

Anti-CD4 Abrogates Rejection and Reestablishes Long-Term Tolerance to Syngeneic Newborn Hearts Grafted in Mice Chronically Infected with *Trypanosoma cruzi*

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Summary

The contribution of autoimmunity in the genesis of chronic Chagas' heart pathology is not clear. In the present study, we show that: (a) BALB/c mice chronically infected with *Trypanosoma cruzi* reject syngeneic newborn hearts; (b) in vivo treatment with anti-CD4 but not anti-CD8 monoclonal antibodies (mAbs) abrogates rejection; (c) CD4⁺ T cells from chronically infected mice proliferate in vitro to syngeneic myocardium antigens and induce heart graft destruction when injected in situ; (d) anti-CD4 treatment of chronically infected mice establishes long-term tolerance to syngeneic heart grafts; and (e) the state of tolerance is related to in vitro and in vivo unresponsiveness of the CD4⁺ T cells. These findings allow us to suggest that autoimmunity is the major mechanism implicated in the rejection of syngeneic heart tissues grafted into the pinna of the ear of mice chronically infected with *T. cruzi*. The similarity of the lesions to those found in humans suggests that autoimmunity is involved in the pathogenesis of chagasic cardiomyopathy in humans. Moreover, this could imply therapeutic strategies by reestablishing long-term tissue-specific tolerance with anti-CD4 mAb treatment, mediating anergy, or deleting the responder CD4⁺ T cells to heart tissue antigens.

Chagas' disease (American trypanosomiasis) is caused by transmission of the protozoan *Trypanosoma cruzi* by a *Triatoma* bug. It is one of the leading causes of death in many countries of Latin America. The disease is characterized by three phases: acute, indeterminate, and chronic (1). The heart is the most severely and frequently affected organ. The degree of the cardiac involvement during the acute phase varies from mild to severe. The acute heart disease, which courses with parasitemia, is characterized histopathologically by foci of myocytolytic necrosis and an impressive mononuclear inflammatory infiltrate directly related to tissular parasitism. The indeterminate phase is usually of long duration (up to 10–30 yr) and is characterized by the absence of histopathological lesions. Progression from the acute to chronic form of Chagas' disease coincides with clearance of parasites from blood stream and tissues. In the chronic phase variable degrees of cardiac hypertrophy and dilatation occur with or without thinning of the apical region (apical aneurism). Foci of myocardial necrosis and degeneration are present with an inflam-

matory infiltrate predominantly composed of mononuclear cells and interstitial fibrosis. Myofibers containing parasites are rarely observed.

The participation of autoimmune mechanisms in the genesis of the chronic myocarditis of Chagas' disease has been suggested and is still a controversial matter (2). Organ-specific autoimmunity is often related to the outcome of many parasitic infections caused by virus, bacteria, and parasites (3–5). Thus, it would be of importance to find out how infection triggers autoimmunity and how the resultant autoimmunity contributes to the disease. This would improve therapeutical tools, thus contributing to the better understanding of the breaking of tissue-specific tolerance.

The present study was undertaken to investigate the autoreactivity against syngeneic heart tissue in vivo by grafting newborn hearts into the pinna of the ear of mice chronically infected with *T. cruzi*. The results demonstrate that syngeneic heart tissues once grafted in normal recipients are accepted, while they are invariably rejected in mice chronically infected

with *T. cruzi*. The rejection is mediated by CD4⁺-bearing T lymphocytes and a state of tolerance can be reestablished by in vivo treatment of chronically infected mice with anti-CD4 mAbs before transplantation.

Materials and Methods

Mice. BALB/c and C57BL/6 mice were bred and maintained under standard conditions in the animal colony at the Department of Immunology, University of São Paulo, Ribeirão Preto, Brazil.

Parasites. The Y and the Colombian strains of *T. cruzi* were used. Blood-derived trypomastigote forms were used in all experiments. Mice were infected subcutaneously with 10³ parasites. The PF strain is nonpathogenic, noninfective, and has been shown to protect mice challenged with virulent *T. cruzi* strains (6). The PF strain is essentially represented by epimastigote forms and is maintained in vitro in Warren's medium. In all experiments in which this strain was used, 2 × 10⁷ parasites/animal/wk were injected during four consecutive weeks.

Chronic Infection. Mice infected with the Y strain of *T. cruzi* were obtained by selecting those animals that survived the acute phase. However, mice infected with the Colombian strain of *T. cruzi* had to be treated during the acute infection with *n*-benzyl-2-nitro-1-amidazolacetamide (Rochagan, Roche Lab., Brazil), orally administered, in a dose of 100 mg/kg/d during 10 d, beginning on the 10th day after infection. 5–6 mo after challenge, mice were considered in the chronic phase, with no parasites in the blood. Control mice were injected with saline instead of the PF, Y, and Colombian strains of *T. cruzi*.

Heart Transplantation Technique. We have modified the method originally described by Fulmer et al. (7). A full description appears elsewhere (8). Briefly, newborn mice were anesthetized by hypothermia, and the heart was immediately excised, rinsed in cold sterile saline (pH 7.2), and implanted into the dorsal base of the pinna of the ear. The implant was subcutaneous, and the surgical incision was gently closed with the aid of forceps. In the present study grafts were sex matched, usually using male recipients. In addition, in many experiments a given recipient had one graft in each ear, a procedure that allowed us to have in the same animal a control and an experimental heart transplant. The majority of grafts in normal syngeneic mice start to present contractile activity after 7–10 d posttransplantation. Rejection was defined macroscopically as the disappearance of the grafted tissue. This corresponded microscopically to perivascular, interstitial, and perimyocytic mixed inflammatory infiltrate (lymphomononuclear cells ± neutrophils) with myocyte necrosis.

Monoclonal Antibodies. Anti-L3T4 (GK1.5) (9), anti-Lyt-2 (53.6.75) (10), and anti-Thy1.2 (HO13.4 from American Type Culture Collection, Rockville, MD) mAbs were obtained in the form of ascitic fluid. Gamma globulin fraction of the ascitic fluid was semi-purified by precipitation at 50% saturation with ammonium sulfate and used for in vivo administration.

