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E Cunha-Neto, … , N Stolf, J Kalil

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Research Article

Heart tissue destruction in chronic Chagas' disease cardiomyopathy (CCC) may be caused by autoimmune recognition of heart tissue by a mononuclear cell infiltrate decades after Trypanosoma cruzi infection. Indirect evidence suggests there is molecular mimicry between T. cruzi and heart tissue. In murine models of CCC, antibodies and CD4+ T cells recognize myosin, the major heart protein. We recently identified a heart-specific epitope of cardiac myosin heavy chain (residues 1442-1447, AAALDK) that is crossreactive with a homologous sequence (AAAGDK) of the immunodominant T. cruzi antigen B13. Furthermore, cardiac myosin-B13 crossreactive antibodies are present in 100% CCC patients vs 14% asymptomatic T. cruzi-seropositive individuals (P = 2.3 x 10(-6)), suggesting a role for molecular mimicry between cardiac myosin and B13 in CCC pathogenesis. In this paper, we obtained heart-infiltrating T cell clones from CCC patients to assess whether molecular mimicry between cardiac myosin and B13 is directly involved in the genesis of heart lesions. We identified T cell clones derived from CCC heart lesions simultaneously responsive to cardiac myosin heavy chain (but not skeletal myosin heavy chain) and B13 T. cruzi protein, but could not find T cell clones primarily reactive to any T. cruzi antigen. Together with the association of myosin-B13 crossreactive antibodies with CCC, the present data strongly suggest the relevance of molecular mimicry between cardiac myosin and the […]

Autoimmunity in Chagas' Disease

Identification of Cardiac Myosin-B13 *Trypanosoma cruzi* Protein Crossreactive T Cell Clones in Heart Lesions of a Chronic Chagas' Cardiomyopathy Patient

Edécio Cunha-Neto,* Verônica Coelho,* Luíza Guilherme,* Alfredo Fiorelli,‡ Noedir Stolf,‡ and Jorge Kalil*
**Laboratory of Transplantation Immunology and ‡Department of Surgery, Instituto do Coração, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, 05403-000, Brazil*

Abstract

Heart tissue destruction in chronic Chagas' disease cardiomyopathy (CCC) may be caused by autoimmune recognition of heart tissue by a mononuclear cell infiltrate decades after *Trypanosoma cruzi* infection. Indirect evidence suggests there is molecular mimicry between *T. cruzi* and heart tissue. In murine models of CCC, antibodies and CD4+ T cells recognize myosin, the major heart protein. We recently identified a heart-specific epitope of cardiac myosin heavy chain (residues 1442–1447, AAALDK) that is crossreactive with a homologous sequence (AAAGDK) of the immunodominant *T. cruzi* antigen B13. Furthermore, cardiac myosin–B13 crossreactive antibodies are present in 100% CCC patients vs 14% asymptomatic *T. cruzi*–seropositive individuals ($P = 2.3 \times 10^{-6}$), suggesting a role for molecular mimicry between cardiac myosin and B13 in CCC pathogenesis. In this paper, we obtained heart-infiltrating T cell clones from CCC patients to assess whether molecular mimicry between cardiac myosin and B13 is directly involved in the genesis of heart lesions. We identified T cell clones derived from CCC heart lesions simultaneously responsive to cardiac myosin heavy chain (but not skeletal myosin heavy chain) and B13 *T. cruzi* protein, but could not find T cell clones primarily reactive to any *T. cruzi* antigen. Together with the association of myosin-B13 crossreactive antibodies with CCC, the present data strongly suggest the relevance of molecular mimicry between cardiac myosin and the *T. cruzi* protein B13 in the pathogenesis of heart lesions in chronic Chagas' disease cardiomyopathy. (*J. Clin. Invest.* 1996. 98:1709–1712.) Key words: autoimmunity • immunology • immunopathology • *Trypanosoma cruzi* • Chagas' disease cardiomyopathy

Address correspondence to Jorge Kalil, Laboratory of Transplantation Immunology, Instituto do Coração, Faculdade de Medicina; Universidade de São Paulo, Av. Dr. Enéas de C. Aguiar, 500 3o. andar, São Paulo, SP, 05403-000, Brazil. Phone: 55-11-2829350; FAX: 55-11-2822354; E-mail: edecunha@spider.usp.br

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Introduction

Chagas' disease (American Trypanosomiasis), caused by the protozoan *Trypanosoma cruzi*, is endemic in many countries of Latin America, where 16–18 million people may be infected. Chronic Chagas' disease Cardiomyopathy (CCC),[1] a dilated cardiomyopathy with a T cell–rich myocarditis that often follows a fatal course, develops in 25–30% of infected individuals 5–30 yr after primary infection (1). The remaining 60–70% chronically *T. cruzi*–infected individuals either remain asymptomatic ("indeterminate" patients) or develop denervation of parietal smooth muscle in the digestive tract (5–10%). As there is no vaccine for Chagas' disease and treatment with anti–*T. cruzi* drugs does not abort the progression of CCC (2), heart disorders are treated only symptomatically.

