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Autoimmunity in Chagas' Disease: Specific Inhibition of Reactivity of CD4$^+$ T Cells against Myosin in Mice Chronically Infected with *Trypanosoma cruzi*

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In this study, we assessed the proliferative response of T cells from mice chronically infected with *Trypanosoma cruzi* to actin, myosin, or *T. cruzi* soluble antigen (SA). We report here that CD4$^+$ T cells from mice chronically infected with *T. cruzi* proliferated in response to myosin but not to actin, whereas cells from naive mice did not proliferate against any of the antigens tested. Antisera raised against myosin- or SA-activated T cells specifically inhibited respectively, the myosin or SA in vitro proliferative response, whereas the response to unrelated antigen remained unimpaired. Sera from chronically infected mice failed to show any significant inhibitory activity. The above findings suggest that autoreactive and *T. cruzi*-reactive T cells belong to different, perhaps nonoverlapping, compartments of the immune cell repertoire of mice chronically infected with *T. cruzi*. The failure of infected mice to trigger the suppressive mechanisms described here might be the primary immune defect leading to breakdown of self-tolerance and unopposed, perhaps tissue-damaging, autoimmunity in experimental Chagas' disease.

Chagas' disease is a chronic, debilitating endemic disease widespread in Central and South America. Its etiologic agent is the protozoan parasite *Trypanosoma cruzi*. One major cause of morbidity and mortality is the cardiac involvement seen in the late phase of the disease, when a chronic, inflammatory cardiomyopathy may lead to congestive heart failure. The lymphocytic infiltration in the absence of detectable parasites in the affected heart tissue, the passive transfer of myocarditis with sensitized T cells in experimental models (9), and the in vitro evidence of lymphocyte cytotoxicity to cardiac fibers in both experimental models (16) and human disease (20) strongly suggest the involvement of autoimmunity in the pathogenesis of chronic Chagas' disease myocarditis (reviewed in references 2, 18, and 19). However, the establishment of a true organ-specific autoimmune nature for Chagas' disease myocarditis awaits the demonstration of discrete organ-specific autoantigens. Data in the literature on defined autoantigenic targets in heart tissue at the level of cellular immunology are scarce (1). The involvement of myocardial contractile proteins in other states of heart-specific autoimmunity, for instance, rheumatic carditis (4) or the chronic myocarditis following coxsackievirus B3 infection (13), makes these proteins likely targets.

Here, we report our studies on the search for an autoantigenic target and the immunomodulation of antiself and antiparasite immune responses in experimental *T. cruzi* infection in the mouse.

MATERIALS AND METHODS

Mice. BALB/c and CBA/J mice (3 months old) were bred in the animal facilities of the Faculdade de Ciências da Saúde, Universidade de Brasília, and maintained on laboratory chow and filtered water ad libitum.

Parasites. Epimastigote culture forms maintained in LIT culture medium and trypomastigotes obtained by in vitro metacyclogenesis in TAUS medium (3) were from the Albuquerque stock of *T. cruzi* cloned at our laboratory. Blood-derived trypomastigotes of the Berenice stock maintained by repeated passage in mice were also used in some experiments.

Infection protocol. BALB/c mice were infected with *T. cruzi* as detailed previously (15). Briefly, mice were intraperitoneally injected with 10^7 washed Albuquerque epimastigotes; this was repeated twice at 2-week intervals. Thirty days later, mice received a trypomastigote challenge (10^4 washed TAUS medium metacyclic forms, intraperitoneally). CBA/J mice were infected with 200 blood-derived Berenice trypomastigotes intraperitoneally. Experiments were performed 120 to 240 days after trypomastigote injection in both cases.

Antigens and mitogens. Concanavalin A (three times crystallized) was purchased from Miles Scientific (Div. Miles Laboratories, Inc., Naperville, Ill.). Phytohemagglutinin was purchased from Wellcome Research Laboratories (Beckenham, England). Chicken egg ovalbumin was purchased from Sigma Chemical Co. (St. Louis, Mo.). Rabbit skeletal muscle actin was prepared as described previously (14), yelding a single sharp band migrating at 43 kilodaltons by sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoretic analysis. Rabbit skeletal muscle myosin was prepared as described by Margossian and Lowey (10), yelding a myosin preparation comparable in purity to rabbit skeletal muscle myosin purchased from Sigma when analyzed by sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis. Mouse cardiac muscle myosin was prepared as described previously (10) and used in some experiments. Contractile proteins were filter sterilized through Millipore membranes (0.2-μm pore size) and stored as a 50% glycerol solution at −20°C until used. Adequate controls showed that glycerol did not interfere with the proliferation assays in concentrations as high as 12.5% (data not shown). *T. cruzi* soluble antigen (SA) was prepared from washed epimasti-