In Vivo T Cell Depletion. In vivo depletion of T cell subsets was achieved by intravenous injection of the corresponding mAb at the dose of 200 µg/mouse, three times per week, starting 1 wk before any other manipulation. The success of T cell subset depletion was ascertained by indirect immunofluorescence microscopy, using the same mAb applied for in vivo treatment and FITC-conjugated mouse anti-rat Ig antibodies (Becton Dickinson & Co., Mountain View, CA) with appropriate dilutions. Depletion was invariably >95% after 2 wk of treatment (usually 200 spleen or

lymph node cells were analyzed). Treatments were maintained for 2 wk after grafting.

Preparation of Non-T Cells. A mixture of spleen and lymph node cells were freed from red blood cells by a brief incubation in 0.84% ammonium chloride, and the remaining viable cells (10⁶/ml) were then incubated with appropriate dilution of a cocktail of anti-L3T4, anti-Lyt-2, and anti-Thy1.2 mAbs at 4°C for 45 min. Cells were washed in BSS and incubated at 37°C for 45 min with baby rabbit complement at a final dilution of 1:10. Such a treatment completely abrogated the capacity of these cells to proliferate to Con A in vitro.

Preparation of T Cells and T Cell Subsets. T cells were obtained from spleens and lymph nodes. Cells were passed over a Sephadex G10 column in order to remove macrophages. The effluent cells were then passed over a nylon wool column, and 20–40% of the load cell number were eluted after a 1-h incubation at 37°C. The nonadherent cells were panned on plastic petri dishes coated with purified anti-mouse Fab antibody. T cells were obtained by 1 h of incubation at 4°C. T cell subsets were obtained by treatment of total T cells either with anti-CD4 or anti-CD8 mAbs and rabbit complement at the appropriated dilutions as described above. Dead cells were further removed by passage over Ficoll gradient. Usually this procedure gave >95% of purity yields as judge by indirect immunofluorescence microscopy, as described above.

In Vitro Cell Culturing. The responding total T cells, CD4 and CD8 subsets, or non T lymphocytes were prepared as above and resuspended in complete medium (RPMI 1640 supplemented with 10% FCS, 5 × 10⁻⁵ M 2-ME, sodium pyruvate, glutamine, nonessential amino acids, and 5 mM hepes buffer) at a concentration of 2 × 10⁵ per culture well together with 2 × 10⁵ irradiated (3,000 rad) spleen filler cells with or without different concentrations of heart antigens in a total volume of 0.2 ml. Each culture was performed in triplicate in flat-bottomed microculture plates and maintained in a humidified atmosphere at 5% CO₂ at 37°C. The cultures were harvested at 96 h after an 8-h pulse with 20 KBq/well of [³H]TdR. Results were calculated from uptake of [³H]TdR and expressed as the mean uptake in cpm of three independent experiments. Myocardium antigens were prepared as described previously (11).

Histological Examination. The ears containing the heart grafts were fixed in Bouin's solution. After fixation, all ears were sectioned transversally across the graft at 2-mm intervals, embedded in paraffin, sectioned, stained with hematoxylin and eosin or Masson's trichrome, and examined under the light microscope. For electron microscopic study, small blocks of tissue were fixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated in acetone, and embedded in araldite. Semithin sections stained with toluidine blue were examined under the light microscope, and a suitable area was selected for preparation of ultrathin sections, which were double stained with uranyl acetate and lead citrate, and examined in an electron microscope at 80 kV (EM 109; Carl Zeiss, Oberkochen, Germany).

To visualize permeability alteration of the myocyte sarcolemma, small pieces of myocardium were immersed in a 1.2% glutaraldehyde (0.3% ruthenium red mixture in 0.1 M cacodylate buffer, postfixed in a 2% osmium tetroxide) 0.3% ruthenium red mixture in 0.1 M cacodylate buffer, dehydrated, and embedded in Epon (12–14). Unstained ultrathin sections selected on the basis of a light microscope were examined in the electron microscope. Since this technique is subjected to artifacts because ruthenium red is introduced during the process of immersion fixation with glutaraldehyde, random sampling and blind evaluation were performed as a control for these effects.

Results

Syngeneic Heart Graft Rejection in Chronically Infected Mice.

Newborn hearts grafted in syngeneic recipients showed grossly visible contractile activity, usually within 2 wk after surgical procedure. The heterotopic hearts persisted up to 100 d. On the contrary, grafting of syngeneic newborn hearts in mice already developing chronic Chagas' disease was consistently rejected and completely absorbed at most by the 20th day posttransplantation. Actually, 70% of the grafts were acutely rejected and disappeared in 2 wk. Similar results were obtained regardless of which *T. cruzi* strain (Y or Colombian) was used to generate chronic infection. Rejection of allogeneic hearts by normal recipients was delayed in relation to infected groups (Fig. 1). Furthermore, it should be pointed out that heart contractile activity was never detected in the organs grafted into chronically infected animals. Syngeneic grafts were not rejected in either normal or PF strain-hyperimmunized mice (Fig. 1). In fact, these grafts persist for long periods (up to 6 mo) without significant changes.

Morphological Findings. Newborn mouse syngeneic hearts transplanted into the ear of normal recipients became vascularized from the auricular artery at the base of the ear. The central cavities of the grafts were filled with adipose tissue (fatty infiltration). Elongated myocardial cells with abundant myofibrils were seen. In most of the areas, myocytes were arranged in parallel bundles. The nuclei were elongated. No