The pathogenesis of heart lesions in CCC has been an issue of much debate. Although heart-infiltrating T cells seem to play a pivotal role in tissue damage, the nature of target antigens in the heart is obscure. The inability to consistently find parasites close to the destructive inflammatory infiltrate in affected heart tissue (3) prompted early investigators to suggest that the inflammatory heart lesion could be of autoimmune nature (4), possibly involving antigenic mimicry between *T. cruzi* and heart antigens. This hypothesis was reinforced by the finding that CD4+ T cells from a murine model of CCC can transfer heart lesions to naive noninfected mice (5). The demonstration of restricted heterogeneity of T cell receptor Vα transcripts in heart biopsies from CCC patients (6) is in line with similar findings in established autoimmune diseases. The recognition of myosin by CD4+ T cells in a murine model of CCC (7) revealed a prime candidate-defined target autoantigen in CCC, because of its abundance (50% of total protein by weight [8], and constitutive presentation by heart interstitial macrophages [9]). Myosin is the major antigenic target in many situations of heart-specific autoimmunity, such as rheumatic fever (10), post–Coxsackie B3 cardiomyopathy (11), and myosin-induced myocarditis (12, 13). Moreover, anticardiac myosin heavy chain (HC) autoantibodies are correlated with chronic inflammatory cardiomyopathy in *T. cruzi*–infected mice (14).

We recently identified a heart-specific epitope (AAALDK) of cardiac myosin HC that is antigenically mimicked by a secondary epitope (AAAGDK) of the immunodominant *T.*

1. *Abbreviations used in this paper:* CCC, chronic Chagas' disease cardiomyopathy; HC, heavy chain.

cruzi antigen B13. Furthermore, cardiac myosin–B13 crossreactive antibodies are present in 100% CCC patients vs 14% asymptomatic *T. cruzi* seropositive individuals ($P = 2.3 \times 10^{-6}$) (15). The association of cardiac myosin HC–B13 crossreactive antibodies with CCC may only be signaling the presence of "helper" T cells of similar specificity. Such hypothetical cardiac myosin–B13 *T. cruzi* antigen crossreactive T cells could mediate tissue damage if located in heart tissue.

Recently, foci of inflammation around scarce *T. cruzi* parasites detected with a sensitive immunoperoxidase technique were observed in postmortem studies of hearts from several CCC patients (16). Authors speculated that the T cells recognizing parasite antigens in situ could be the ultimate effectors of heart tissue damage. However, as the overwhelming majority of microscope fields show myocarditis in the absence of *T. cruzi* forms even with sensitive detection techniques, it would remain to be proven whether T cells in areas devoid of living forms of *T. cruzi* would also be recognizing parasite antigens.

This study aims at identifying the nature of the antigen(s) recognized by heart-infiltrating T cells from a CCC patient, whether heart or parasite derived, and thus contribute to testing the autoimmune/antigenic mimicry theory of pathogenesis.

Methods

Patient. Heart-infiltrating T cells were obtained from two samples of a routine workup transvenous endomyocardial biopsy of the 35-yr-old male patient JGS, diagnosed as severe CCC (severe heart failure and dilated cardiomyopathy with a positive serology for *T. cruzi*, all other causes excluded) who was on the waiting list for cardiac transplantation. The fragments were collected for in vitro T cell culture with informed consent. Sample collection procedures have been cleared by the Committee of Ethics of Instituto do Coração, HCFMUSP. His HLA-DR type was 1, 13 (6). His serum contained cardiac myosin–B13 protein crossreactive antibodies as described (15).

