The pathogenesis of Chagas’ disease: when autoimmune and parasite-specific immune responses meet*

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ABSTRACT
Chagas’ disease is a major health problem in Latin America, where it constitutes one of the leading causes of heart failure. About one fourth of Trypanosoma cruzi-infected individuals develop chronic chagasic cardiomyopathy (CChC), the most severe form of the disease. CChC is histologically characterized by the presence of multifocal inflammatory infiltrates in the heart, composed mainly by mononuclear cells, usually adhered to myocytes and leading to myocytolysis, and frequently by interstitial fibrosis. The pathogenesis of CChC is still unclear, despite intense investigations both in human beings and in animal models of the disease. Although tissue parasitism is rare in the chronic phase of infection, an immune response targeted to persistent parasites or parasite antigens is suggested, by some authors, as the pathogenic mechanism of CChC. Other researchers affirm that the lack of correlation between tissue parasitism and intensity of inflammation suggests, along with the presence of autoreactive immune responses, that CChC results from the action of an autoimmune response. Herein we review reports from the literature and our own data, which together indicate, on one hand, the participation of parasite-specific immune responses and, on the other hand, clearly demonstrate the participation of heart-specific immune responses in the pathogenesis of CChC. Moreover, multiple factors may determine whether an individual in the indeterminate form of the disease will develop CChC. The mechanisms by which T. cruzi breaks immunological tolerance to heart antigens are also discussed.

Key words: Chagas’ disease, myocarditis, autoimmunity, Trypanosoma cruzi, delayed-type hypersensitivity.

INTRODUCTION
Infection with Trypanosoma cruzi causes American trypanosomiasis or Chagas’ disease, which progresses in three consecutive phases. An acute phase, characterized by intense parasitism and blood parasitemia occurs after parasite transmission. This is followed by an asymptomatic or indeterminate phase, in which no clinical symptoms are observed. Most of the individuals will remain in this phase of the infection throughout the rest of their lives. About 30% of them, however, develop a chronic phase, after time periods ranging from a few months to decades (Dias and Coura 1997). The majority of cases of chronic disease consists of a progressively debilitating chronic cardiomyopathy, for

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which there is no current cure. One of the leading causes of heart failure in several Latin American countries, the chronic chagasic cardiomyopathy (CChC) is characterized by an intense myocarditis, which may lead to heart enlargement, and frequently by arrhythmia, causing, in concert, heart malfunction and death (Köberle 1968).

The pathological basis of CChC has been a matter of intense debate in the last decades. That it is immunologically mediated has been clearly shown by experiments in athymic nude mice, in which infection by *T. cruzi* causes strikingly high tissue parasitism without heart inflammation (Soares et al. 2001a and Figure 1). The specificity of the pathogenic immune response, however, is the source of the controversy. One main hypothesis proposes that the pathogenic inflammatory response is exclusively directed to *T. cruzi* antigens at sites of parasite persistence (Tarleton 2001, Higuchi 1997). The second main hypothesis is that CChC is an autoimmune disease triggered in some individuals by *T. cruzi* infection (Cunha-Neto and Kalil 1995, Leon and Engman 2001). Here, points of view against and in favor of these two hypotheses will be presented, together with evidence that both *T. cruzi*- and heart-specific responses may be important in the pathology of CChC.

**T. CRUZI INFECTION AND THE MURINE MODEL**

*T. cruzi* is a hemoflagellate protozoan parasite capable of infecting different cell types in the mammalian host. It has, frequently, a preference to invade muscle cells, including heart muscle cells. In the acute phase of infection, parasites are easily found replicating in different tissues and organs, as well as circulating in the blood. As adaptive immune responses are stimulated, the high tissue parasitism is controlled, but never eradicated. Host and pathogen will then live in equilibrium, similar to other parasitic infections, such as leishmaniasis and toxoplasmosis. This equilibrium may be broken, for instance, in a state of immunosuppression, when patent parasitism may reappear (Sartori et al. 1998, Galhardo et al. 1999).

