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Depletion of T-Cell Subpopulations Results in Exacerbation of Myocarditis and Parasitism in Experimental Chagas' Disease

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The contribution of T-cell subpopulations to immunopathology in murine *Trypanosoma cruzi* infection was studied by using in situ localization of lymphocytes and in vivo depletion of T-lymphocyte populations. $CD8^+$ T cells were the major lymphocyte population in the inflamed hearts of C3H/HeSnJ mice infected with the Sylvio X10/4 clone of *T. cruzi* at all time points of the acute and chronic phases of the infection examined. Depletion of $CD8^+$ and/or $CD4^+$ T cells beginning on day 20 of the infection resulted in a moderate decrease in the inflammation and an increase in parasite burden in the hearts of mice at day 30 of infection. Longer-term depletion, beginning at day 20 and extending as long as 200 days of infection, resulted in an increased inflammatory response in the heart. A large proportion of the inflammatory cells in the hearts of anti-CD8- or anti-CD4- and anti-CD8-treated mice were $Thy1^+$ and $CD4^-$ $CD8^-$. At 200 days of infection, the increased inflammation was accompanied by an increase in the parasite load in the heart. These results show that T-cell subset depletion does not prevent the inflammatory response associated with acute and chronic *T. cruzi* infection. The increased parasite load in T-cell-depleted mice also demonstrates the participation of these T-cell subsets in regulation of parasite load throughout the course of the infection. The increased inflammatory response despite T-cell depletion and in association with increased numbers of tissue parasites suggests that intracellular parasites are a driving force behind the inflammatory response in chronic murine *T. cruzi* infection.

Trypanosoma cruzi is a protozoan parasite of many mammalian species, including humans, and is the causative agent of Chagas' disease, a syndrome characterized by inflammation and degeneration of cardiac and smooth muscle. The disease irreversibly affects internal organs, primarily the heart, esophagus, colon, and peripheral nervous system, and is one of the most important public health problems in Latin America. *T. cruzi*-induced acute heart disease is characterized by foci of myocyte necrosis and inflammation directly related to tissue parasitism (2). In the chronic stage of infection, widely distributed foci of myocarditis are present with myocytolysis and fibrotic replacement of the myocardium (2).

The exact relationship between *T. cruzi* infection and Chagas' disease is not known; however, both direct (parasite-mediated tissue destruction) and indirect (mostly immunological or autoimmune) etiologies have been proposed. Support for an immunological basis of pathology in chronic *T. cruzi* infection comes mainly from the difficulty in detecting intracellular and blood forms of *T. cruzi* in patients with chronic infection (3) and from the reported modulation of pathology in immunocompromised-animal models (13, 18, 25, 31).

In previous studies we have sought to define the role of the immune system in the inflammatory response that occurs in the heart during *T. cruzi* infection (27, 29, 31). Using a mouse model of the infection and disease, we have characterized the lymphocyte populations in the acute inflammatory lesions (27). The majority of these infiltrating cells are $CD8^+$ T cells and a significant but lesser number are $CD4^+$ T cells. In this study we asked two questions: (i) what is the composition of the inflammatory response in the hearts of mice with chronic *T. cruzi* infections (i.e., experimental Chagas' disease), and (ii) can the symptoms of the chronic disease state be ameliorated by depletion of specific T-cell populations in these infected mice? We now report that as in the acute infection, $CD8^+$ T cells are the major lymphocyte population in the inflamed myocardium in chronic disease, and the ratio of $CD8^+$ to $CD4^+$ cells in inflammatory sites remains constant throughout the course of infection. Furthermore, we show that depletion of $CD8^+$ and $CD4^+$ T cells increases susceptibility to infection but moderates myocardial inflammation during the acute phase. However, continual depletion of $CD8^+$ and/or $CD4^+$ cells in the postacute and chronic phases of the infection results in exacerbation of chronic pathology and enhanced myocardial parasitism.

MATERIALS AND METHODS

Parasites. The Brazil strain of *T. cruzi* was maintained by serial passage in C3H/HeSnJ mice, and the Sylvio X10/4 clone of *T. cruzi* (17) was maintained by serial passage in bovine embryo skeletal muscle cell cultures (21). Parasitemia levels were determined by hemacytometer counting of parasites in diluted tail vein blood. Tissue parasitism is expressed as the number of pseudocysts per square millimeter of sectioned myocardium. The area of sectioned myocardium was determined by using the Image-1 software (Universal Imaging Corporation, West Chester, Pa.).

Animals, infection, and antibody treatment. C57BL/6J female or C3H/HeSnJ male mice were infected with 10^3 Brazil strain or 10^6 Sylvio X10/4 *T. cruzi* trypomastigotes, respec-

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tively. For depletion of T-cell populations, antibody treatments were begun either on the day of infection or at various times postinfection. Mice received intraperitoneal injections of 0.5 mg of anti-CD4 (GK1.5) and/or anti-CD8 (H35.17.2) purified monoclonal antibodies three times per week in the first week and once a week thereafter. For long-term depletion studies, 124 male 6-week-old C3H/HeSnJ mice were infected intraperitoneally with 10^6 tissue-culture-derived trypomastigotes of the Sylvio X10/4 clone of *T. cruzi* and were equally divided into four groups (nondepleted, CD4 depleted, CD8 depleted, and CD4 and CD8 depleted). Antibody treatments were continued until the time of sacrifice at either 30, 60, 120, or 200 days postinfection. The degree of lymphocyte depletion was monitored by avidin-biotin-peroxidase complex antibody staining of spleen sections (27) or by flow cytometric analysis as previously described (28). All mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and were maintained in microisolator units (Lab Products, Maywood, N.J.) in a specific-pathogen-free environment.

Antibodies. Monoclonal antibodies used for in vivo cell depletion (H35.17.2, anti-CD8 [cell line the gift of Richard Titus, Harvard School of Public Health], and GK1.5, anti-CD4 [from the American Type Culture Collection {ATCC} Rockville, Md.]) were harvested from supernatants of hybridomas grown in PFHMII protein-free medium (GIBCO, Gaithersburg, Md.) (30). Antibodies used in immunohistochemistry either were obtained commercially (RM-4-5, anti-CD4 [from PharMingen Laboratories, San Diego, Calif.]) or were used as culture supernatants from cells grown in RPMI 1640 plus 10% fetal bovine serum (30.H12, anti-Thy1.2 [ATCC]; RA3-6B2.1, anti-CD45R [cell line the gift of Robert Coffman, DNAX Research Institute, Palo Alto, Calif.]); 53.6.72, anti-CD8 [ATCC]; H57-597, anti-alpha/beta T-cell receptor [anti-alpha/beta TCR] [ATCC]; and PF136, anti-natural killer cell [ATCC]). Purified normal rat immunoglobulin G (IgG) or hamster IgG (Sigma Chemical Co., St. Louis, Mo.) was used as a negative control. Biotinylated, mouse tissue-adsorbed rabbit anti-rat IgG or anti-hamster IgG (Vector Laboratories, Burlingame, Calif.) was used as the secondary antibody.

Immunohistochemistry. Tissues for immunohistochemistry were processed by acetone fixation and plastic embedding as previously described (27). Heart and a small portion of spleen (positive control) were collected in RPMI 1640 at 4°C and fixed at −20°C in acetone (100% ACS grade) overnight. Tissue samples were infiltrated with catalyzed acrylic monomer (JB-4 kit; Polysciences, Inc., Warrington, Pa.) and allowed to polymerize at 4°C for several hours. Three-micrometer serial sections were cut and air dried for 1 h at room temperature immediately before being stained.

Immunohistochemical staining of plastic sections was performed by the three-step avidin-biotin-peroxidase complex method (27). Serial sections of tissue samples were stained with monoclonal antibodies against either Thy1, CD8, CD4, CD45R, alpha/beta TCR, or NK1.1.