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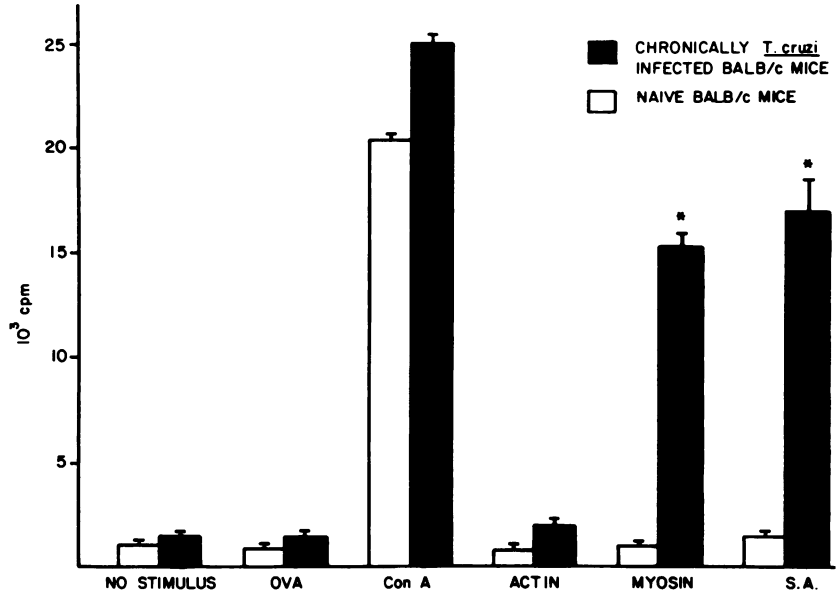

FIG. 1. Lymphocyte proliferative responses of BALB/c mice chronically infected with *T. cruzi*. OVA, Ovalbumin, 150 μg/ml; Con A, concanavalin A, 5 μg/ml; actin, 150 μg/ml; myosin, 150 μg/ml; SA, 100 μg/ml. Results are expressed as mean cpm ± standard deviation. Ten consistent repeat experiments were performed. *, $P < 0.01$ between naive and infected mice.

gote forms of the Ernestina stock cloned at our laboratory as described previously (20).

Reagents and monoclonal antibodies. [^{3}H]thymidine (25 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), protein A-Sepharose 4B and CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.), and frozen guinea pig complement (Cedarlane Laboratories) were used. Culture supernatants of the following hybridomas were used as a source of monoclonal antibodies against lymphocyte surface antigens: GK 1.5 (anti-L3T4) was kindly supplied by M. E. Dorf (Boston, Mass.); HO 13.4 (anti-Thy-1.2) and anti-Lyt-2 were gifts from D. Umetsu and R. DeKruyff (Palo Alto, Calif.).

Lymphocyte cultures and proliferation assay. Axillary, inguinal, and popliteal lymph nodes were teased into single-cell suspensions in ice-cold Earle balanced salt solution (pH 7.4) supplemented with 2% fetal calf serum (Flow Laboratories, Inc., McLean, Va.), washed, and suspended in RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin-streptomycin (Flow Laboratories), 12.5 mM HEPES (*N* - 2 - hydroxyethylpiperazine-*N'* - 2 - ethanesulfonic acid) buffer (pH 7.4), 0.2% $NaHCO_3$, and 10% fetal calf serum (Hyclone Laboratories, Tulsa, Okla.). The cells were cultured (10^5 per well) in 96-well flat-bottom microdilution culture plates (Falcon; Becton Dickinson Labware, Oxnard, Calif.) in the presence of various concentrations of the appropriate antigen. Positive (concanavalin A or phytohemagglutinin) and negative (unrelated foreign protein or culture medium alone) controls were included in all experiments. Cells were incubated for 72 h in a humidified, 37°C, 5% CO_2 atmosphere, pulsed with 0.5 μCi of [^{3}H]thymidine per well, and harvested 18 h later with a cell harvester. [^{3}H]thymidine incorporation was assessed by standard liquid scintillation techniques.