The evaluation of the factors responsible for the development or control of pathogenic responses has been hindered by the high variability of the parasite: different *T. cruzi* strains display distinct biological behaviors (Andrade and Magalhães 1996). It has been reported that a single individual was simultaneously infected by different strains of *T. cruzi*, each showing a preference for specific tissues (Vago et al. 2000). To overcome the difficulties of carrying out experiments with human material, several researchers have chosen to investigate the pathological mechanisms of CChC in a mouse model of infection. Decades of investigations using this experimental model have taught us that both host and parasite genetic backgrounds contribute to disease development. Thus, certain mouse strains will develop CChC after infection with some strains of *T. cruzi*, but not with others (Andrade 1990, Andrade et al. 1985). A model that closely resembles the human disease has been studied in our laboratories. This model uses the Colombian strain of *T. cruzi*, a strain isolated from a patient with severe CChC (Federici et al. 1964). Infection of BALB/c mice with Colombian strain trypomastigotes, using an infective inoculum of 100 parasites, similar to what occurs in natural transmission (Brener et al. 2000), reproduces the three phases of infection observed in human beings. In the acute phase of infection, high parasitemia and intense myocarditis is observed, followed by a period of nearly complete regression of heart inflammation. However, four to five months after the onset of parasitemia, an intense and progressive inflammatory response in heart tissue is found.

**PARASITE PERSISTENCE AND INFLAMMATION**

The first aspect to be analyzed is the scarce parasitism found in the chronic phase of infection. Several studies have demonstrated that *T. cruzi* parasites or antigens can be found, although rarely, in individuals with chronic infection (Barbosa Jr and Andrade 1984, Jones et al. 1993, Palomino et al.
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Fig. 1 – Degree of parasitism and number of inflammatory cells in hearts of T. cruzi-infected nu/nu mice. Normal BALB/c nu/nu mice or reconstituted with splenocytes were infected with 100 Colombian strain T. cruzi. Each column represents the mean number of inflammatory cells per mm² (A) or the mean number of parasite nests per cm² (B) in heart sections from three mice. Vertical bars represent the standard deviations of the means.

2000). However, the analyses of human heart fragments have failed to show a correlation between intensity of inflammation and of parasitism, even with the use of highly sensitive techniques, such as PCR or immunohistochemistry (Palomino et al. 2000, Olivares-Villagomez et al. 1998). In fact, this remarkable feature of the disease was immediately pointed out 90 years ago by Vianna (1911), the excellent pathologist working in collaboration with Carlos Chagas. Vianna described that the intensity of inflammatory response observed in hearts of individuals with chagasic cardiopathy was not proportional to the degree of tissue parasitism. More importantly, in CChC patients, rare parasites are also found in other organs free of significant inflammation, whereas in the myocardium, they are found associated with chronic inflammatory response and fibrosis (Barbosa Jr and Andrade 1984, Mortara et al. 1999). This has also been observed in the mouse model of CChC (Buckner et al. 1999). If parasite persistence were the only factor driving the pathological response, one would expect a clear association between parasitism and inflammation, which would be manifested in all parasitized organs. The fact that this is not so is a clear evidence that the chronic inflammation is not merely caused by a T. cruzi-specific immune response.

DELAYED-TYPE HYPERSENSITIVITY AND CChC

The inflammatory reaction observed in CChC has a delayed-type hypersensitivity (DTH)-like aspect, with focal inflammation composed mainly of mononuclear cells (Andrade 1983). DTH reactions to T. cruzi antigens were shown in vivo in human beings (Mayer and Pifano 1941, Tschudi et al. 1968) and in the mouse model using crude T. cruzi extracts (Gonzales-Cappa et al. 1968), as well as purified T. cruzi antigens, such as the immunodominant SAPA antigen (Nasser et al. 1997) and cruzipain (Laderach et al. 1996). The DTH responses are mediated by CD4⁺ T cells, as adoptive transfer of this cell population purified from T. cruzi-infected mice to naïve mice also transferred the ability to mount a local DTH reaction (Hontebeirye-Joskowicz et al. 1987). As pointed out above, the lack of association between tissue parasitism and inflammation suggest that the pathogenic response in CChC is not only directed against T. cruzi antigens but also to self antigens. However, anti-T. cruzi responses can also induce lesions and contribute to CChC. In the

Acute phase of infection, destruction of intracardiac parasympathetic fibers occur, which may in part be responsible to some of the complications observed in the chronic phase of infection (Ribeiro-dos-Santos et al. 1976). This neuronal destruction is probably mediated by inflammatory reactions promoted by anti-\textit{T. cruzi} responses targeted to \textit{T. cruzi} antigens adsorbed on the neuronal cell surface (Ribeiro-dos-Santos and Hudson 1980a,b).