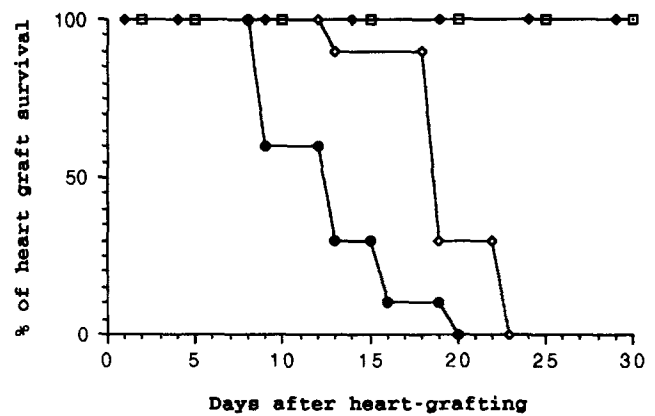


Figure 1. Engraftment or rejection of syngeneic newborn hearts in different groups of mice. Normal BALB/c mice (□), PF strain-hyperimmunized BALB/c mice (◆), or BALB/c mice chronically infected with the Colombian strain of *T. cruzi* (●) were grafted with syngeneic newborn hearts in both ears. A group of normal BALB/c mice was grafted with C57BL/6 newborn hearts as an allogeneic control (◇). Groups were of 10 animals each.

mitotic figures were seen. The interstitial space was diffusely widened, particularly around vessels, due to collagen fibrils, fibroblasts, and areas of mononuclear cells (Fig. 2). These hearts showed a mature ultrastructural appearance with par-

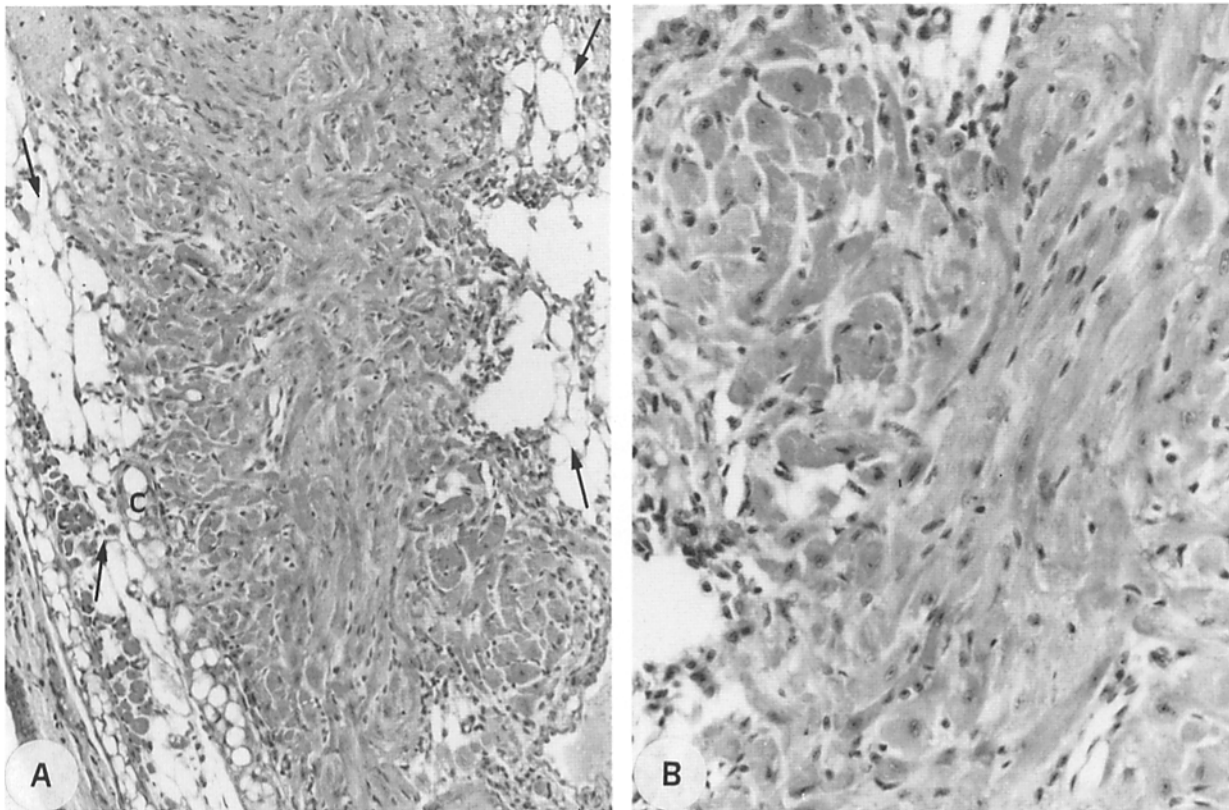


Figure 2. Newborn myocardium 2 mo after transplantation into the ear of syngeneic control mice. In most of the areas, the myocytes are arranged in parallel bundles. The nuclei are elongated. No mitotic figures are seen. The interstitial space is diffusely widened due to mild interstitial fibrosis. Fatty infiltration (arrows) is noted at the ventricular cavity and the periphery of the graft. s, skin; c, ear cartilage. Hematoxylin and eosin; A, $\times 170$; B, $\times 340$.

allel bundles of myofibrils alternating with rows of mitochondria and differentiated intercalated discs. The findings in the myocardium stained with ruthenium red were similar to those previously described (12-14). None of the cardiac cells had been penetrated by the tracer, and the density was confined to the extracellular space, particularly the pericellular material and collagen bundles.

The syngeneic heart grafted into the ear of chronically infected mice showed a marked inflammatory infiltrate composed of lymphomononuclear cells associated with multiple

and diffuse foci of myocytolytic necrosis (Fig. 3), quite similar to the pattern of allogeneic heart grafts (Fig. 4). When the relationship between mononuclear cells and cardiocytes was examined by electron microscope, a close proximity between the two cells could be seen. The sarcolemma of the myocardial cells had a wavy outline. The basal lamina was loosely adherent, with a relatively low electron density and finely granular appearance. The mononuclear cells commonly showed convolution of their surface in the vicinity of the myofibrils. Numerous cytoplasmic projections come into close apposi-

