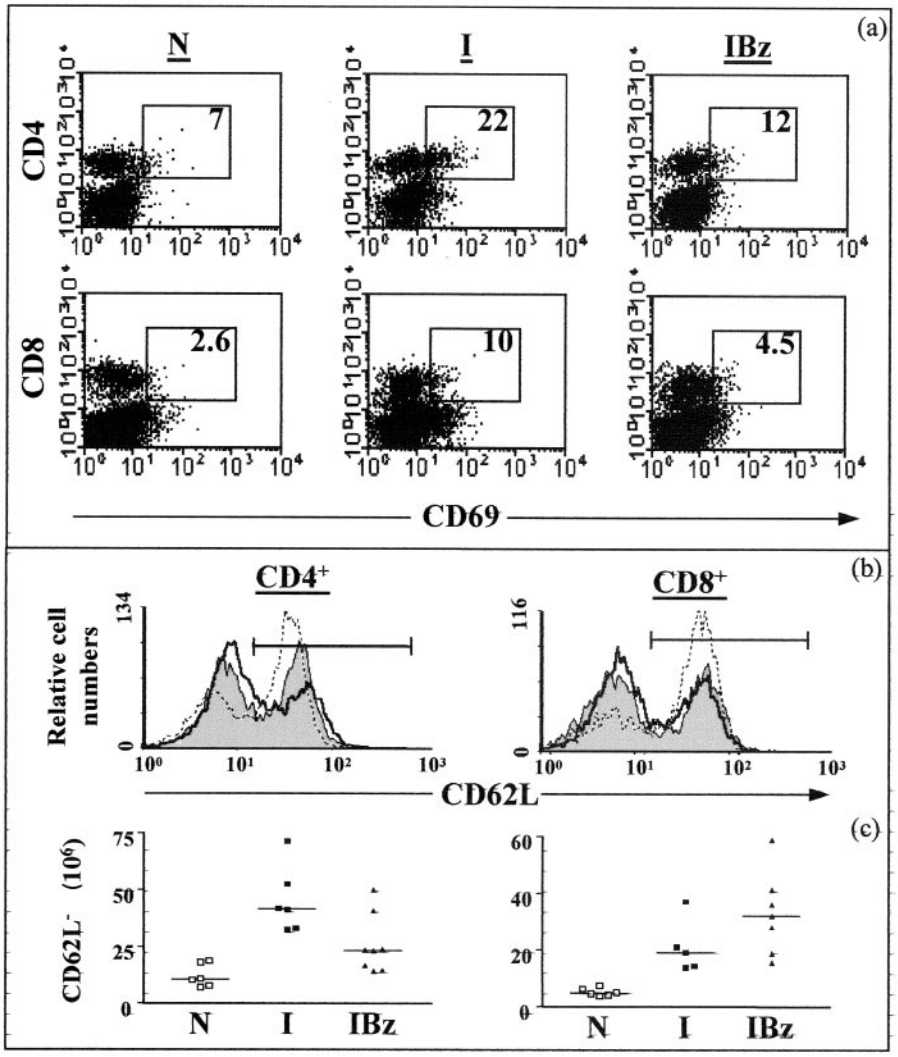

FIG. 4. Diminished CD69 expression in $CD4^+$ and $CD8^+$ lymphocytes together with downregulation of CD62L expression on $CD8^+$ lymphocytes after infection and benznidazole treatment. Cytofluorometric studies were performed with noninfected (N), infected, nontreated (I), and infected, benznidazole-treated (IBz) mice. (a) A representative dot plot of CD69 expression versus CD4 or CD8 expression is shown, with the percentages of $CD4^+CD69^+$ or $CD8^+CD69^+$ cells indicated inside the inner square boxes; data are from four experiments with three to four mice per group. Positive staining was calculated by defining a positivity marker from the end of the unrelated immunoglobulin isotype control fluorescence. (b) Representative histograms of CD62L fluorescence intensity in $CD4^+$ and $CD8^+$ lymphocytes from noninfected (N; dotted lines), infected, nontreated (I; solid lines), and infected, benznidazole-treated (IBz; shaded curves) mice in a representative experiment. The solid horizontal lines depict positive staining defined by an immunoglobulin isotype control. (c) Individual absolute $CD4^+CD62L^-$ and $CD8^+CD62L^-$ numbers are shown, with solid horizontal lines representing median values. CD62L phenotyping was done in three experiments, with similar results, by using three mice from each group.

Two weeks of treatment that commenced 1 week after infection was used, since we aimed to study changes in the peripheral lymphoid organs during the acute phase of the infection. This approach allowed analysis to take place after the appearance of bloodstream parasites but before the death of the infected, nontreated group (the mortality rate is high during the third week after infection) (37). The parasitological parameters found in our experimental therapeutic model confirmed published data in terms of the reduction of the level of parasitemia and the increase in the rate of survival after treatment (2, 31).

Assessment of cellularity in the peripheral lymphoid organs revealed clear expansion of spleen lymphocytes on day 9 postinfection in both the nontreated, infected and the treated, infected mice, suggesting that the increased level of parasitemia is crucial to trigger splenomegaly. Although this expansion waned at day 14 after infection in the nontreated group, benznidazole-treated mice showed a sustained splenomegaly. Interestingly, further analysis of the T-cell subsets in the spleens of infected, benznidazole-treated mice demonstrated that $CD8^+$ lymphocytes are the subpopulation that is preferentially expanded.