Evaluation of inflammation. Scoring of inflammation was performed on the same serial sections used for the immunophenotyping. The sections were stained with hemotoxylin and eosin and scored according to the extent of inflammation (normal, 0; focal, 1; multifocal, 2; diffuse with partial wall involvement, 3; and total wall involvement, 4) as previously described (27). Cells (nuclei) other than muscle fibers, fibroblasts in endomysium, perimysium, and capillaries were counted. These cells included lymphocytes, macrophages, polymorphonuclear cells, fibroblasts, plasma cells, and mast cells.

Statistical analysis. Data were analyzed with StatView SE software on a Macintosh computer. One-factor analysis of variance was used to determine the significance level for numbers of infiltrating cells resulting from antibody treatment. The significance level between groups was calculated by using Scheffe's F test. The nonparametric Kruskal-Wallis test was used to determine the significance level for inflammation scores resulting from antibody treatment. The change in CD8/CD4 ratio through the course of infection was examined by using the chi-square test. Significant differences were judged at the 0.05 level.

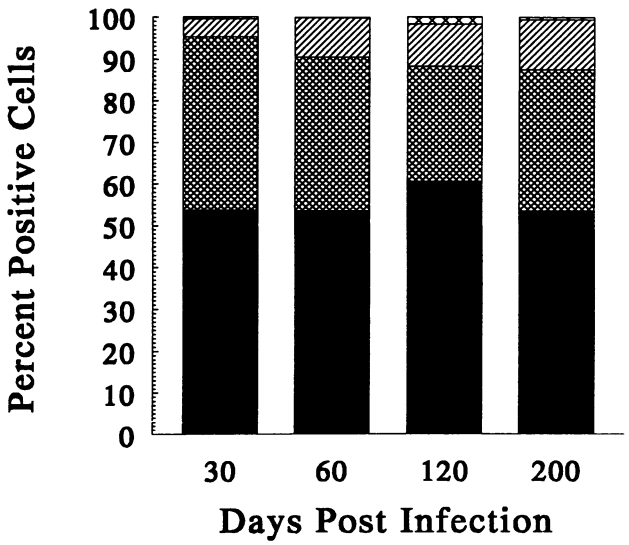

FIG. 1. The relative proportions of lymphocytes in the heart as identified by immunohistochemistry stay constant during the course of *T. cruzi* infection. The data indicate the percentage of cells positive for each marker relative to the total number of the cells stained in serial sections for all of the antibodies tested. Nine sets of serial sections for each of eight mice were examined for each time point. ■, Thy1.2; ▩, CD8; ▨, CD4; ⊠, CD45R.

RESULTS

We had previously reported that $CD8^+$ T cells dominate the inflammatory lesions in both the heart and skeletal muscle of mice with acute *T. cruzi* infections (27). In this study, we have extended this examination of inflammatory heart lesions to include the postacute and chronic stages of infection in the mouse. Similar to that in the acute infection, the postacute- and chronic-stage inflammatory response is characterized by a predominance of $CD8^+$ T cells, relatively fewer $CD4^+$ cells, and virtually no B cells (Fig. 1). The relative proportion of these cell types stays nearly static from day 30 of the infection through day 200. Examinations of selected mice at later time points (200 to >350 days postinfection) show a similar pattern of inflammatory cells. The change in the ratio of $CD4^+$ to $CD8^+$ cells throughout the infection was not statistically significant ($P > 0.05$).

Hypotheses on the etiology of Chagas' disease, as well as some experimental data, suggest that a variety of natural or artificially induced immunodeficiencies might modulate or prevent the chronic inflammation associated with *T. cruzi* infection. To determine the role of T-cell subsets in the inflammatory tissue response in experimental Chagas' disease, we depleted $CD4^+$ and/or $CD8^+$ T cells from infected mice. In order to avoid the mortality associated with depletion of T-cell

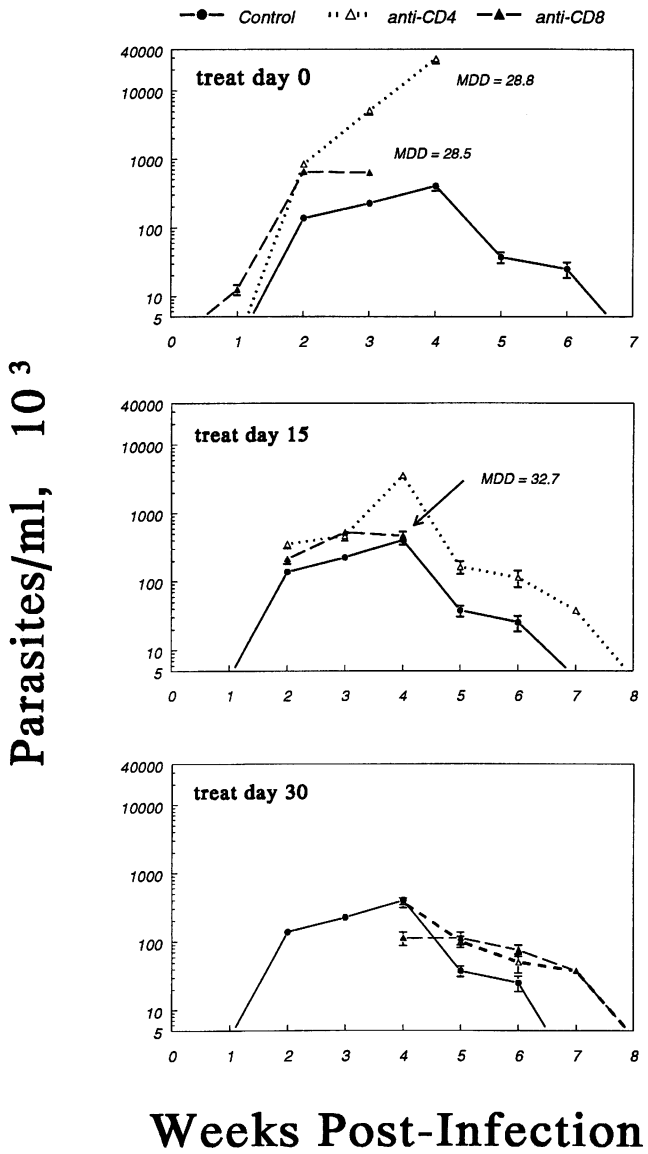

FIG. 2. Effect of delaying anti-CD4 or anti-CD8 antibody treatment on parasitemia levels and longevity in C57BL/6J mice infected at time zero with 10^3 blood-form trypomastigotes of the Brazil strain of *T. cruzi* (six animals per group). ●, control; △, anti-CD4; ▲, anti-CD8. MDD, mean number of days to death.

populations prior to or at the beginning of *T. cruzi* infection (4, 24, 28), we first sought to determine how soon after the infection depleting antibodies could be administered without affecting parasitemia and mortality. Therefore, mice were infected with the Brazil strain of *T. cruzi* and then treated with either anti-CD4 or anti-CD8 antibodies beginning at either day 0, 15 (midway into the peak of parasitemia), 30 (peak parasitemia), or 60 (postacute) of the infection. As previously reported, depletion of either T-cell subpopulation at the initiation of infection results in high-level parasitemias and the death of all animals before day 30 of infection (Fig. 2, top panel). Delaying the initiation of antibody treatment for $CD8^+$ T-cell depletion until day 15 of infection results in moderately higher parasitemia levels and death of all treated mice before day 35 of infection (Fig. 2, middle panel). Interestingly, mice treated to deplete $CD4^+$ T cells exhibit significantly higher parasitemia levels than either CD8-depleted or nontreated mice but are nevertheless able to control the parasitemia and survive the infection. Delaying antibody treatment until day 30 of infection had only a marginal effect on parasitemia levels, and both anti-CD4- and anti-CD8-treated mice survived the acute stage of infection (Fig. 2, bottom panel). Similar results were obtained when antibody treatment was initiated on day 60 of infection, with no increase in parasitemia or change in longevity in either CD4- or CD8-depleted mice (data not shown).