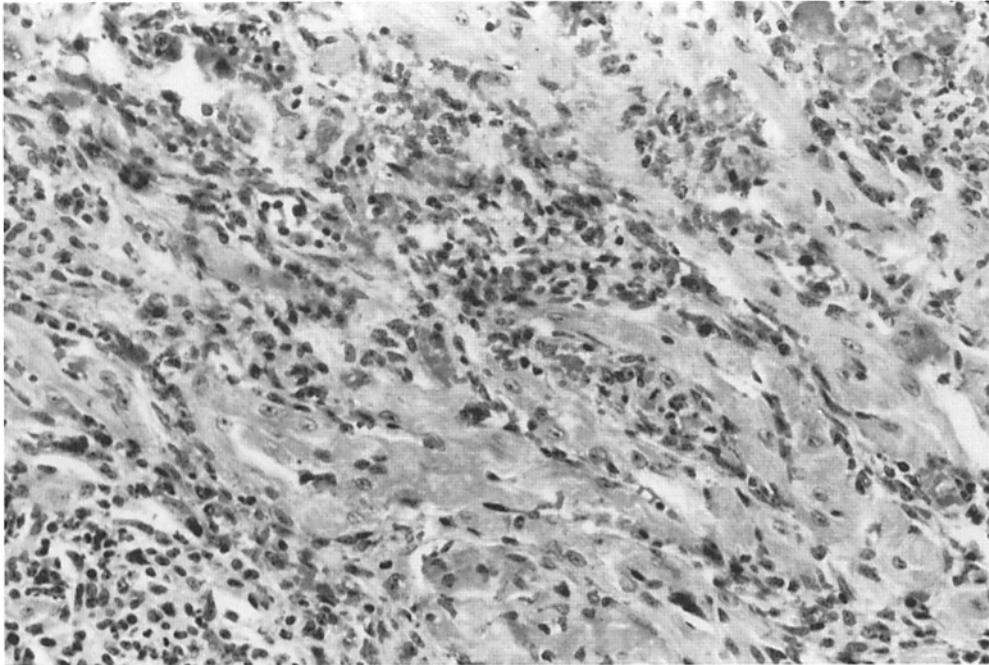


Figure 3. Syngeneic newborn myocardium 13 d after implantation in BALB/c mouse chronically infected with *T. cruzi*. Focal myocytolytic necrosis and extensive interstitial lymphomononuclear infiltrate and interstitial fibrosis. Hematoxylin and eosin; $\times 410$.

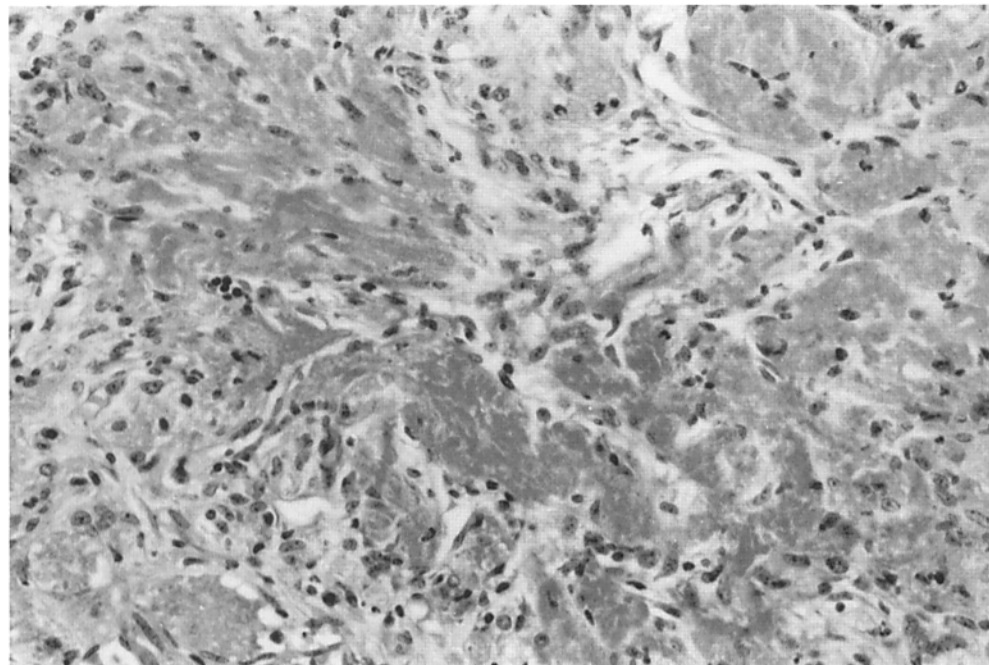


Figure 4. C57BL/6 newborn myocardium 13 d after implantation in normal BALB/c mouse. Diffuse lymphomononuclear infiltrate associated with severe interstitial replacement fibrosis. Hematoxylin and eosin; $\times 410$.