The lymphocyte expansion in infected mice (24, 25) is also accompanied by an activation-induced cell death process, as

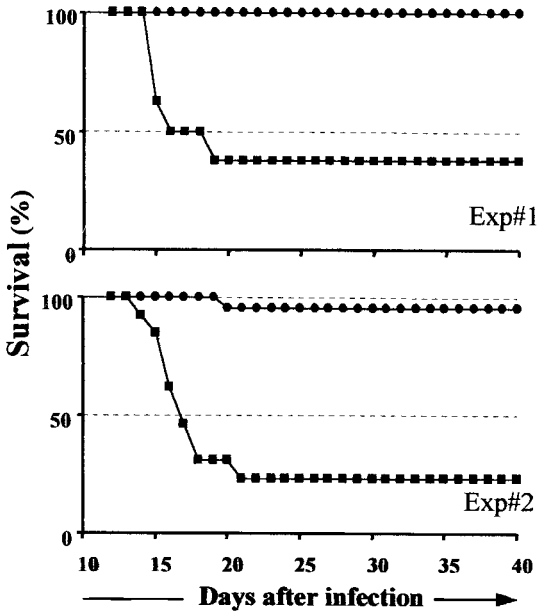

FIG. 5. Benznidazole treatment confers immune protection against a secondary challenge with *T. cruzi*. The survival rate depicts data for 40 infected, benznidazole-treated, reinfected mice (filled circles) and 21 primary infected control mice of the same age (filled squares) in two independent experiments.

described previously (21, 22). We postulate that an imbalance in apoptosis could also be taking place in the infected animals receiving treatment. In this regard, we demonstrated that infected mice receiving benznidazole therapy had a partial inhibition of the increased rate of splenocyte apoptosis seen in the infected group. These findings suggest that a block in activation-induced cell death that occurs during infection and benznidazole treatment might be occurring concomitantly with the expansion of specific lymphocyte subsets.

Although benznidazole treatment was able to block the expansion of recently activated $CD69^+$ T cells in infected hosts, it also sustained a selective expansion of $CD44^+CD62L^{-/low}CD8^+$ T cells, a phenotype indicating the gain of an effector and memory function (27). In this regard, immunological memory after parasitological cure was suggested to be sustained by the splenocytes of infected, benznidazole-treated mice (3), with detection of *T. cruzi* antigens, but not intact parasites, in the spleen germinal centers (4). Moreover, it was shown that larger numbers of activated T lymphocytes were found in the peripheral blood of patients in the chronic phase of Chagas' disease, including benznidazole-treated and cured patients (14). The higher rates of proliferative responses of peripheral blood mononuclear cells against parasite antigens seen in treated and cured patients reinforce the presence of long-term memory T cells (6).

However, one possibility is that the presence of large numbers of $CD8^+$ T cells bearing an effector phenotype does not result in protection. The inflammatory response in the heart could be intensified, particularly due to elevated levels of IFN-γ production by these expanded cells. In fact, patients with cardiac alterations produce more IFN-γ than individuals in an indeterminate phase, indicating a possible role of this cytokine in the physiopathology (7). Accordingly, we recently demonstrated that perforin-knockout mice are more susceptible to *T. cruzi* infection, with the animals dying with intense myocarditis, which comprises elevated numbers of IFN-γ-positive $CD8^+$ lymphocytes (18).

Another possibility is that expanded numbers of $CD8^+$ T cells actively participate in parasite control. It was previously demonstrated that the absence of $CD8^+$ lymphocytes leads to higher levels of parasitemia and tissue parasitism (39), and it was suggested that this higher level of susceptibility is related to lower levels of IFN-γ production (30). Additionally, benznidazole treatment seems to correlate directly with a lower-level inflammatory response in the heart, with reductions in the amounts of extracellular matrix components deposited occurring concomitantly with smaller numbers of inflammatory infiltrates in the myocardium of infected and benznidazole-treated mice (4, 34). Fewer electrocardiographic changes and better clinical conditions as well as decreased serologic titers are also observed in patients with chronic Chagas' disease after treatment (41).

Although the direct participation of $CD8^+$ T lymphocytes was not defined, we demonstrated that benznidazole-treated mice acquire complete resistance in a reinfection process. Importantly, our results are distinct from those of a previous study, in which nifurtimox (another trypanocidal drug) rendered infected mice more susceptible to reinfection (9). This discrepancy may be related to the use of a different parasite strain and mice of a different lineage or may indicate the antipodal actions of these drugs upon the immune system during *T. cruzi* infection.

Altogether, these data suggest that benznidazole treatment of infected mice both interrupts in vivo parasite replication directly and induces new immune regulation during *T. cruzi* infection. Therefore, the effectiveness of any treatment regimen should take into account not only the trypanocidal activity of a specific drug. Comprehensive and long-term studies should also consider the repercussions of these treatments on the immune system of the infected host at late phases of the infection.

ACKNOWLEDGMENTS

We thank Solange L. de Castro (Laboratory of Cell Biology, Oswaldo Cruz Institute) and Scott B. Snapper (Massachusetts General Hospital-Harvard Medical School) for critical reading of the manuscript. We also thank Rodrigo C. Oliveira and Wilson Savino (Osvaldo Cruz Foundation) for interesting discussions.

This work received financial support from Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro, Conselho Nacional de Desenvolvimento Tecnológico, and Instituto Oswaldo Cruz.

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