Antigens. Purified human cardiac and skeletal muscle myosin were obtained from normal human heart ventricular tissue and intercostal skeletal muscle, respectively, from a cadaveric organ donor as described (17). Affinity-purified recombinant immunodominant *T. cruzi* antigens B13 and B12 (18), CRA and FRA (kindly provided by Dr. S. Goldenberg, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, reference 19) and JL5 (kindly provided by Dr. Mariano Levin, INGEBI/CONICET, Buenos Aires, Argentina, reference 20) are β-galactosidase fusion proteins (proposed for use in serodiagnosis of *T. cruzi* infection). As a means to further purify each antigen preparation (and get rid of myosin light chains), proteins were subject to sodium dodecyl sulphate–7.5% polyacrylamide gel electrophoresis (21) and transferred to nitrocellulose sheets (22). Adequate protein bands were excised and prepared as nitrocellulose suspensions for use in T cell proliferation assays (23). Nonrecombinant β-galactosidase and "clean" nitrocellulose strips were processed similarly and used as negative controls. Whole *T. cruzi* trypomastigote lysate was obtained by five cycles of deep freezing–thawing of trypomastigotes derived from *Y* strain–infected LLC-MK_2 monolayers (24).

Heart-derived T cell clones. Heart-infiltrating T cell clones were generated without prior exposure to antigen in vitro, using a protocol that selects IL-2 receptor–expressing in vivo–activated T cells. Endomyocardial biopsy fragments were finely minced and cultured in 96-well flat-bottom culture plates (Falcon Labware, Cockeysville, MD) in DME with 10% normal human serum (HS), further supplemented with 20 U/ml human recombinant IL-2 (Hoffmann-La Roche, Nutley, NJ), in the presence of autologous irradiated (5,000 R) PBMC (10^5/well) as described (25, 26). Lymphoblasts were cloned by limiting dilution in 96-well flat-bottom culture plates on DME + 10% HS, in the presence of 20 U/ml IL-2, PHA (10 μg/ml), and irradiated PBMC, 10^5/well (26). Grown clones were subject to antigen-induced proliferation assays (2×10^4/well) in triplicate wells of 96-well microtiter plates, in the presence of 10^5 HLA-DR compatible (DR1, 13) irradiated PBMC in complete medium. 48 h later, wells were pulsed with 1 μCi 3H-thymidine (Amersham Corp., Arlington Heights, IL) for 18 h. [^{3}H]thymidine incorporation was measured at the MATRIX 96 direct beta counter (Packard, Canberra, Australia). This system employs a gaseous mixture rather than conventional liquid scintillation media for counting beta emissions, with a lower counting efficiency generating universally lower cpm values on background and subject. However, stimulation indexes remain proportional to those obtained with conventional liquid scintillation media. Student's *t* test was used to compare means of cpm values in T cell proliferation assays with means of cpm from negative controls. Surface phenotype of T cell clones was identified by FACS® immunostaining with OKT3, OKT4, and OKT8 monoclonal antibodies performed with a FACScan® (Becton Dickinson & Co., San Jose, CA).

Results

Heart-derived CD4+ T cell clones simultaneously recognize cardiac myosin and B13 protein. We assayed 18 T cell clones obtained from CCC patient JGF, in two different cloning experiments. In spite of the 2:1 predominance of CD8+ to CD4+ T cells by immunoperoxidase staining at endomyocardial biopsy (27), all T cell clones obtained expressed a CD3+CD4+CD8- phenotype, a disparity to be discussed elsewhere (Cunha-Neto, E., F. Albuquerque, L. Guicherme, V. Coelho, B. Ianni, C. Mady, F. Baccal, F. Bocchi, J. Kalil, manuscript in preparation). We found two T cell clones capable of simultaneously recognizing human cardiac myosin HC and B13 recombinant *T. cruzi* protein, clones E2O5 and E2.17, each obtained in a separate cloning experiment (Table I). The fact that only 2 out of 18 T cell clones displayed responses towards any putative relevant antigen is in line with the fact that typically 1–10% of T cells present in a delayed-type hypersensitivity immune reaction actually respond to the triggering antigen (28, 29). Although the proliferative responses were significantly higher than controls ($P < 0.01$), absolute cpm values were low. Both the expansion and cloning protocol in the absence of in vitro selection with the test antigen (28, 30), and the use of antigen-bound nitrocellulose particles (23, 31), have been described as generating low proliferative values. Furthermore, the gas-based direct β emission counter used has a lower counting efficiency than conventional liquid scintillation β counters. Neither of the other immunodominant recombinant *T. cruzi* antigens, nor the highly heterogeneous whole *T. cruzi* trypomastigote lysate induced any significant proliferation by any of the tested clones. Fig. 1 displays the stimulation indexes of clones E2O5 and E2.17 against cardiac and skeletal myosin heavy chains and B13 protein. It is interesting to note the tissue specificity of myosin recognition by T cell clones; while both clones proliferated to cardiac myosin, neither proliferated significantly to human skeletal muscle myosin.