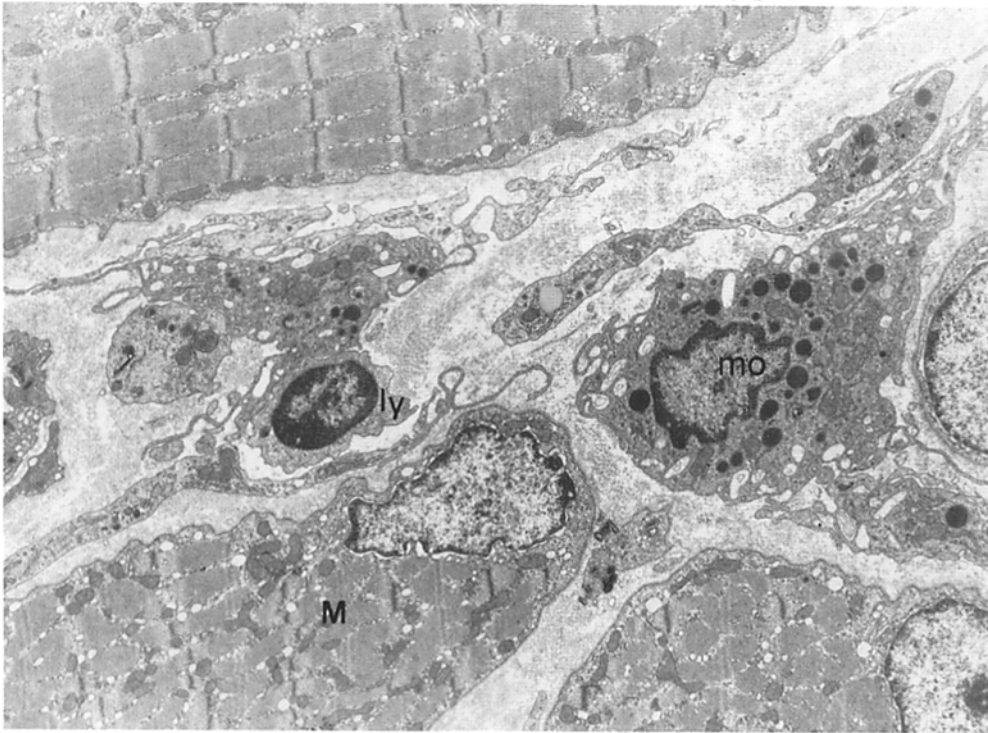


Figure 5. Electron micrograph of BALB/c newborn myocardium 14 d after implantation in syngeneic mouse chronically infected with *T. cruzi*. The interstitial space is widened by cellular infiltration of macrophages (*mo*), activated lymphocytes (*ly*), and collagen fibers in the vicinity of myocytes (*M*). Uranyl acetate and lead citrate; $\times 6,100$.

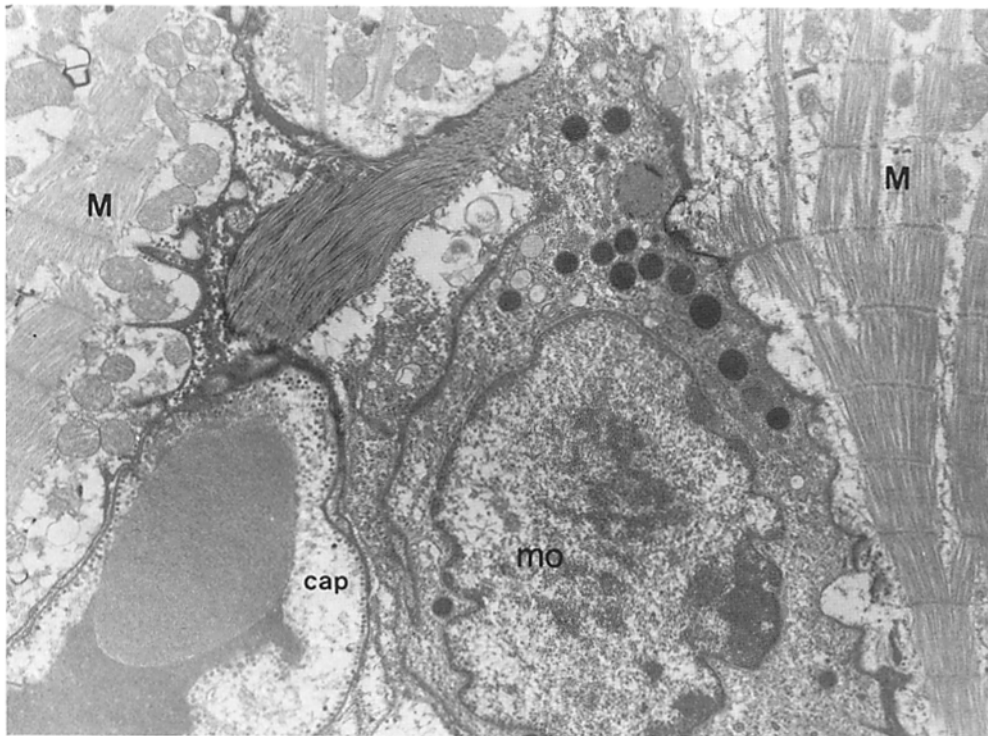


Figure 6. Electron micrograph of BALB/c newborn myocardium 16 d after implantation in syngeneic mouse chronically infected with *T. cruzi*. The cytoplasmic components of a macrophage (*mo*) adjacent to myocytes (*M*) have taken up ruthenium red as evidenced by their density. The myofibril at the right of the figure shows especially intense ruthenium red staining of myofibrils, mitochondria, and T tubules, predominantly in the subsarcolemmal zone close to the macrophage. *cap*, capillary. Unstained section; $\times 7,300$.

tion with the myocytes (Fig. 5). The ruthenium red staining in syngeneic myocardium transplanted into chronically infected mice demonstrated, similarly to the allogeneic transplanted hearts, that the cytoplasmic components of myocytes were labeled at the site of macrophage contact, denoting alteration of the plasma membrane permeability (Fig. 6).

Differential Effects of In Vivo T Cell Subset Monoclonal Antibody Depletion on the Rejection of Syngeneic Heart Grafts. We have analyzed the cell population that was necessary for the rejection of syngeneic heart grafts in chronically infected mice by depletion of CD4⁺ or CD8⁺ single-positive T lymphocytes with mAbs. Fig. 7 shows that rejection of newborn

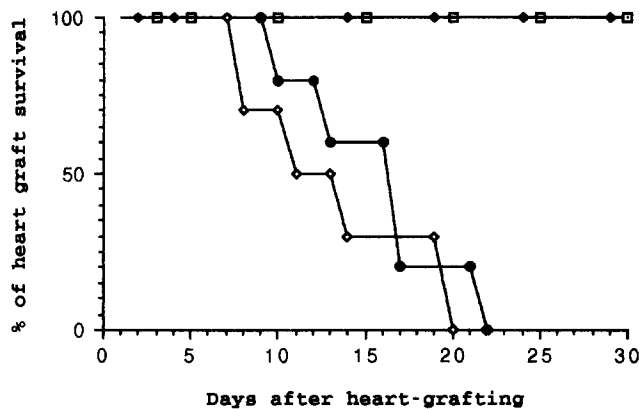


Figure 7. Engraftment of syngeneic newborn hearts in chronically infected mice receiving anti-CD4 mAb treatment. Normal BALB/c mice (□), BALB/c mice chronically infected with Colombian strain of *T. cruzi* receiving anti-CD8 mAb therapy (◇), or BALB/c mice chronically infected with the Colombian strain of *T. cruzi* receiving anti-CD4 mAb therapy (◆), were grafted with syngeneic newborn hearts in both ears. A group of normal BALB/c mice was grafted with C57BL/6 newborn hearts as an allogeneic control (●). Groups were of 10 animals each.

heart tissue grafted into chronically infected mice could be prevented by treating the animals with anti-CD4 mAb 2 wk before transplantation. In contrast, all mice receiving anti-CD8 treatment rejected the heart grafts within 20 d after transplantation.