Generation of antisera against antigen-specific lymphocytes. After 72 h of incubation in vitro in the presence of myosin or SA, 10^6 lymph node cells from *T. cruzi*-infected or naive mice were injected into the tail veins of naive 3-month-old syngeneic BALB/c or CBA/J mice. Thirty days later, their sera were collected, filter sterilized, and stored at −20°C until used.

In some experiments, myosin-specific suppressive antiserum was passed through a protein-A-Sepharose 4B immunoaffinity column. The eluate was collected and used as a source of myosin-suppressive immunoglobulin G.

Assay of inhibition of proliferation by antiserum. The assay to determine the inhibition of proliferation by antiserum was performed by supplementing lymphocyte cultures as described above with 0.5% of either antiserum, and the percent inhibition was determined in comparison with reference wells supplemented with naive cell antiserum. In some experiments, sera from mice chronically infected with *T. cruzi* were also assayed for inhibition. Percent inhibition was calculated according to the following formula: % inhibition = [(reference well cpm above background − inhibitory antiserum cpm above background)/reference well cpm above background] × 100 where cpm is counts per minute.

Complement-dependent antibody cytotoxicity. Lymph node cells (10^6/ml) were incubated for 1 h with the appropriate antibodies plus guinea pig complement at 37°C in the absence of antigen, washed, and subsequently incubated with antigen or mitogen for proliferation assays; controls lacking antibody were always included.

Statistical analysis. All experiments were performed as triplicate wells. The significance of the difference between means ± standard deviations was determined by Student's *t* test.

RESULTS

Antigen-induced proliferation of lymphocytes from infected mice. We found that lymphocytes from BALB/c mice chronically infected with *T. cruzi* showed significant proliferative responses to myosin and SA but not to actin in any of the concentrations tested (50 to 250 μg/ml). Lymphocytes from naive mice did not proliferate against any of the antigens tested (Fig. 1). To exclude the possibility that myosin reactivity could be a result of sensitization by heart antigen

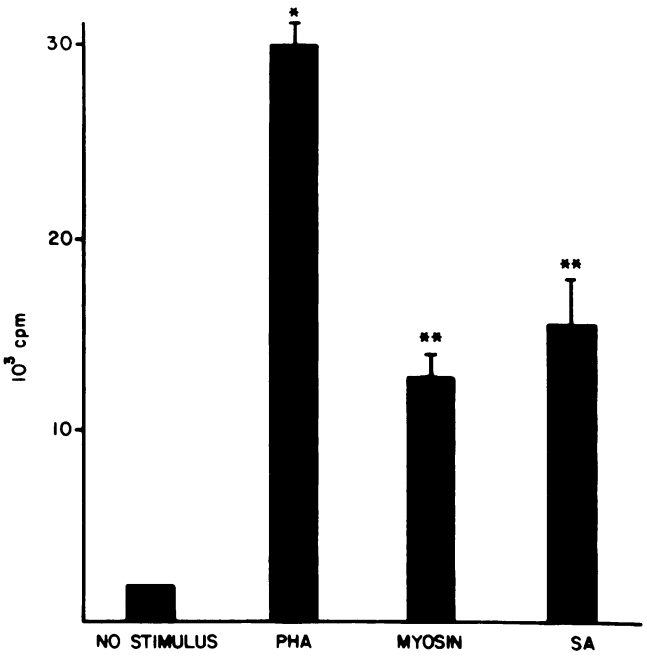

FIG. 2. Lymphocyte proliferative responses of CBA/J mice chronically infected with *T. cruzi*. PHA, Phytohemagglutinin, 10 μg/ml. Other substances, See legend to Fig. 1. Results are expressed as mean cpm ± standard deviation. Five consistent repeat experiments were performed. *, $P < 0.01$ between stimulated and background wells. **, $P < 0.05$ between stimulated and background wells.