Discussion

In this study, we identified T cell clones derived from CCC heart lesions simultaneously responsive to cardiac myosin HC and B13 *T. cruzi* protein. However, we could not identify T cells responding to none of several immunodominant recombinant *T. cruzi* antigens as well of a total *T. cruzi* lysate. The potential participation of molecular mimicry between cardiac

Table I. Proliferation Assay of T Cell Clones Derived from Endomyocardial Biopsy of CCC Patient JGF

	Control	PHA	*T. cruzi* lysate	‡Nitro-cellulose control	‡Human cardiac myosin	‡Human skeletal myosin	‡B13	‡JL5	‡CRA	‡FRA	‡β-gal
cl. E2B6	8±2.9	7490±699	8±3.3	17±5.4	23±3.3	9±0.5	7±2.4	6±2.1	9±2.1	11±2.1	8±2.2
cl. E2F3	27±3.7	10199±4500	35±7	13±1.2	19±2.4	14±3.4	14±7.8	17±3.9	20±5.9	19±3.7	14±3.3
cl. E2F9	10±1.2	4834±303	15±7.1	18±3.3	34±6.5	15±2.4	7±1.7	15±0.9	15±2.2	10±1.9	16±3.6
cl. E2J4	12±1.7	4945±441	12±4.6	16±0.5	26±3.7	12±2.9	17±8.3	22±0.9	13±2.6	20±6.5	24±9.8
cl. E2K6	25±4.5	1052±216	44±7.4	52±17	48±1.2	ND	37±9	ND	ND	ND	40±5.4
cl. E2K10	21±3.3	231±50	20±3.1	29±6.8	31±2.6	ND	20±8.7	ND	ND	ND	32±2.6
cl. E2M2	88±11.8	4299±105	132±27	29±9.1	58±3.6	48±5.3	60±13.1	68±4.3	100±15.8	69±11	44±7.3
cl. E2O4	16±4.1	1575±130	17±4.9	26±3.1	17±1.2	ND	21±1.2	ND	ND	ND	22±10
cl. E2O5	51±6.2	5502±1154	20±2.9	34±6.6	173*±38	49±6.8	135*±23	23±7.4	28±0.8	23±10	36±15
cl. E2.3	20±1.6	457±58.5	35±11.2	47±5.1	70±13	ND	59±9.2	ND	ND	ND	40±13
cl. E2.6	20±1.2	3201±167	57±29	13±5.9	25±7.5	13±5.2	56±15	30±8.4	24±6.4	24±0.8	34±8.1
cl. E2.9	94±21.7	1734±218	36±4.3	168±117	104±8.8	93±6.4	84±6.9	51±9.6	58±4.8	70±12.3	91±20
cl. E2.11	45±2.9	1528±834	39±13.1	49±5.4	68±11	95±25	39±2.8	32±5.7	55±7.9	46±5.7	61±12.3
cl. E2.12	88±12	1357±157	51±6.8	88±19	130±16	109±20	72±4.8	59±8.5	65±8.8	79±22	91±6.1
cl. E2.14	80±16	1905±405	99±31	79±4.5	121±7.9	112±11	87±12	69±0.8	98±15	92±16	117±11
cl. E2.17	85±22	880±60	112±17	49±10	150*±25	103±27	138*±11	51±15	51±12	98±15	75±34
cl. E2.40	86±27	2370±206	143±36	175±35	139±12	182±41	180±42	137±36	124±23	169±31	284±73

Numbers are expressed as counts per minute±SD. *$P < 0.01$, experimental vs control. ‡Antigens were added as nitrocellulose particles and should be compared with nitrocellulose control.

myosin and B13 *T. cruzi* protein in the pathogenesis of CCC, already suggested by the identification of CCC-associated cardiac myosin–B13 crossreactive antibodies (15), was further supported by the finding of CCC heart-infiltrating crossreactive T cell clones.

Such crossreactive T cells could be the providers of T cell help for the production of cardiac myosin–B13 crossreactive antibodies found in 100% of CCC patients (15). As all evidence suggests that heart-infiltrating T cells are the ultimate effectors of heart tissue damage in CCC, the finding of cardiac myosin–B13 crossreactive T cell clones in the heart lesion site of a CCC patient may further implicate such T cells in the genesis of CCC heart lesions. The isolation of T cells reactive to candidate self-antigens in the target organ of autoimmune lesion, as has been done for rheumatic heart disease (26), Graves' thyroid disease (28, 32), and autoimmune hepatitis (29), has been taken as important evidence for the establishment of the autoimmune origin of a disease, according to criteria proposed by Rose and Bona (33).