Reestablishment of Heart Tissue-specific Tolerance in Chronically Infected T. cruzi Mice by In Vivo Treatment with Anti-CD4 mAb Chronically infected mice that received in vivo administration of anti-CD4 antibodies and heart grafts thereafter were not able to reject these transplants even after anti-CD4 mAb withdrawal and reconstitution of the CD4 T cell compartment. To better demonstrate this phenomenon, we treated a group of chronic infected mice with anti-CD4 mAb for 2 wk as described above, and after 2 mo posttreatment, we grafted these mice with newborn hearts. Table 1 shows that normal mice had accepted the majority of syngeneic grafts (19/20) over a period of 100 d postengraftment and do reject

Table 1. *In Vivo Pretreatment with Anti-CD4 mAb Reestablishes Heart Tissue Tolerance*

Experimental groups	Anti-CD4	Incidence of graft survival	
		Syngeneic	Allogeneic*
Normal	-	19/20 (100)	0/20 (22)
Normal	+	20/20 (100)	0/20 (23)
Chronically infected	-	0/20 (20)	0/20 (19)
Chronically infected	+	18/20 (100)	0/20 (23)

* Hearts from newborn C57BL/6 mice were grafted in BALB/c mice from the different experimental groups. Numbers shown in parentheses represent the total time of graft observation in days.

allografts in the maximum 22 d posttransplantation. Previous treatment with anti-CD4 mAb did not block allograft rejection. In contrast, chronically infected mice that received anti-CD4 mAb accepted 90% of syngeneic grafts (18/20) over a period of 100 d of observation, but are still able to reject full allogeneic heart transplants at most 23 d postengraftment. Histopathology of these mice have confirmed that no inflammatory reaction was present in the tolerant groups.

In Situ Injection of T Cells from Chronically Infected or Previously Anti-CD4-treated Mice into Heart Grafts. Thereafter, we investigated the ability of T cells and their subpopulations from normal, chronically infected, or *T. cruzi*-hyperimmunized mice to damage hearts grafted in normal recipients. When total T cells, CD4, or CD8 T lymphocyte subsets from normal or *T. cruzi*-hyperimmune mice were injected adjacently to hearts that have been grafted in naive syngeneic recipients, no significant alteration occurred. The grafts persisted up to 100 d, and contractile activity was present. In some groups the incidence of graft survival was <100%, here, the few reabsorbed transplants can be considered normal events, due to technical problems. In contrast, injection of total T cells or CD4⁺ T lymphocytes from chronically infected *T. cruzi* mice resulted in a complete destruction of the organs usually within 4 d after injection, and invariably 100% of the grafts were completely reabsorbed by 7 d postinjection. On the contrary, CD8⁺ T lymphocytes from the same donor group were not effective in inducing graft damage. We further injected into the grafts CD4⁺ T cells from chronically infected mice that had been previously treated with anti-CD4 mAb and naturally reconstituted thereafter, in order to check if such a reactivity would persist. We found that no graft destruction could be induced by these cells and grafts persisted for long periods (Table 2). Moreover, histopathology revealed a dramatic inflammatory reaction surrounding and within the grafts, when CD4 or total T cells from chronically infected mice were injected (Fig. 8).

In Vitro Proliferative Responses of T Lymphocytes from Chronically Infected T. cruzi Mice to Myocardium Antigens. Purified CD4⁺, CD8⁺, total T cells, or non-T lymphocytes from nontreated chronically infected mice, or chronically infected mice previously treated with anti-CD4 mAb and naturally repopulated thereafter, were cultured in the presence of syngeneic heart antigens and irradiated filler cells. Fig. 9 shows that purified CD4⁺ or total T lymphocytes from nontreated chronically infected mice are able to proliferate to myocardial antigens; also, the optimal concentration of the antigen was 20 μg/ml (not shown). CD8⁺ T cells did not respond to any concentration of myocardial antigens as well as the population where T cells were eliminated. T cells of chronically infected mice 2 mo posttreatment with anti-CD4 were not able to proliferate to myocardial antigens. Control cells either from normal or *T. cruzi*-immunized mice did not show any proliferative activity to heart antigens (not shown).

Discussion

The pathogenesis of chronic Chagas' heart disease remains incompletely understood. Different mechanisms have been

Table 2. Injection of Different Cell Populations into Heart Grafts

Donor groups	Cell population*	Incidence of graft survival	Period of observation
Normal	Total T cells	18/20	d 100
	CD4	20/20	100
	CD8	19/20	100
	Non-T cells	20/20	100
Hyperimmunized	Total T cells	19/20	100
	CD4	20/20	100
	CD8	18/20	100
	Non-T cells	17/20	100
Chronically infected	Total T cells	0/20	7
	CD4	0/20	7
	CD8	20/20	100
	Non-T cells	18/20	100
Anti-CD4-tolerized mice	Total T cells	20/20	100
	CD4	18/20	100
	CD8	19/20	100
	Non-T cells	19/20	100

* Purified cell populations from the different experimental groups were injected within the hearts grafted in normal recipients at a cell number of 5×10^4 in a total volume of $10 \mu\text{l}$. Hyperimmunized mice were inoculated four times in intervals of 7 d with 2×10^7 epimastigote forms of the PF strain of *T. cruzi* and challenged 30 d after the last inoculation with 10^3 trypomastigote forms of the Y or Colombian strains of *T. cruzi*.

proposed. The lesions could be due to continuing parasitic invasion of the heart, local release of toxins, or both. The low levels of parasitemia and the very rare findings of tissue parasitic pseudocysts are arguments against the hypothesis

that myocardial lesions are directly correlated with the presence of parasites. The chronic cardiomyopathy of Chagas' disease could be a neurogenic form of heart disease promoted by destruction of the parasymphathetic ganglion cells in the