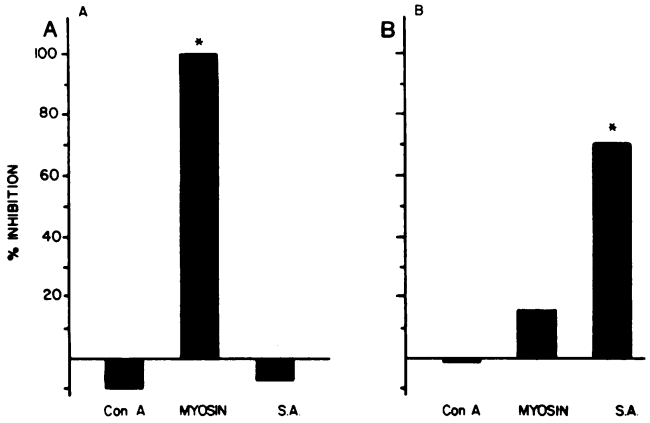

FIG. 3. Effect of antisera against myosin- or SA-responsive cells on lymphocyte proliferative responses of BALB/c mice chronically infected with *T. cruzi*. For the concentrations of stimulating substances, see the legend to Fig. 1. (A) Antiserum against BALB/c myosin-responsive cells. (B) Antiserum against BALB/c SA-responsive cells. Results are expressed as percent inhibition of [^{3}H]thymidine incorporation. Background, 120 ± 22 cpm; maximal proliferation to myosin, 10,747 ± 1,020 cpm; maximal proliferation to SA, 2,900 ± 190 cpm. Ten consistent repeat experiments were performed. *, $P < 0.01$ between experimental and control.

carried over by LIT medium epimastigotes or the result of a particular combination of mouse strain and *T. cruzi* stock, we assayed lymph node cells from CBA/J mice infected intraperitoneally in a single injection with 200 blood-derived Berenice stock trypomastigotes. Again, myosin and SA significantly stimulated the proliferation of lymphocytes from chronically infected CBA/J mice (Fig. 2).

Owing to the scarcity of material and the low yield of purified material, a limited number of assays were performed with murine cardiac myosin. All four BALB/c mice chronically infected with *T. cruzi* tested showed significant lymphocyte proliferative responses to autologous cardiac myosin (data not shown). The proliferative response to myosin was abolished by pretreatment with monoclonal anti-Thy-1.2 or anti-L3T4 antibody plus complement but not by monoclonal anti-Lyt-2 antibody plus complement, thus indicating that our assays are indeed measuring helper-inducer CD4$^+$ T-cell proliferation (data not shown).

Antigen-specific immunomodulation of myosin of SA proliferative responses. In a second set of experiments, we observed the effect of anti-immune cell antisera on the antigen- or mitogen-driven proliferation of lymphocytes from *T. cruzi*-infected mice. In these experiments, we supplemented the culture medium with 0.5% serum from either (i) mice injected with lymph node cells from syngeneic naive mice or (ii) mice injected with lymph node cells from syngeneic *T. cruzi*-infected mice, after the lymph node cells were subjected to 72 h of culture in vitro in the presence of the appropriate antigen (myosin or SA). Antiserum against myosin-responsive BALB/c cells completely inhibited proliferation of lymphocytes from *T. cruzi*-infected BALB/c mice in response to myosin but not in response to SA or concanavalin A (Fig. 3A). Antiserum against SA-responsive BALB/c cells significantly, but not completely, inhibited proliferation of lymphocytes from *T. cruzi*-infected BALB/c mice in response to SA with no significant inhibition of the myosin or concanavalin A responses (Fig. 3B). Interestingly, the inhibition of myosin responses by myosin-suppressive antisera was invariably more powerful than the inhibition of SA responses by SA-suppressive antisera; 50% inhibition of proliferative responses titrated fourfold higher for myosin than for SA in the respective antisera (data not shown). When we assayed antisera raised in CBA/J mice against CBA/J myosin-responsive cells, we also observed complete inhibition of myosin-specific lymphocyte proliferation of syngeneic *T. cruzi*-infected mice (Fig. 4).

We next assayed the complement-dependent lymphocytotoxic activity of the BALB/c anti-myosin-responsive-cell antiserum. Preincubation of BALB/c cells with the immunoglobulin G fraction (50 μg/ml) of myosin-suppressive antiserum plus complement caused some degree (ca. 60%) of inhibition of myosin-specific proliferation (4,155 ± 248 cpm versus 10,150 ± 1,233 cpm) with no effect on phytohemagglutinin-specific proliferation (21,135 ± 1,587 cpm versus 19,934 ± 2,130 cpm) of lymphocytes from *T. cruzi*-infected mice.

In an attempt to determine whether antigen-specific serum suppressive factors are an operative immunoregulatory mechanism in chronic murine *T. cruzi* infections, we assessed the effect of sera from *T. cruzi*-infected BALB/c mice on syngeneic lymphocyte proliferation against myosin, SA, or phytohemagglutinin. Sera from chronically infected mice showed no significant suppressive activity (Fig. 5).