The DTH aspect of inflammatory reactions in CChC suggests that Th1 cells participate in the pathogenesis of this disease. In fact, an association between intensity of CChC and production of high levels of IFN-\(\gamma\) in human infection has been shown (Bahia-Oliveira et al. 1998). To investigate the role of IFN-\(\gamma\) T-cell responses in the pathogenesis of experimental CChC, we studied the course of \textit{T. cruzi} infection in IL-4-deficient BALB/c mice, in which Th1 responses are accentuated. Infection of these mice with Colombian strain \textit{T. cruzi} caused higher acute phase parasitemia and tissue parasitism than the infection of IL-4 +/+ control mice (Soares et al. 2001a). These findings correlated with the production of IFN-\(\gamma\), since IL-4 –/– mice had higher levels of this cytokine than IL-4 +/+ mice. Interestingly, despite the lower number of parasites, IL-4 –/– mice had an early and more intense myocarditis than IL-4 +/+ mice (Figure 2). These results indicate a dual role of IFN-\(\gamma\) in \textit{T. cruzi} infection, where this cytokine has a protective role in parasitism control, but, on the other hand, acts as a critical mediator of CChC. Moreover, they reinforce the lack of association between intensity of inflammation and degree of tissue parasitism in CChC. This lack of association is also observed when \textit{T. cruzi}-infected young and aged mice are compared. Despite of having lower parasitemia and mortality early in the infection, aged mice have more intense myocarditis in the chronic phase of infection than young mice (Cardillo et al. 1993). This phenomenon may also be associated with a decreased Th2 response, as aged mice have impaired Th2-dependent immune responses (Smith et al. 2001).

**AUTOIMMUNE RESPONSES IN CChC**

Proving a disease to be of autoimmune aetiology is not an easy task. To classify a disease as autoimmune, one needs to show not only the presence of an immune response against self antigens, but also clinical or experimental evidence of the primary role of this response in the pathogenesis of tissue lesions.

Immune responses against self antigens in human and experimental Chagas’ disease were demonstrated by several research groups. Antibodies against a number of antigens, including antigens expressed in cardiac (McCornick and Rowland 1989, Cunha-Neto et al. 1995) and nervous (Ribeiro-dos-Santos et al. 1979, Van Voorhis and Eisen 1989) tissues, and UsnRNPs (Bach-Elias et al. 1998), among others, have been detected during \textit{T. cruzi} infection (reviewed in Kierszenbaum 1999).

However, autoantibodies are commonly found after infection with different pathogens, without any implications regarding an autoimmune pathology (Argov et al. 1989, Daniel-Ribeiro and Zanini, 2000).

As for autoantibodies, there is a growing number of reports indicating a pathogenic role of IgG antibodies, present in the sera of chagasic patients, against muscarinic and adrenergic receptors of cardiomyocytes (Borda et al. 1984, Sterin-Borda et al. 1991). The production of autoantibodies against beta1 and beta2-adrenoreceptors correlated with primary electrical cardiac abnormalities (Chiale et al. 1995). It was also demonstrated that sera from chronic chagasic individuals interfere with electric and mechanical activities of embryonic myocardial cells \textit{in vitro} (Costa et al. 2000, Kaplan et al. 1997) and affect cardiac electrogensis and conduction when perfused in the rabbit heart (de Oliveira et al. 1997). These alterations in heart-cell function seem to result from the binding of antibodies to \(\beta\)-adrenergic and M2-cholinergic receptors on the myocardial cell surface (Costa et al. 2000, de Oliveira et al. 1997, Kaplan et al. 1997). Thus, by stimulating the production of autoantibodies against certain
self-antigens, *T. cruzi* infection may cause disturbances in heart cell functions, which may lead to complications associated with CChC.

Autoreactive T lymphocytes specific to heart or nerve tissue antigens were also found in chagasic mice and patients (Rizzo et al. 1989, Cunha-Neto et al. 1996, Hontebeyrie-Joskowicz et al. 1987). A first indication of a pathogenic role of self-reactive T-cell responses in CChC was obtained by our group using a heterotopic heart transplant model (Ribeiro-dos-Santos et al. 1992). In this report, newborn syngeneic hearts transplanted in the ears of *T. cruzi*-infected mice were rejected, while the control hearts transplanted in ears of normal mice engrafted and started beating after seven to ten days. The rejection of normal, syngeneic heart transplant was mediated by CD4+ T cells, as the transfer of this T cell population purified from spleens of chagasic mice, but not of CD8+ T cells, rendered normal recipients capable of rejecting syngeneic transplants. A few years later, however, Tarleton et al. (1997) published a report in which they describe that, in a different infection model (using Brazil and Sylvio strains of *T. cruzi* to infect C57Bl/6 and C3H mice), rejection of syngeneic heart transplants did not occur in chronic chagasic mice. Graft rejection was only observed when the transplant was performed during the acute phase of infection (and parasites could be found in the heart graft), or when *T. cruzi* antigen was injected into the transplanted tissue. These observations lead the authors to the conclusion that an anti-*T. cruzi* response was responsible for the destruction of the heart transplant. Thus, in some mouse models, parasite-specific immune responses seem to be the only cause of CChC (Tarleton et al. 1997), whereas this does not seem to be the case in other mouse models (Ribeiro-dos-Santos et al. 1992, Buckner et al. 1999) and in human beings (Barbosa Jr and Andrade 1984, Mortara et al. 1999).