In order to study the role of T-cell subset depletion in inflammation, mice were infected with *T. cruzi* and at 20 days postinfection were treated with antibodies to deplete $CD4^+$ and/or $CD8^+$ T cells. Antibody treatments were continued until the time of sacrifice at either 30, 60, 120, or 200 days postinfection. The success of the T-cell depletion was monitored by immunohistochemical analysis of spleen and heart tissues and by flow cytometric analysis of splenocytes. Figures 3 and 4 show an example of the effect of anti-CD8 treatment on lymphocyte populations in the spleens (Fig. 3) and hearts (Fig. 4) of mice infected for 30 days. $Thy1^+$, $CD4^+$, and $CD45R^+$ cells are clearly evident in the serial sections of the spleen, but as previously reported (27), $CD4^+$ and $CD45R^+$ cells are less numerous in the heart. However, virtually no $CD8^+$ cells remain in either the spleen or the heart after 10 days of antibody treatment. The depletion protocol was also effective for mice treated with anti-CD4 antibody or the combination of anti-CD4 and anti-CD8 antibodies, as demonstrated by enumeration of positively staining cells in the inflammatory sites (Fig. 5). Similar results were obtained at other time points in the infection, although the depletion of $CD8^+$ T cells was slightly less effective at later time points (e.g., days 120 and 200) (data not shown). All animals in the control and anti-CD4- or anti-CD8-treated groups survived the infection and course of treatment. However, approximately half of the doubly depleted (anti-CD4- and anti-CD8-treated) mice died before the scheduled date of sacrifice.

Despite the absence of $CD8^+$ and/or $CD4^+$ T cells in the anti-CD8 and doubly depleted mice, $Thy1^+$ cells are still prominent in both the spleen and heart (Fig. 4 to 6). In an attempt to further determine the phenotypes of these inflammatory cells in the anti-CD8-treated mice, an additional set of animals was infected with *T. cruzi* and either left untreated or treated with anti-CD8 beginning on day 20 postinfection. These animals were sacrificed on day 30 of the infection, and the hearts were examined for the presence of $Thy1^+$, $CD4^+$, $CD8^+$, and $CD45R^+$ cells as before and in addition were examined for beta TCR, and NK markers. The majority of the inflammatory cells in the hearts of the CD8-depleted mice were doubly negative T cells as determined from the high percentage of both $Thy1^+$ and beta TCR expression and the low number of $CD4^+$ or $CD8^+$ T cells (Table 1). In addition, the anti-CD8-treated mice demonstrated an increase in the number of NK1.1-bearing cells relative to those in the untreated mice.

The effect of T-cell subset depletion on inflammation and myocardial damage was monitored by two measures: a routine histological analysis which takes into consideration the presence of necrotic myocardial fibers and the number and size of inflammatory foci (inflammatory index) and a more objective counting of the number of nonmuscle nuclei (presumably inflammatory-cell nuclei) in the interstitium of the heart. The myocardial inflammatory score and the number of inflammatory cells are highest during the early acute stage of the infection in untreated mice (Fig. 6 and 7, day 30 control).

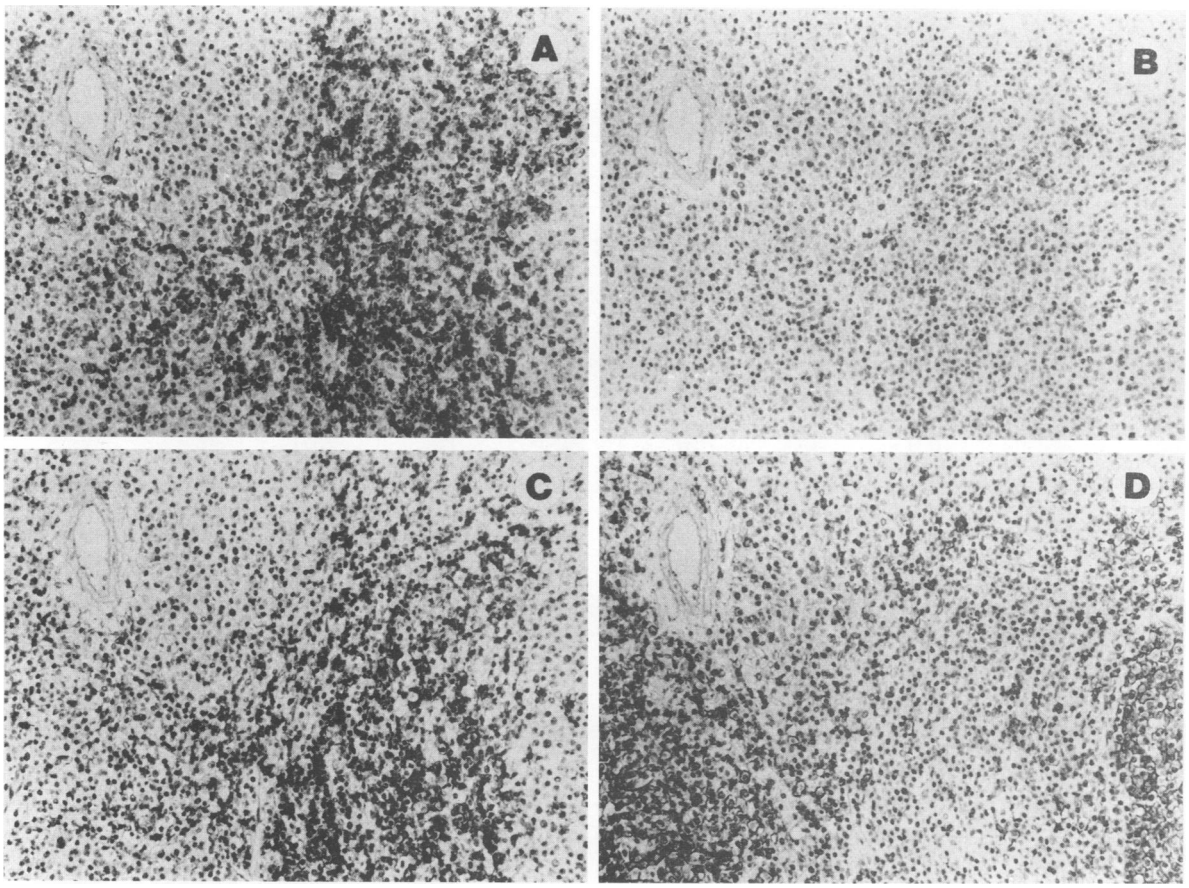

FIG. 3. Effectiveness of antibody injection for depletion of $CD8^+$ T cells in the spleen. The ability of the antibody treatment protocol to deplete T-cell populations was tested by analysis of spleens at 30 days postinfection with immunohistochemistry. Serial sections were stained with anti-Thy1.2 (A), anti-CD8 (B), anti-CD4 (C), and anti-CD45R (D). Note the lack of positive cells in the section stained with anti-CD8 antibody and the different location of the cells stained with anti-Thy1.2 and anti-CD4 antibodies relative to those stained with anti-CD45R antibody, corresponding to different areas of the spleen.

Similar to earlier findings (20, 21), myocardial lesions during the acute phase were characterized by necrosis of muscular fibers and the presence of mononuclear cell infiltrates. As expected, CD8 depletion and combined CD8 and CD4 depletion resulted in significant decreases in both inflammation scores ($P < 0.05$) and numbers of infiltrating cells in foci ($P < 0.05$) during the acute infection. The decrease in both parameters resulting from CD4 depletion was not statistically significant ($P > 0.05$).

Mice which had received continuous antibody treatment since day 20 of infection demonstrated an increase in the severity of myocarditis in the postacute phase of the infection as determined by both inflammatory scores and the numbers of infiltrating cells (Fig. 6 and 7). These increases were particularly prominent in the mice sacrificed on day 200 of the infection. The myocardial inflammation was characterized by destruction of myocardial fibers, interstitial fibrosis, and diffuse lymphocytic infiltration. Anti-CD4 treatment resulted in the most marked and consistent increase in chronic myocardial inflammation (Fig. 6 and 7) ($P < 0.05$). Anti-CD8 and anti-CD8 plus anti-CD4 treatments yielded a less-consistent outcome; these treatments resulted in statistically significant increases in inflammation scores but not in the numbers of infiltrating cells in foci. The disparity between the two parameters may indicate more-widespread inflammation throughout the heart tissue rather than increased inflammatory cells in a particular focus. Therefore, despite the significant reduction in $CD4^+$ and $CD8^+$ T cells obtained by using the treatment protocol, a concomitant decrease in the chronic pathology was not observed.