Genetic restriction of serum suppressive factor activity. Searching for an element of genetic restriction in the interaction of the suppressive factors with antigen-responsive cells, we studied the effect of antiserum from a given strain on homologous antigen-specific proliferative responses of an allogeneic strain of mice. BALB/c anti-myosin-responsive-cell antiserum had no effect on CBA/J myosin-specific responses. Conversely, CBA/J antiserum had no effect on BALB/c myosin-specific responses (Fig. 6). These data also

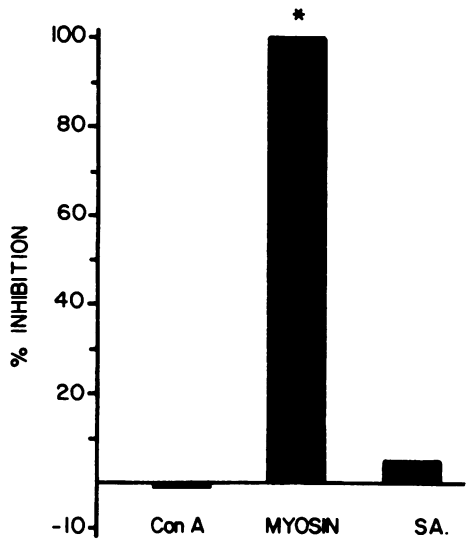

FIG. 4. Effect of antiserum against myosin-responsive cells on lymphocyte proliferative responses of CBA/J mice chronically infected with *T. cruzi*. For concentrations of stimulants, see the legend to Fig. 1. Results are expressed as percent inhibition of [^{3}H]thymidine incorporation. Background incorporation, 3,150 ± 150 cpm; maximal proliferation to myosin, 12,580 ± 1,760 cpm. Four consistent repeat experiments were performed. *, $P < 0.01$ between experimental and control. Con A, Concanavalin A.

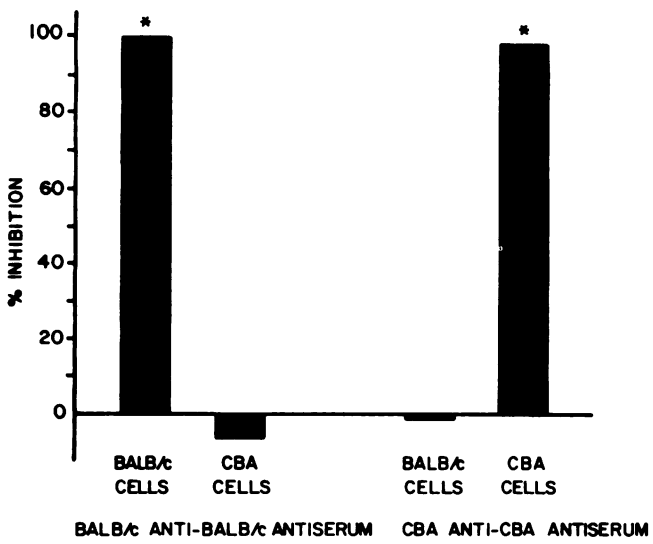

FIG. 6. Genetic restriction of myosin-specific serum suppressive factors. Results are expressed as percent inhibition of maximal proliferation to myosin. Background, 1,170 ± 153 cpm; maximal proliferation to myosin, 12,580 ± 1,760 cpm. *, $P < 0.01$ between experimental and control.

rule out the possibility that the inhibitory activity of the antisera is due to antigen-binding antibodies, since antimyosin antibodies, if present in the antisera, would bind free myosin irrespective of the genetic background of the assayed lymphocytes, thus readily inhibiting allogeneic lymphocyte proliferative responses to myosin. That anti-myosin-responsive-cell antiserum lacks any detectable myosin-binding activity was indicated by the inability of a CNBr-activated Sepharose 4B-coupled affinity column with myosin suppressive antiserum to bind any amount of the myosin applied to it (data not shown).

DISCUSSION

The results showed that BALB/c and CBA/J mice chronically infected with *T. cruzi* have CD4$^+$ T lymphocytes