To further demonstrate the role of autoreactive T cells in the pathogenesis of experimental CChC, we generated a CD4+ T-cell line from the spleen of a chronic chagasic mouse (Ribeiro-dos-Santos et al. 2001). This T-cell line was raised by repeated *in vitro* stimulation with syngeneic heart extract. After eight months of culture, this T-cell line strongly reacted with heart extracts from different sources (syngeneic, allogeneic and xenogeneic). Interestingly, this T-cell line also reacted with *T. cruzi* antigen, although the culture was maintained free of...
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Fig. 3 – Heart sections of BALB/c mice after T. cruzi infection and/or cardiac antigen immunization. A. Focal and discrete inflammatory infiltration by mononuclear cells 30 days after infection with low virulence T. cruzi Y strain clone (100 parasites). B. Inflammatory mononuclear infiltration with myocytolytic lesions, incipient fibrosis and absence of parasites in a cardiac antigen-hyperimmunized mouse 30 days after infection with T. cruzi Y strain clone (100 parasites). C. Myocarditis by mononuclear cells, frequently adhered to myofibers and inducing myocytolysis eight months after infection with T. cruzi Colombian strain (100 parasites). The finding of parasite in this phase of infection is very rare. D. Focal and discrete myocarditis by mononuclear cells in mouse tolerized by injection of a myosin-enriched cardiac fraction eight months after infection with T. cruzi Colombian strain. E. Intense carditis by mononuclear cells with massive destruction of cardiac cells and presence of interstitial fibrosis four months after immunization with myosin-enriched fraction. F. Intense interstitial fibrosis with few remaining intact cardiac fibers and intense inflammatory infiltration by mononuclear cells four months after immunization with a myosin-enriched fraction. A-D. H&E, 400×. E. H&E, 200×. F. Masson’s trichrome stain, 400×.

parasites by treatment with amphotericin B. More importantly, this anti-heart T-cell line caused the destruction of myoblasts in vitro as well as syngeneic heart transplant rejection when injected into the transplanted heart. It proliferated in the presence of T. cruzi and heart antigens, and presented a Th1 cytokine profile upon stimulation with these antigens. More importantly, the adoptive transfer of this T-cell line induced intense myocarditis in athymic mice (nu/nu), which is extremely sensitive to T. cruzi infection, in the complete absence of T. cruzi.

AUTOIMMUNE MYOCARDITIS

The inflammatory reaction found in CChC is similar to that of autoimmune myocarditis induced by immunization with cardiac myosin, characterized by a multifocal inflammatory infiltrate composed by mononuclear cells and interstitial fibrosis (Figure 3E and F). This similarity lead us and others to compare the immune responses of mice infected with T. cruzi and/or immunized with heart antigens. Leon et al. (2001) demonstrated that sera from A/J mice (highly susceptible to autoimmune myocarditis) contain antibodies which strongly recognized heart antigens, including myosin, in the acute phase of T. cruzi infection. Additionally, T. cruzi-infected A/J mice developed DTH response against cardiac myosin. Based on these observations, the authors concluded that an autoimmune response is triggered by T. cruzi early in the infection. Due to the high virulence of T. cruzi for A mice, the evaluation of hearts from these mice in the chronic phase of infection is difficult. However, A mice treated with benznidazole during the acute phase of infection survive and progress to the chronic phase of infection (authors’ unpublished observation). Using this approach, we found that A mice have intense myocarditis after three months of infection with the Colombian-strain T. cruzi, whereas BALB/c mice have nearly absent inflammation at this time after infection. This result shows that A mice are more susceptible than BALB/c mice both for induction of experimental autoimmune myocarditis and for development of CChC, suggesting a common basis for both diseases. Moreover, hyperimmunization (eight injections) of BALB/c mice with low doses of heart antigen does not induce autoimmune myocarditis. However, two months after a challenge with Y strain T. cruzi (100 parasites), these mice had intense myocarditis, whereas infected-control mice have nearly normal hearts (Soares et al. 2001b; Figure 3 A and B). Hyperimmunized BALB/c IL-4 –/– mice, on the other hand, developed mild, but clearcut myocarditis, which was also aggravated after T. cruzi infection. One explanation for these findings is that T. cruzi infection breaks the tolerance to heart antigens, which is kept under control by mechanisms probably dependent on IL-4.
WHAT ARE THE MECHANISMS BY WHICH
*T. CRUZI* BREAKS IMMUNOLOGICAL TOLERANCE?