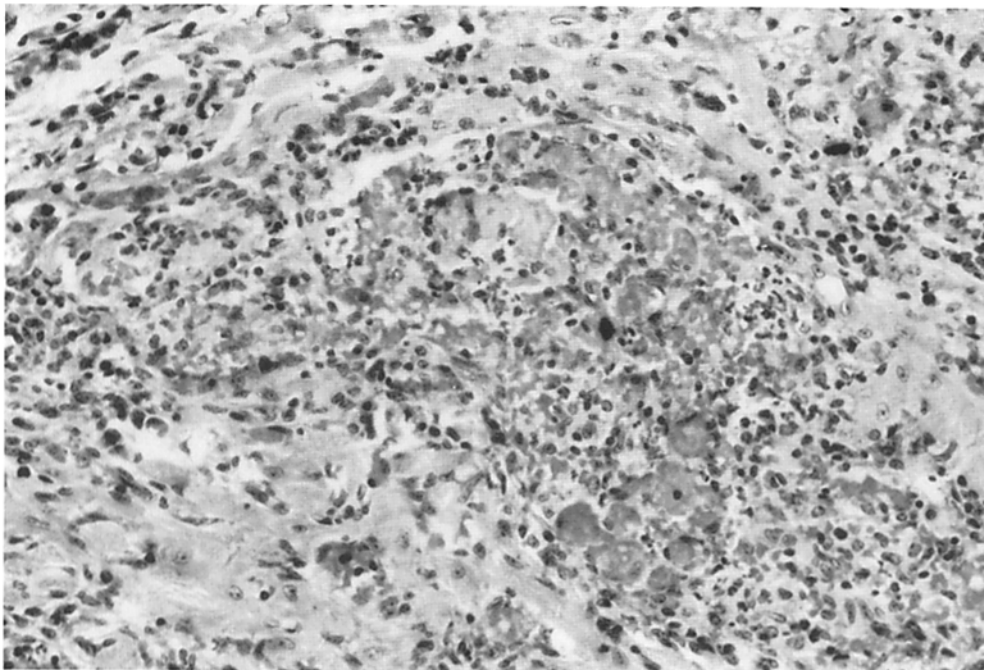


Figure 8. BALB/c newborn myocardium implanted in normal syngeneic mouse 4 d after injection of 5×10^4 purified CD4⁺ T cells from mice chronically infected with *T. cruzi*. Severe and diffuse lymphomononuclear infiltrates replace the myocardium. Extensive myocyte necrosis associated with mild fibrosis. Hematoxylin and eosin; $\times 410$.

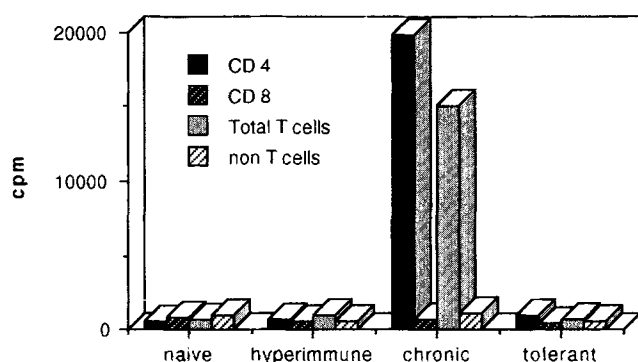


Figure 9. CD4⁺ T cells from chronically infected BALB/c mice, tolerized with anti-CD4, show unresponsiveness to myocardial antigens. Purified CD4⁺, CD8⁺, total T cells, or non-T cells from naive mice, mice hyperimmunized with PF strain of *T. cruzi*, mice chronically infected with the Colombian strain of *T. cruzi*, or mice that had been treated with anti-CD4 were cultured (2×10^5 /well) together with irradiated filler cells (2×10^5 /well) in the presence of heart antigens from syngeneic animals (a previous study showed that 20 μ g/ml was the optimal antigen concentration) for 4 d with [³H]thymidine added for the final 8 h. Data are expressed as the mean of triplicate wells in cpm. Data represent one of five similar experiments.

heart (15). Results of histologic studies have shown a reduction in the number of cardiac parasympathetic neurons proportional to the pathologic changes in the heart (15). These observations have been challenged by investigators who have found neither neuronal damage in the early stage of the disease nor a relationship between neuronal counting and the progression of the disease (16). Functional studies in humans have shown that patients may have alterations in the parasympathetic autonomic control of the heart without cardiovascular symptoms (17). In contrast, results of recent studies have demonstrated that myocardial damage may precede functional cardiac parasympathetic injury in patients with Chagas' disease (18). Recently, microvascular abnormalities have been postulated to play a significant role in the pathogenesis of chronic Chagas' heart disease (1, 6).

The participation of autoimmune mechanisms as the main cause of chagasic cardiomyopathy has been postulated (2, 19). An important goal is the establishment of an experimental model that could provide strong evidence in support of the hypothesis and allow a reliable readout of immune manipulations by which different sets of lymphocytes could be implicated in the generation of the disease.

In the present work we have clearly demonstrated that mice chronically infected with *T. cruzi* were able to reject syngeneic heart grafts. The rejection of syngeneic hearts grafted in chronically infected adult mice was faster when compared with the full allogeneic situation. This was in striking contrast to those organs grafted into normal or *T. cruzi*-hyperimmunized syngeneic recipients, which can persist for >6 mo. The histological study of heart tissue grafted into chagasic mice revealed a persistent and intense mononuclear inflammatory infiltrate, quite similar to the pattern obtained in the allogeneic conditions, thus indicating that cellular mechanisms are implicated.