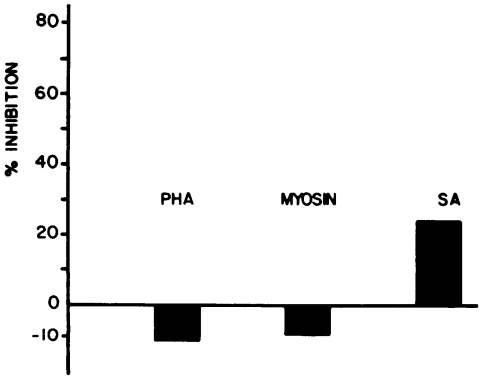

FIG. 5. Effect of pooled sera from BALB/c mice chronically infected with *T. cruzi* on lymphocyte proliferative responses of BALB/c mice chronically infected with *T. cruzi*. Sera from infected mice were assayed for inhibitory activity. For concentrations of stimulants, see the legends to Fig. 1 and 2. Results are expressed as percent inhibition of maximal proliferation. Background incorporation, 1,155 ± 523 cpm; there is no statistically significant suppression. PHA, Phytohemagglutinin.

responsive to myosin. This reactivity seems to be associated with the natural history of chronic *T. cruzi* infection in the mouse, rather than being an artifact resulting from LIT culture medium antigen carry-over or biased choice of a mouse strain and *T. cruzi* stock. We do not know as yet whether this reactivity is (i) a consequence of cross-reactivity between *T. cruzi* antigens and myosin or (ii) an autoimmune phenomenon dependent only on the exposure of previously sequestered antigens by damaged heart cells. Although we could neither exclude nor confirm the existence of cross-reactivity between *T. cruzi* antigens and myosin, we were able to modulate the autoimmune response apparently without interfering significantly with the immune response against *T. cruzi*. It is possible to induce in naive mice serum factors that specifically inhibit in vitro different compartments of the immune repertoire associated with chronic *T. cruzi* infection of the mouse.

The development of down-regulating suppressor mechanisms is essential for the homeostatic control of the course and size of specific immune responses and for the maintenance of immunological tolerance to self-constituents (5). Soluble antigen-specific suppressor factors present in serum might be (i) antigen-directed, nonimmunoglobulin, soluble suppressor-T-cell-derived factors found in the sera of mice hyperimmune to a given antigen (6, 7); (ii) T-cell antigen receptor-directed anti-idiotype–anticlonotype-like antibodies (8, 17); or (iii) antigen-specific antibody or immune complex in antibody excess (21). The latter possibility is virtually ruled out in our system, as detailed in the Results section. The finding that antiserum plus complement pretreatment partially inhibits antigen-specific proliferation (see above) suggests that the inhibitory activity lies partially in complement-fixing antibodies directed at clonally expressed antigen-recognition structures on the T-cell surface; but other molecules (non-complement-fixing antibodies and/or soluble suppressor-T-cell-derived factors) would be necessary to account for the complete inhibition seen in the other experiments (Fig. 3, 4, and 6). Further supporting suppressor factor heterogeneity in our system, preliminary results

show that antigen-specific serum suppressive activity can be recovered in both the immunoglobulin G and non-immunoglobulin G fractions (L. V. Rizzo, E. Cunha-Neto, and A. Teixeira, unpublished observations).

Mice chronically infected with *T. cruzi*, however, are themselves unable to trigger such antigen-specific suppressive mechanisms, despite the continued in vivo exposure of their immune systems to expanded numbers of myosin-reactive and SA-reactive T cells. Failure to trigger such suppressive mechanisms might be the primary immune defect leading to breakdown of tolerance to myosin and unopposed, perhaps tissue-damaging, autoimmunity in chronic *T. cruzi* infection in the mouse.

It is also possible that the defect in antigen-specific suppressive mechanisms is not restricted to the observed myosin and SA responses but is rather a generalized defect in tolerance induction mechanisms associated with chronic *T. cruzi* infection. Extensive autoantibody formation has been described in both acute and chronic phases of experimental and human *T. cruzi* infection. In the acute phase, it has been associated with polyclonal B- and T-cell activation (11) and might persist in the chronic phase as a consequence of such a generalized defect in immune mechanisms of self-tolerance.

Although the pathogenetic role of myosin-responsive $CD4^+$ T cells in chronic *T. cruzi* infection myocarditis has not been determined, myosin might still be a major autoantigen in this setting, despite being an intracellular component. The heavy chain of cardiac myosin was found to be the major autoantigen in coxsackievirus B3 postinfectious autoimmune myocarditis (13). In addition, genetically predisposed mice develop myocarditis following immunization with cardiac myosin in complete Freund adjuvant (12). If myosin autoantigenicity proves to be important in the pathogenesis of chronic Chagas' disease myocarditis, antigen-specific immunomodulation might be a means for controlling the progression of autoimmune heart lesions. Further experiments are necessary to determine the pathogenetic role of myosin-responsive T cells and to study the in vivo feasibility of antigen-specific immunomodulation.

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