The data in Fig. 2 suggest that the protocol of anti-CD4 and anti-CD8 antibody treatment used in these experiments did not affect the level of the acute-phase parasitemia. However, we had not previously determined the effect of short- or long-term $CD4^+$ and/or $CD8^+$ T-cell depletion on levels of tissue parasites. Anti-CD4 or anti-CD8 antibody treatment had little effect on tissue parasitism on day 30 postinfection, but combined treatment with both antibodies resulted in an eightfold increase in tissue parasite load (Fig. 8). This increase may account for the high mortality in this experimental group. During later stages of the infection, the tissue parasite load decreases in all groups (note the 10-fold difference in the scales for the day 30 group and the other groups). However, the antibody treatments resulted in an increase in the number of pseudocysts at these later time points. This increase was especially noticeable at day 200, when the anti-CD8-treated group had a nearly twofold increase in tissue pseudocysts and the anti-CD4 and anti-CD4-CD8 treatment groups experienced nearly fourfold increases.

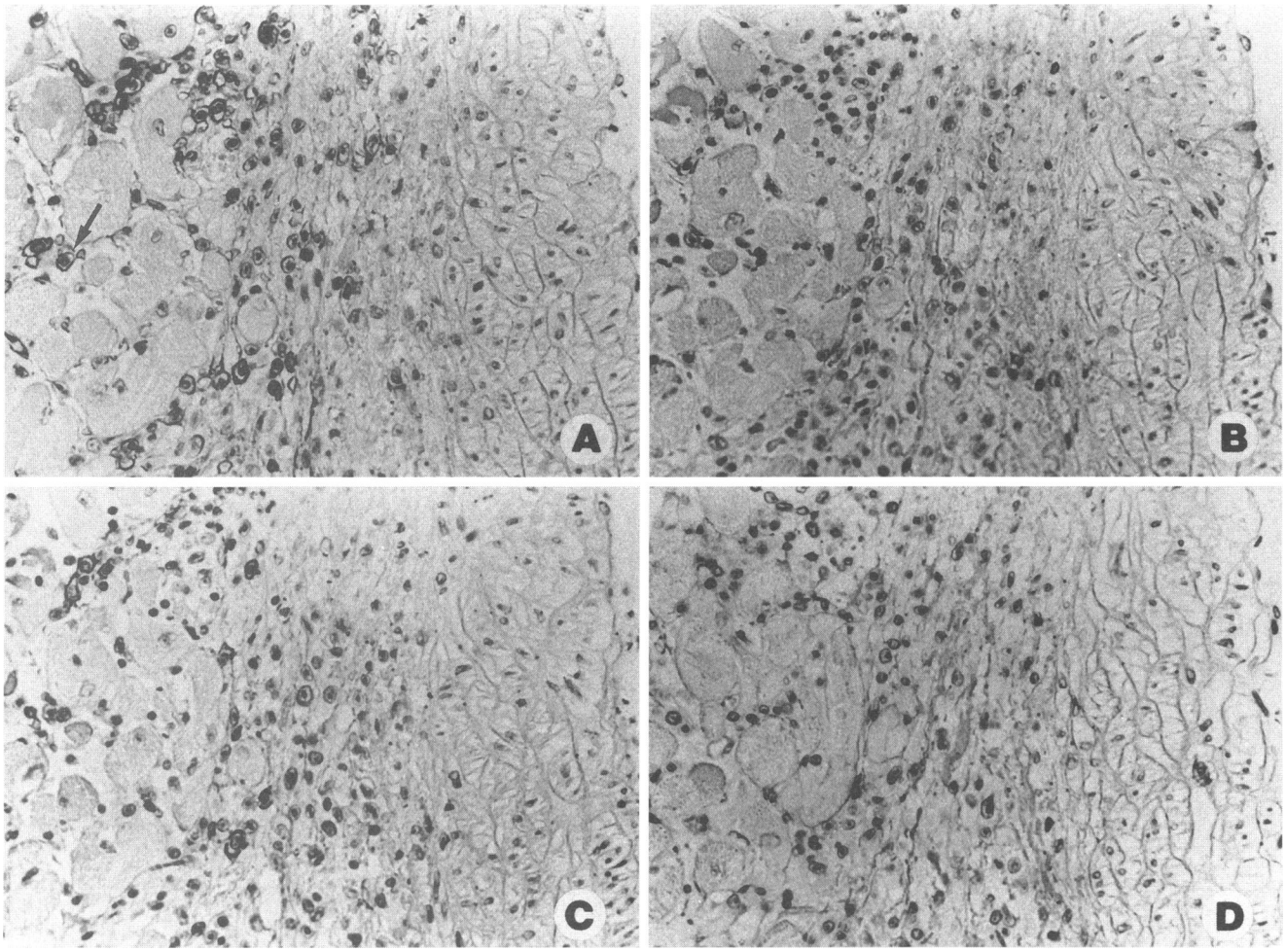

FIG. 4. Effectiveness of antibody injection for depletion of $CD8^+$ T cells in the heart. The ability of the antibody treatment protocol to deplete T-cell populations was tested by analysis of hearts at 30 days postinfection with immunohistochemistry. Serial sections were stained with anti-Thy 1.2 (A), anti-CD8 (B), anti-CD4 (C), and anti-CD45R (D). Positively stained cells are identified by a distinct dark nucleus, clear cytoplasm, and dark cell surface halo (arrow in panel A).

DISCUSSION

Host immune responses have been hypothesized to contribute to both the control of the parasite in the acute phase of *T. cruzi* infection and the occurrence and severity of disease in the chronic stage of the infection. Experimental evidence for a role of the immune system in both parasite control and pathogenesis comes largely from studies with experimental animals, with the mouse model being used most extensively. In this study, we have used infection with the Sylvio X10/4 strain of *T. cruzi* in C3H/HeSnJ male mice because of the similarities of this combination of parasite and mouse strain to the infection and disease in humans. These similarities include an acute phase characterized by low to undetectable parasitemia and low mortality and a chronic phase with inflammation and tissue damage focused in the heart, with resulting heart dysfunction (20, 21).

The present study provides the first in situ investigation of the lymphocytic composition in inflamed myocardium throughout the course of *T. cruzi* infection and the first attempt to manipulate the course of experimental Chagas' disease by prolonged administration of anti-T-cell antibodies. Our results demonstrate that as in the acute infection (27), $CD8^+$ T cells dominate the lymphocyte infiltrate in the inflamed myocardium of mice in the postacute and chronic stages of *T. cruzi* infection. The lymphocyte composition in inflammatory foci remains relatively constant throughout the entire course of the infection, with relatively few B cells and macrophages present. A predominance of $CD8^+$ T cells in chronic myocarditis has also been reported in autopsy specimens from chagasic patients (11), reinforcing the validity and clinical significance of the present study.

The high representation of $CD8^+$ and, to a lesser extent, $CD4^+$ T cells in the chronic inflammatory lesions suggested that depletion of these populations of cells might alleviate the chronic disease. This conclusion is based in part on the hypothesis that the chronic disease state is primarily a result of an autoimmune process and not a direct result of parasite invasion or the antiparasite immune response. Genetic immunodeficiencies, such as the nude phenotype (13, 24, 32) or knockout of the beta-2-microglobulin (31) or class II major histocompatibility complex (29a) genes, and depletion of T cells (4, 19, 24, 28) are all known to result in a decreased inflammatory reaction at the expense of higher parasitemia levels and mortality in the acute phase of the infection.