A set of experiments were done to determine the phenotype of the cells involved in the phenomenon of syngeneic heart rejection. In vivo depletion of CD4⁺, but not CD8⁺, T cells abrogates rejection. Heart grafts were retained by chronically infected mice during the course of anti-CD4 mAb treatment. After 2 mo of stopping antibody injections, the CD4⁺ population returned to normal levels, as judged by indirect immunofluorescence (not shown). However, grafts were not rejected. Instead they persisted for >6 mo, indicating that the recipients became tolerant. Thereafter, to differentiate between authentic tolerance and graft adaptation due to loss of the acute inflammatory reaction just after transplantation, a group of chronically infected mice was treated with the same mAb for 2 wk, and 2 mo after withdrawal of the antibody and reconstitution of the CD4⁺ compartment, heart grafts were implanted. These grafts were not rejected, surviving for >6 mo, clearly showing that a state of tolerance had been reestablished. In contrast, allogeneic grafts were not accepted and have been rejected in the maximum of 22 d posttransplantation. Anti-CD4 mAb was successfully used in similar experiments dealing with induction of tolerance to islet allografts in the mouse, or allogeneic heart grafts in the rat (20, 21).

The role of CD4 molecules in T cell activation has been analyzed. However, there has been much controversy in this regard over the part played by the CD4 glycoprotein. Recently, it has been reported that when preceded by ligation of CD4, signaling through TCR- α/β results in mature T cell unresponsiveness in vitro, due to induction of activation-dependent cell death by apoptosis (22). In contrast, it has been observed that in vivo treatment with anti-CD4 mediates proliferative clonal anergy of V β 11 T cells during allotransplantation tolerance induction to pancreatic islets of Langerhans (23). The self reactivity of CD4⁺ T cells from chronically infected mice could also be shown by in vitro studies, where these lymphocytes proliferate in the presence of heart antigens plus irradiated filler cells. Non-T cell populations or CD8⁺ T lymphocytes failed to show any activity in this assay. Moreover, CD4⁺ T cells obtained from mice previously treated with anti-CD4 could not reject syngeneic heart grafts when injected in situ, thus indicating that tolerance after anti-CD4 treatment correlates with the disappearance of myocardial reactivities in vitro and in vivo. Our results do not allow further distinction between deletion or proliferative anergy as the basis for tolerance in this model, since in vitro unresponsiveness to myocardium antigens detected after in vivo anti-CD4 treatment could not be reverted by addition of IL-2-rich supernatants (not shown), and no molecular marker can be used, so far.

In vivo studies with CD4⁺ T cells from chronically infected mice have shown that these cells are able to mediate syngeneic heart graft destruction when injected in situ, whereas CD8⁺ or non-T cells were not effective. The bulk of results establishes that autoreactivity is restricted to the CD4⁺ T cell compartment, which is clearly different from allogeneic skin graft rejection, that has been attributed to both subsets of T lymphocytes (24, 25). This is in agreement with other experimental models of organ-specific autoimmune

diseases in which CD4⁺ T cells play a major role in the induction of tissular lesions (26–28). Importantly, this is also in keeping with the data showing that the majority of T cells infiltrating the heart in both acute and chronic phases of experimental *T. cruzi* infection actually correspond to CD4⁺ T cells (29). Besides, these cells have been reported to modulate antibody production (30), macrophage activation (31, 32), and peripheral nerve cell injury (33). One might still argue that the results obtained by injecting cells in situ was a consequence of previously in vivo activated CD4⁺ T lymphocytes, independently of their putative antiheart antigens specificity. Actually, this does not seem to be the case since CD4⁺ T cells taken from 15-d infected mice, which have been described to be already activated in vivo (34), did not lead to any lesion when injected within the heart grafts. Furthermore, using the in situ injections, we could demonstrate the presence of CD4⁺ T lymphocytes against heart tissues as soon as 25–30 d after initial infection, but not earlier (not shown). In addition, in vivo treatment with anti-IFN- γ mAb, one of the important lymphokines released by activated inflammatory CD4⁺ T cells (35), was not able to block the graft damage induced by these cells (not shown).

It is known that expression of CD4 correlates with the ability of the cell to recognize antigens bound to class II molecules (36, 37) and that CD4 binds to class II molecules coaggregates with the TCR/CD3 complex and aids the activation of T cells. However, since there is no formal demonstration that myocardial cells can express class II molecules on the membrane, it would be difficult to accept that the CD4⁺ T cell could account for the final effector cytotoxic cell activity. The ruthenium red staining suggests that the mononuclear cell with an activated macrophage appearance could be the final effector cell that destroys the cardiomyocytes. In this case, CD4⁺ T cells would recognize myocardial antigens together with class II expressed on resident tissue macrophages or dendritic cells, thus activating these CD4⁺ T cells with consequent production of interleukins such as

the early T lymphocyte activation 1 (Eta-1) protein (38). This could initiate an inflammatory process leading to the destruction of heart tissue.

One interesting question raised from these data refers to the triggering of those autoreactive CD4⁺ T lymphocytes. Molecular mimicry between heart and *T. cruzi* antigens could eventually be responsible for further stimulation of such a reactivity (39). However, *T. cruzi*-hyperimmunized mice were not able to reject syngeneic heart grafts, and CD4⁺ T cells from these mice could not destroy the grafts when injected in situ or proliferate in vitro in the presence of heart antigens. The acute infection with massive lesions of the target organ would be necessary to provoke the break of tissue-specific tolerance. For instance, during the acute phase of the disease, *T. cruzi* antigens would decorate the myocardium cells and augment tissue damage induced by the immune response against the parasite itself (40). Furthermore, polyclonal activation of T and B cells is an important component during the acute phase of *T. cruzi* infection. It has been suggested that such an activation could play an important role in the development of autoimmune disease *a posteriori* (34). So far, we favor the hypothesis that extensive tissue damage and immune dysfunction during the acute infection, rather than cross-reactivity between *T. cruzi* and self antigens, are absolutely necessary for the appearance of organ-specific autoimmunity during the chronic phase.

In conclusion, the findings in the present study allow us to suggest that autoimmunity is the major mechanism implicated in the rejection of syngeneic heart tissues grafted into the pinna of the ear of mice chronically infected with *T. cruzi*. The similarity of the lesions to those found in humans suggests that autoimmunity is involved in the pathogenesis of chagasic cardiomyopathy in humans. Moreover, this could imply therapeutic strategies by reestablishing long-term tissue-specific tolerance with anti-CD4 mAb treatment, mediating anergy, or deleting the responder CD4⁺ T cells.

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