The inability of cardiac myosin–B13 protein crossreactive T cell clones to recognize skeletal muscle myosin may be due to the fact that cardiac and skeletal myosin HCs only share 75% homology at the protein level (data not shown). Thus, the B13 crossreactive T cell epitope in cardiac myosin seems to be a heart-specific epitope. This is in line with the heart specificity of tissue damage in CCC and further corroborates the relevance of such T cell clones. For that matter, it is known that the B13 crossreactive antibody epitope of cardiac myosin is specific of the cardiac isoform (15).

The lack of T cell clones reactive towards either the other immunodominant recombinant *T. cruzi* antigens tested or towards the *T. cruzi* trypomastigote lysate is intriguing. As the biopsy samples studied were devoid of *T. cruzi* themselves, our results suggest that, at least in the areas of heart tissue free

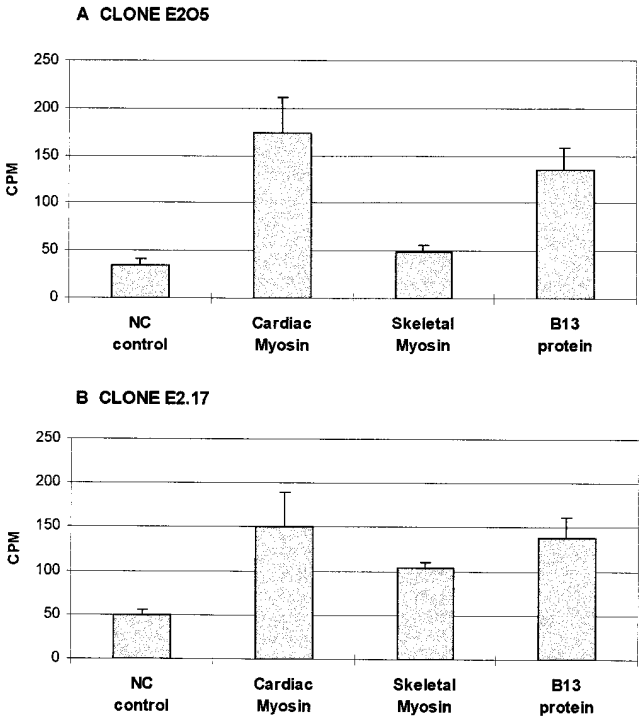

Figure 1. Proliferative response of myosin–B13 crossreactive CD4+ T cell clones derived from endomyocardial biopsy of patient CCC patient JGF. (*A*) Cardiac myosin vs nitrocellulose control, $P = 0.007$ (Student's *t* test), skeletal myosin vs nitrocellulose control, $P = 0.089$ (Student's *t* test), and B13 protein vs nitrocellulose control, $P = 0.004$ (Student's *t* test). (*B*) Cardiac myosin vs nitrocellulose control, $P = 0.006$ (Student's *t* test), skeletal myosin vs nitrocellulose control, $P = 0.055$ (Student's *t* test), and B13 protein vs nitrocellulose control, $P = 0.001$ (Student's *t* test).

from the parasite, the direct antigenic stimulus of *T. cruzi* may not be the determining event for the lymphocytic infiltrate.

Thus, in natural infection, crossreactive CD4+ T cell clones sensitized in the periphery by macrophages presenting *T. cruzi* B13 antigen after endocytosis of the parasite could become activated upon recirculation to the heart tissue, where myosin heavy chain epitopes are abundantly and constitutively presented by class II MHC molecules on heart interstitial macrophages, as demonstrated in mice by Smith and Allen (9). From then on, CD4+ T cells might initiate and maintain a typical delayed-type hypersensitivity reaction in heart tissue, by the release of inflammatory cytokines and cellular recruitment.

This paper showed the first demonstration of immunological crossreactivity/molecular mimicry at the level of heart-infiltrating T cells between a molecularly defined *T. cruzi* antigen and a molecularly defined heart-specific protein in Chagas' cardiomyopathy. Together with the cardiac myosin–B13 crossreactive antibody found to be strongly associated with CCC (15), the present data strongly suggest the relevance of molecular mimicry between cardiac myosin and the *T. cruzi* protein B13 in the pathogenesis of heart lesions in chronic Chagas' cardiomyopathy. The identification of defined target antigens in molecular mimicry may turn CCC into a model for other human autoimmune diseases (like type I diabetes, multiple sclerosis, or rheumatoid arthritis) not so clearly associated with an infectious agent (34). Future experiments involving analysis of clones from a larger number of patients, cytokine expression pattern, and passive transfer experiments in murine models may be able to yield definite proof for or against the pathogenetic role of cardiac myosin–B13 crossreactive T cells in CCC.

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