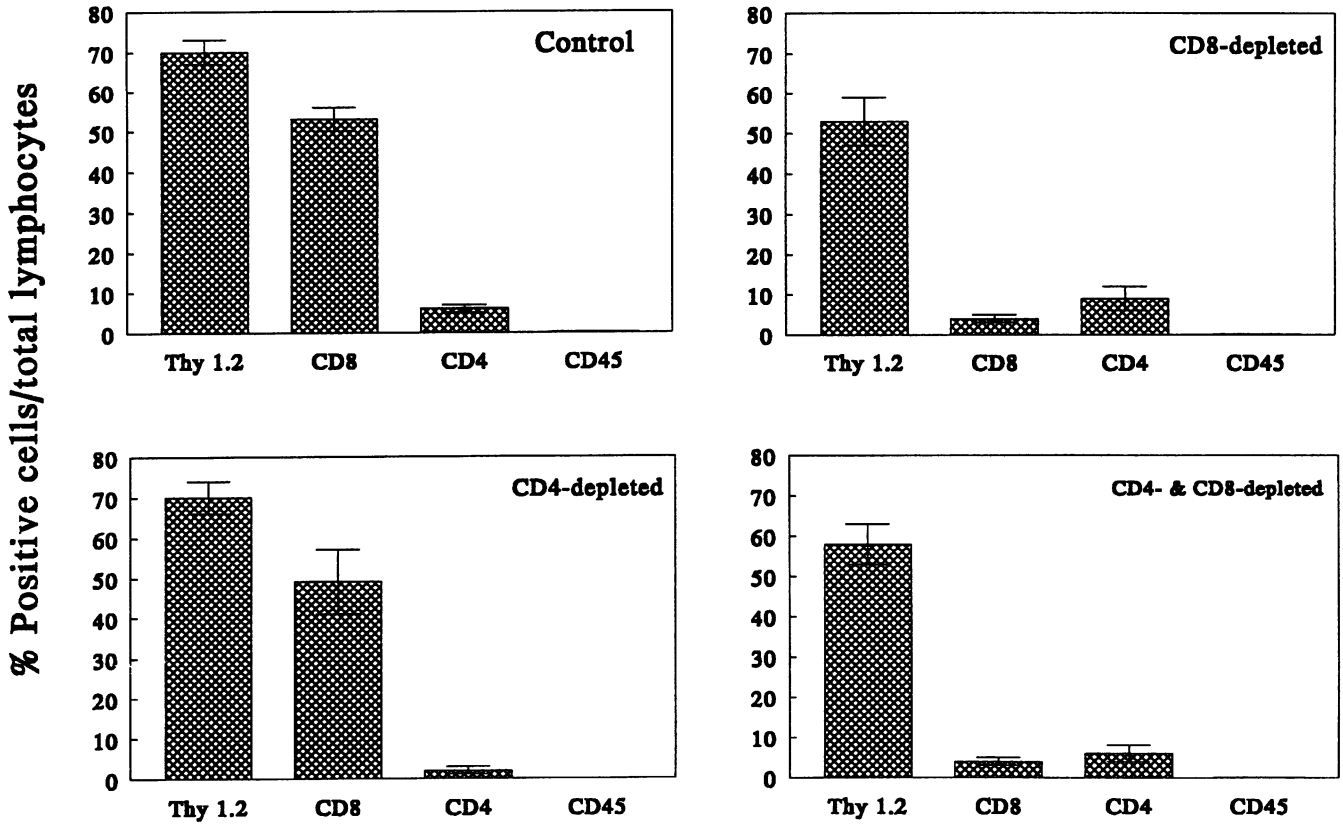

FIG. 5. Effect of antibody treatment on the composition of the cellular infiltrates in the hearts of day 30-infected mice. Hearts from mice which had received no treatment (Control) or anti-CD8 (CD8-depleted), anti-CD4 (CD4-depleted), or both anti-CD4 and anti-CD8 (CD4- & CD8-depleted) antibodies were stained to determine the presence of Thy1.2$^+$, CD8$^+$, CD4$^+$, or CD45R$^+$ cells. Data are expressed as mean percentages (± standard error) of positively stained lymphocytes among the total number of lymphocytes in myocardial lesions. Each datum point represents an average of 12 sets of serial sections from six to eight mice.

However, the high acute-phase mortality in CD4-depleted (4, 19, 24) and CD8-depleted (28) mice has prevented the study of the role of these T-cell subsets in the development of chronic disease. In order to avoid this problem, we delayed the depletion of CD4$^+$ or CD8$^+$ T cells until day 20 of infection, a protocol which we had found to result in no increase in parasitemia or mortality in C57BL/6J mice infected with the Brazil strain of *T. cruzi* (Fig. 2).

Consistent with these previous studies, we report here that a 10-day course of anti-T-cell antibody treatment starting on day 20 of *T. cruzi* infection results in a decrease in the cardiac inflammatory response in the acute phase. However, the long-term depletion of CD4$^+$ and/or CD8$^+$ T cells in *T. cruzi*-infected mice results in both an increased tissue parasite burden and tissue inflammation in the chronic stage. Immunosuppressants such as cyclosporin A (7), cyclophosphamide (8, 10), or gamma irradiation (8), as well as human immunodeficiency virus infections (12, 23), have been reported to reactivate chronic infections. Thus, the increased tissue parasite burden in chronically infected mice depleted of CD4$^+$ and/or CD8$^+$ T cells is not necessarily surprising. What is somewhat surprising is that mice depleted of the T-cell populations which normally predominate in the inflammatory lesions actually exhibit an increase in the cardiac inflammatory response. This increase is documented by both an increase in the inflammatory index and an increase in the number of nuclei per field (a more objective and quantitative measure of the number of inflammatory cells).

TABLE 1. Phenotypes of inflammatory cells in the hearts of untreated and anti-CD8-treated mice infected for 30 days with *T. cruzi*

Treatment[a]	% Cells positive for[b]:					
	Thy1	CD8	Alpha/beta TCR	CD4	NK1.1	CD45R
Control	80.8 ± 5.9	73.5 ± 7.2	74.8 ± 2.7	6.8 ± 5.2	3.2 ± 2.1	1.5 ± 1.9
Anti-CD8	80.7 ± 6.7	7.9 ± 1.8	82.0 ± 7.8	12.2 ± 1.2	21.8 ± 7.4	2.6 ± 2.9

[a] C3H/HeSnJ mice were infected with 10^6 Sylvio X10/4 *T. cruzi* trypomastigotes and were either untreated or treated with anti-CD8 antibodies as described in Materials and Methods beginning on day 20 of infection. All tissues were obtained from mice killed at day 30 of the infection.

[b] Mean (± standard deviation) percentage of positive cells for each marker relative to the total number of inflammatory cells. Each data set represents 12 different foci in each of three mice per group.

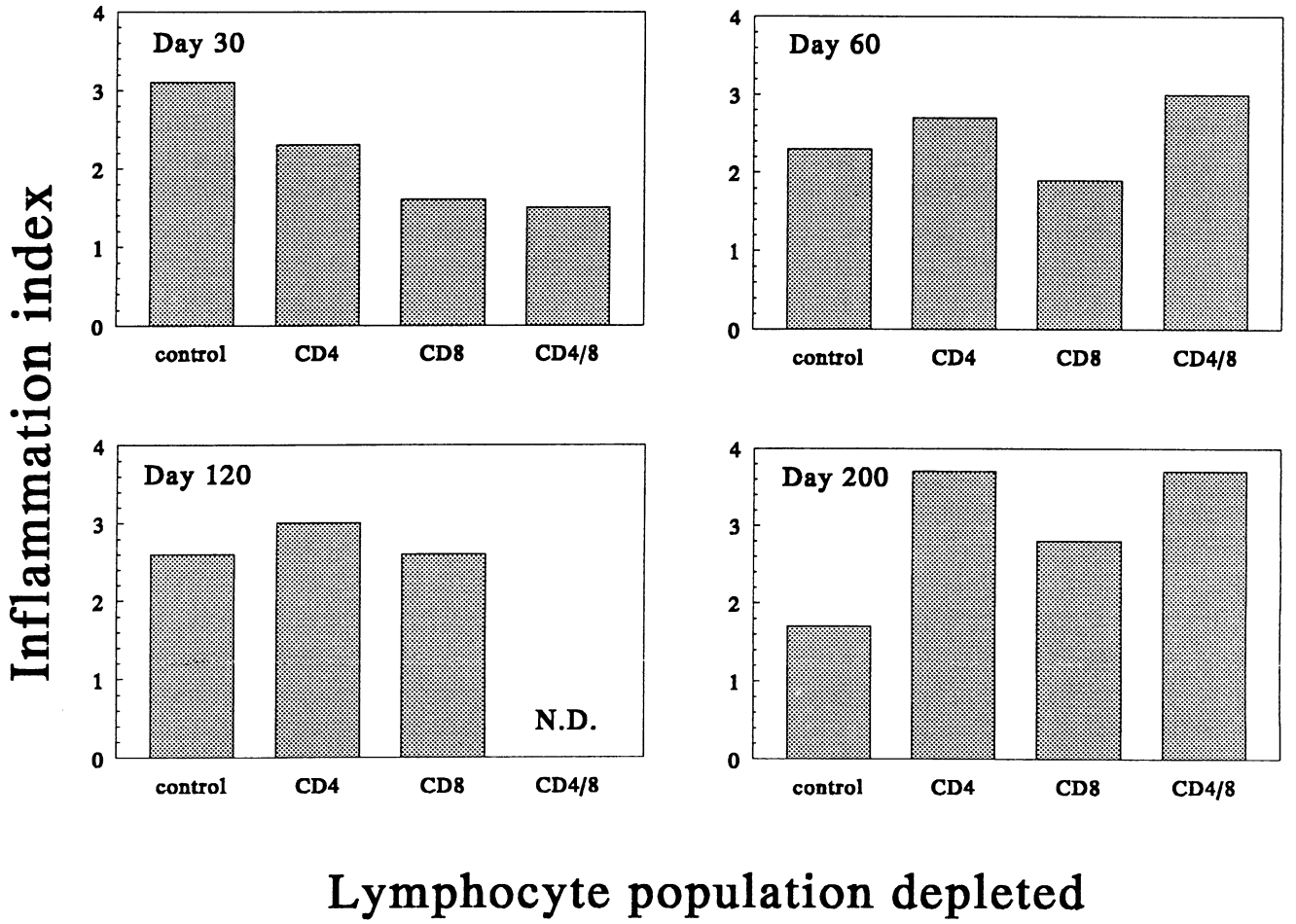

FIG. 6. Score of myocardial inflammation in *T. cruzi*-infected C3H mice depleted of the indicated lymphocyte populations. Each datum point represents the mean of nine sets of serial sections from each of group. Because of higher mortality in the doubly depleted group, data for this group were not collected on day 120 of the infection (N.D.).

Most previous studies have focused on the pathogenic role of $CD4^+$ T cells in experimental Chagas' disease (6, 14, 15, 22, 25, 26). Passive-transfer experiments have shown that $CD4^+$ cells from chronically infected mice cause peripheral nerve and liver pathology in naive animals (15, 26). While our data are not inconsistent with a pathogenic function for $CD4^+$ T cells in the inflammatory response, it seems clear that in both human and murine chagasic lesions, $CD4^+$ T cells are a minor population. However, the number of $CD4^+$ T cells in the lesions may not reflect their true importance to the disease process. Indeed, we find a near absence of inflammatory responses in acutely infected mice lacking either $CD8^+$ (31) or $CD4^+$ (29a) cells because of gene knockout, perhaps suggesting that the $CD4^+$ population is necessary for induction of the heavily $CD8^+$ infiltrate, at least in the acute stage of the infection. Strong support for the participation of $CD4^+$ cells in *T. cruzi*-induced immunopathogenesis comes from heart transplant studies in which grafted heart tissues were rejected by syngeneic mice with chronic *T. cruzi* infection. Treatment of the transplant recipients with anti-CD4 or anti-major histocompatibility complex class II antibodies prevented heart graft destruction (22). Data from the present study failed to show modulation of myocardial inflammation resulting from long-term CD4 and CD8 depletion in chronically *T. cruzi*-infected mice. Rather, continual administration of anti-CD4 and -CD8 antibodies resulted in an increase in the inflammatory response, thus exacerbating the chronic myocarditis.

The data reported herein provide support for two additional conclusions concerning the role of the immune system in *T. cruzi* infection and Chagas' disease. First, it seems clear from this study that both $CD4^+$ and $CD8^+$ T cells are required for the long-term immune control of *T. cruzi*, particularly at the level of intracellular parasites. In the absence of either one of these populations, tissue parasite levels increase. The preponderance of $CD8^+$ T cells in inflammatory sites as well as the dependence of the murine host on $CD8^+$ as well as $CD4^+$ T cells for control of the parasite load in both acute and chronic infection reinforces the need for further study of the specific mechanisms of immune control used by anti-*T. cruzi*-specific $CD8^+$ T cells and the identities of the parasite antigens which they recognize.

Second, our data suggest an association between increased parasite burden and increased inflammatory response in the chronic infection. This relationship between enhanced inflammation and increased tissue parasite load is particularly surprising considering that the cells which normally compose the majority of the inflammatory response have been depleted. The increase in inflammation, despite T-cell depletion and in

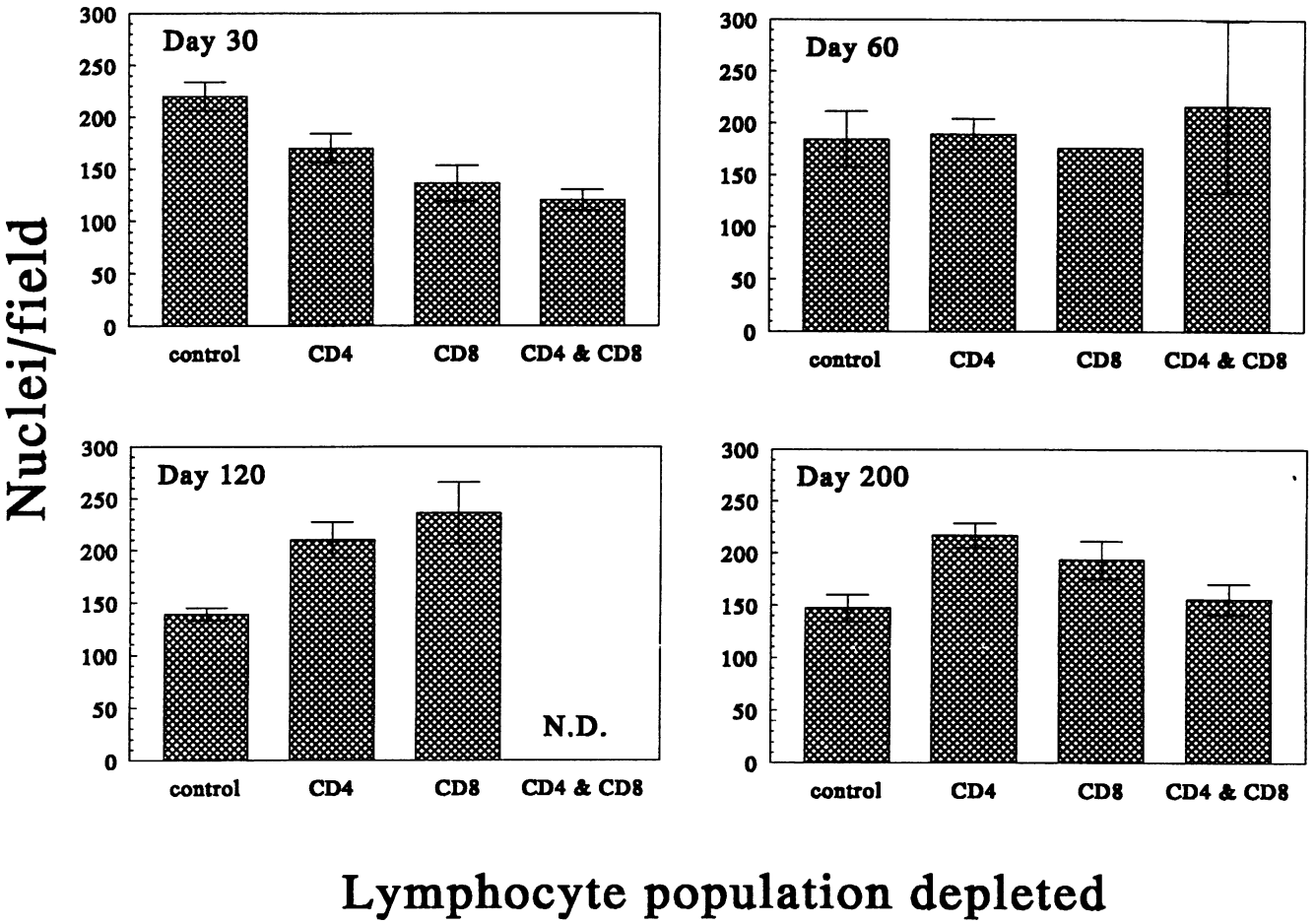

FIG. 7. Enumeration of nonmyocardial cells infiltrating the hearts of *T. cruzi*-infected mice depleted of the indicated lymphocyte populations. Each datum point represents the mean of nine sets of serial sections from each of group. Because of higher mortality in the doubly depleted group, data for this group were not collected on day 120 of the infection (N.D.).

association with increased parasite load, suggests that it is the parasite rather than autoantigens which provides the stimulus for the chronic inflammatory response. While such a conclusion is counter to the widely accepted autoimmune etiology for Chagas' disease, it does not specifically exclude a role for the immune response in the chronic chagasic disease process. For example, it is possible that it is the parasite which initiates and sustains the inflammatory response, establishing an environment which then promotes immune system-mediated damage. In the absence of the parasite, the conditions which promote immune system-mediated tissue damage (e.g., cytokine production and enhanced major histocompatibility complex and adhesion molecule expression) would no longer exist, and the chances of tissue damage might decrease.

A number of recent studies lend further support to the association between tissue parasites and chronic disease. Most significantly, Jones et al. report a high association of *T. cruzi* DNA (as detected by PCR) with cardiac lesions in autopsy specimens from fatal chronic chagasic cardiopathy and the absence of detectable parasite DNA in heart specimens from seropositive cadavers which lacked evidence of chagasic cardiopathy (16). Antigens of *T. cruzi* have also been reported to persist in the lesions of chronically infected mice even when morphologically detectable parasite-infected cells are difficult to detect (5). Decreasing the parasite burden in the chronic stage of *T. cruzi* infection has also been shown to result in a reversal of disease. For example, a regression of inflammatory lesions and fibrosis has been reported in chronically infected mice undergoing chemotherapeutic treatment for *T. cruzi* infection (1). In all cases the degree of regression correlates with the success of the treatment, with parasitologically cured mice showing the most marked reversal of pathologic signs.

One question left unanswered by the present study is the origin, specificity, and function of the doubly negative $Thy1^+$ cells which populate the acute and chronic inflammatory sites of the anti-CD8-treated and doubly depleted mice. Studies with short-term-CD8-depleted mice (day 30 of infection) suggest that the majority of these $CD4^-$ $CD8^-$ $Thy1^+$ cells are beta TCR^+ and NK^-. In addition, the hearts of infected mice receiving short-term anti-CD8 antibody treatment also show a substantial increase in the numbers of NK cells relative to those in untreated mice. Our studies do not allow us to distinguish between true doubly negative T cells (9) which may be recruited into myocardial lesions and T cells whose surface molecules may be modulated following antibody cross-linking of CD8 or CD4 glycoproteins.

Data from this study provide solid evidence for the role of $CD4^+$ and $CD8^+$ T cells in immune control of *T. cruzi* in the acute and chronic stages of the infection and additional support for the hypothesis that the parasite load is a determin-

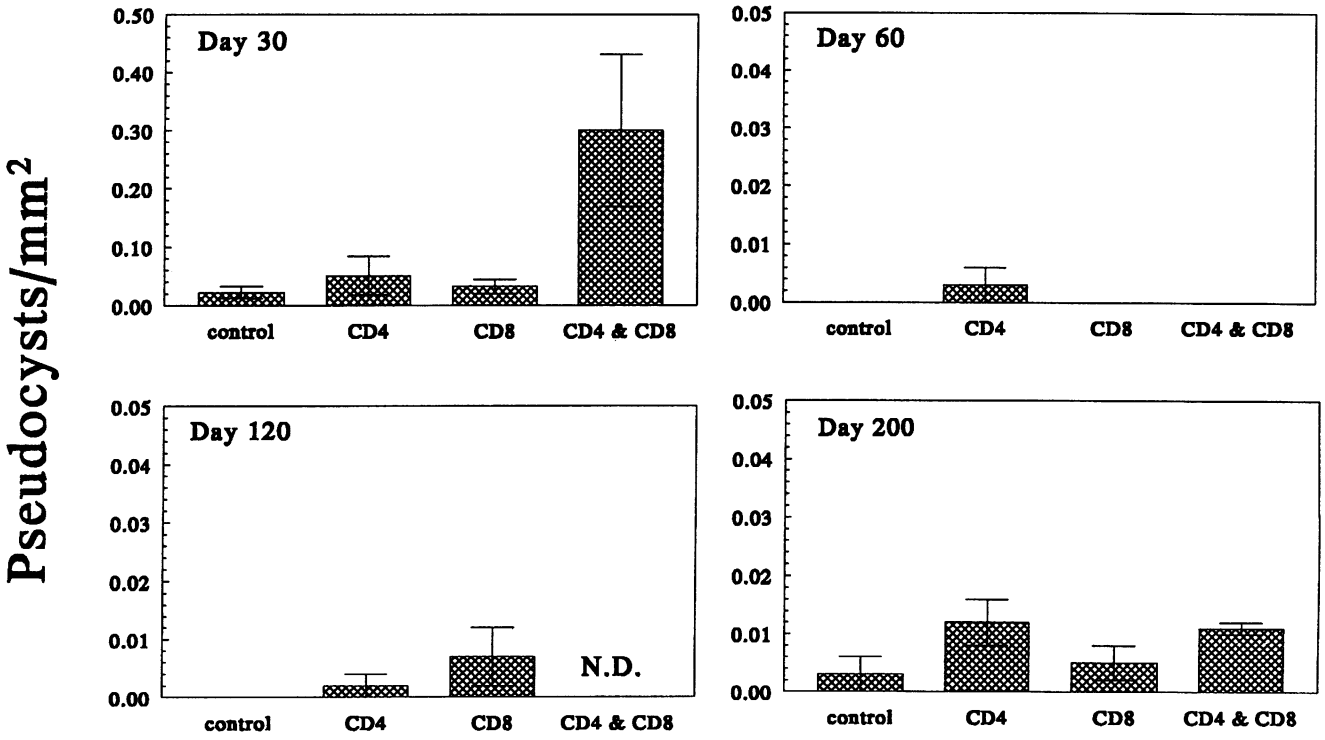

FIG. 8. Effect of antibody treatment on myocardial parasitism. Parasitism in myocardial tissue is expressed as the number of *T. cruzi* pseudocysts per square millimeter of heart tissue section. Each datum point represents the average for six mice (± standard error).

ing factor in the severity of chronic chagasic disease. If both of these hypotheses are true, attempts to alleviate chronic disease symptoms should focus on minimizing the parasite load either through chemotherapy or through vaccination to enhance the antiparasite immune response.

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REFERENCES

1. **Andrade, S. G., G. S. Stocker, A. S. Pimentel, and J. A. Grimaud.** 1991. Reversibility of cardiac fibrosis in mice chronically infected with Trypanosoma cruzi, under specific chemotherapy. Mem. Inst. Oswaldo Cruz **86:**187–200.
2. **Andrade, Z. A.** 1983. Mechanism of myocardial damage in Trypanosoma cruzi infection. CIBA Found. Symp. **99:**214–233.
3. **Andrade, Z. A.** 1991. Pathogenesis of Chagas' disease. Res. Immunol. **142:**126–129.
4. **Araujo, F.** 1989. Development of resistance to *Trypanosoma cruzi* in mice depends on a viable population of L3T4$^+$ (CD4$^+$) T lymphocytes. Infect. Immun. **57:**2246–2248.
5. **Ben Younes-Chennoufi, A., M. Hontebeyrie-Joskowicz, V. Tricottet, H. Eisen, M. Reynes, and G. Said.** 1988. Persistence of Trypanosoma cruzi antigens in the inflammatory lesions of chronically infected mice. Trans. R. Soc. Trop. Med. Hyg. **82:**77–83.
6. **Ben Younes-Chennoufi, A., G. Said, H. Eisen, A. Durand, and M. Hontebeyrie-Joskowicz.** 1988. Cellular immunity to Trypanosoma cruzi is mediated by helper T cells (CD4+). Trans. R. Soc. Trop. Med. Hyg. **82:**84–89.
7. **Boullon, F., A. Sinagra, A. Riarte, M. Lauricella, J. Barra, M. Besanson, C. Lejour, B. O. Lopez, R. Favaloro, and E. L. Segura.** 1988. Experimental cardiac transplantation and chronic Chagas' disease in dogs. Transplant. Proc. **20:**432–437.
8. **Brener, Z., and E. Chairi.** 1971. The effects of some immunosuppressive agents in experimental chronic Chagas' disease. Trans. R. Soc. Trop. Med. Hyg. **65:**629–636.
9. **Brooks, E. G., D. P. Wirt, R. M. Goldblum, S. Vaidya, M. T. Asuncion, J. C. Patterson, C. F. Ware, and G. R. Klimpel.** 1990. Double negative (CD4 − CD8 −) T cells with an alpha/beta T cell receptor. Non MHC-restricted cytolytic activity and lymphokine production. J. Immunol. **144:**4507–4512.
10. **Cabeza Meckert, P. M., J. G. Chambo, and R. P. Laguens.** 1988. Modification of the pattern of infection and evolution of cardiopathy in experimental Chagas' disease after treatment with immunosuppressive and trypanocidal drugs. Medicina (Buenos Aires). **48:**7–11.
11. **d'Avila Reis, D., E. M. Jones, S. Tostes, Jr., E. R. Lopes, G. Gazzinelli, D. G. Colley, and T. L. McCurley.** 1993. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor-alpha+ cells and dominance of granzyme A+, CD8+ lymphocytes. Am. J. Trop. Med. Hyg. **48:**637–644.

12. **Ferreira, M. S., S. A. Nishioka, A. Rocha, A. M. Silva, R. G. Ferreira, W. Olivier, and S. Tostes, Jr.** 1991. Acute fatal Trypanosoma cruzi meningoencephalitis in a human immunodeficiency virus-positive hemophiliac patient. Am. J. Trop. Med. Hyg. **45:** 723–727.
13. **Goncalves da Costa, S. C., P. H. Lagrange, B. Hurtrel, I. Kerr, and A. Alencar.** 1984. Role of T lymphocytes in the resistance and immunopathology of experimental Chagas' disease. Ann. Immunol. (Inst. Pasteur) **135C:**317–332.
14. **Hontebeyrie-Joskowicz, M.** 1991. Murine Trypanosoma cruzi infection: a role for TH2 cells in the immunopathology of chronic infection. Res. Immunol. **142:**141–143.
15. **Hontebeyrie-Joskowicz, M., G. Said, G. Milon, G. Marchal, and H. Eisen.** 1987. L3T4+ T cells able to mediate parasite-specific delayed-type hypersensitivity play a role in the pathology of experimental Chagas' disease. Eur. J. Immunol. **17:**1027–1033.
16. **Jones, E. M., D. G. Colley, S. Tostes, E. R. Lopes, J. C. Vnencak, and T. L. McCurley.** 1993. Amplification of a Trypanosoma cruzi DNA sequence from inflammatory lesions in human chagasic cardiomyopathy. Am. J. Trop. Med. Hyg. **48:**348–357.
17. **Miles, M. A.** 1974. Cloning of Trypanosoma cruzi. Trans. R. Soc. Trop. Med. Hyg. **68:**256–260.
18. **Minoprio, P., A. Coutinho, S. Spinella, and J. M. Hontebeyrie.** 1991. Xid immunodeficiency imparts increased parasite clearance and resistance to pathology in experimental Chagas' disease. Int. Immunol. **3:**427–433.
19. **Minoprio, P., H. Eisen, M. Joskowicz, P. Pereira, and A. Coutinho.** 1987. Suppression of polyclonal antibody production in Trypanosoma cruzi-infected mice by treatment with anti-L3T4 antibodies. J. Immunol. **139:**545–550.
20. **Postan, M., J. J. Bailey, J. A. Dvorak, J. P. McDaniel, and E. W. Pottala.** 1987. Studies of Trypanosoma cruzi clones in inbred mice. III. Histopathological and electrocardiographical responses to chronic infection. Am. J. Trop. Med. Hyg. **37:**541–548.
21. **Postan, M., J. A. Dvorak, and J. P. McDaniel.** 1983. Studies of Trypanosoma cruzi clones in inbred mice. I. A comparison of the course of infection of C3H/HEN− mice with two clones isolated from a common source. Am. J. Trop. Med. Hyg. **32:**497–506.
22. **Ribeiro-dos-Santos, R., M. A. Rossi, J. L. Laus, J. S. Silva, W. Savino, and J. Mengel.** 1992. Anti-CD4 abrogates rejection and reestablishes long-term tolerance to syngeneic newborn hearts grafted in mice chronically infected with Trypanosoma cruzi. J. Exp. Med. **175:**29–39.
23. **Rosemberg, S., C. J. Chaves, M. L. Higuchi, M. B. Lopes, L. H. Castro, and L. R. Machado.** 1992. Fatal meningoencephalitis caused by reactivation of Trypanosoma cruzi infection in a patient with AIDS. Neurology **42:**640–642.
24. **Rottenberg, M., R. L. Cardoni, R. Anderson, E. L. Segura, and A. Orn.** 1988. Role of T helper/inducer as well as natural killer cells in resistance to Trypanosoma cruzi infection. Scand. J. Immunol. **28:**573–582.
25. **Russo, M., N. Starobinas, P. Minoprio, A. Coutinho, and J. M. Hontebeyrie.** 1988. Parasitic load increases and myocardial inflammation decreases in Trypanosoma cruzi-infected mice after inactivation of helper cells. Ann. Inst. Pasteur/Immunol. **139:**225–236.
26. **Said, G., M. Joskowicz, A. A. Barreira, and H. Eisen.** 1985. Neuropathy associated with experimental Chagas' disease. Ann. Neurol. **18:**676–683.
27. **Sun, J., and R. L. Tarleton.** 1993. Predominance of CD8+ T cells in the inflammatory lesions of mice with acute Trypanosoma cruzi infection. Am. J. Trop. Med. Hyg. **48:**161–169.
28. **Tarleton, R. L.** 1990. Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with Trypanosoma cruzi. J. Immunol. **144:**717–724.
29. **Tarleton, R. L.** 1991. The role of T-cell subpopulations in experimental Chagas' disease. Res. Immunol. **142:**130–134.
29a. **Tarleton, R. L., et al.** Unpublished data.
30. **Tarleton, R. L., and A. M. Beyer.** 1991. Medium-scale production and purification of monoclonal antibodies in protein-free medium. BioTechniques **11:**590–593.
31. **Tarleton, R. L., B. H. Koller, A. Latour, and M. Postan.** 1992. Susceptibility of beta-2-microglobulin-deficient mice to Trypanosoma cruzi infection. Nature (London) **356:**338–340.
32. **Trischmann, T., H. Tanowitz, M. Wittner, and B. Bloom.** 1978. Trypanosoma cruzi: role of the immune response in the natural resistance of inbred strains of mice. Exp. Parasitol. **45:**160–168.