The main proposed mechanisms by which a microorganism could trigger an autoimmune disease are the sharing of epitopes (molecular mimicry) between the pathogen and the host, and the inflammation and tissue damage caused by the infection, leading to release self antigens, recruitment of inflammatory cells and/or production of immunomodulators and expression of surface molecules, which could, in concert, trigger or sustain an autoimmune response (Davies 1997, Fairweather et al. 2001).

One of the better characterized example of molecular mimicry between *T. cruzi* and a heart antigen was demonstrated by Cunha-Neto and co-workers. These investigators showed the presence of antibodies in chagasic patients which reacted both with cardiac myosin and B13, a *T. cruzi* antigen (Cunha-Neto et al. 1995). More recently, T-cell clones cross-reacting with cardiac myosin and B13 were also obtained from hearts of CChC patients (Cunha-Neto et al. 1996). Most of these T-cell clones are CD4+ and secrete high levels of IFN-γ (Abel et al. 2001). However, there is no demonstration of a role against B13 in the pathogenesis of CChC, neither in human beings, nor in experimental models. In fact, it has been recently suggested that there is no evidence supporting a role for molecular mimicry in the pathogenesis of autoimmune diseases triggered by microorganisms, in general (Benoist and Mathis 2001).

*T. cruzi* could also act triggering or enhancing an autoreactive response kept under control by suppressive mechanisms. First, by its ability to infect cardiac fibers, it causes intense damage, antigen release and inflammation in the acute phase of infection. Second, *T. cruzi* may affect the properties of professional antigen-presenting cells, such as macrophages and dendritic cells. In this regard, it was demonstrated that *T. cruzi* molecules can modulate the production of pro-inflammatory cytokines by macrophages (Almeida et al. 2000), and affect the maturation of dendritic cells (Van Overtvelt et al. 1999). Third, the immune response against *T. cruzi* antigens could also influence the induction of self-reactive responses. In experimental *T. cruzi* infection, high levels of IFN-γ are produced during the acute phase of the disease (Hoft et al. 1993, Zhang and Tarleton 1996). This production may result from the parasite intensely stimulating macrophages to produce IL-12 and IL-18, two potent IFN-γ inducing factors (Frosch et al. 1996, Meyer zum Büschenfelde et al. 1997, Camargo et al. 1997). Additionally, the immunodominant *T. cruzi* trans-sialidase superfamily antigens induce Th1-type responses both in human beings and in mice (Millar and Kahn 2000, Ribeirão et al. 2000). Thus, the persistence of parasites could sustain the release of self antigens, the presence of small inflammatory foci in the heart and the production of inflammatory cytokines, which may, in concert, potentiate a self-reactive response. The possibility of re-infection with *T. cruzi* of individuals living in endemic areas may also influence the development of CChC, as new loads of parasites may induce release of autoantigens and soluble mediators. In fact, it has been reported that the frequency of infected individuals developing CChC has decreased in areas where parasite transmission has been controlled (Dias and Coura 1997).

CAN WE BLOCK THE PROGRESSION OF CChC?

Understanding the mechanisms of pathology in CChC may help the development of a therapy for prevention or intervention on the disease progress. Based on the results obtained with heart antigen immunization, we used the mouse model to test protocols of immunomodulation of CChC by induction of tolerance to heart antigens. Tolerance was induced by co-administration of anti-CD4 monoclonal antibodies and a myosin-enriched fraction, prepared from syngeneic hearts, before infection with Colombian strain *T. cruzi*. Hearts from tolerized mice had no or very discrete myocarditis eight months after infection, whereas hearts from non-tolerized mice had intense inflammation (Pontes-de-Carvalho...
et al. 2001; Figure 3C and D). This result clearly demonstrates a role for an autoimmune component in CChC, which could be prevented or inhibited by induction of tolerance to heart antigens.

CONCLUDING REMARKS
Perhaps the most intriguing feature of CChC is the fact that most T. cruzi-infected individuals will survive throughout their lives without any manifestations of the disease. This may be due to many factors, such as the parasite strain, the host genetic background, the number of re-infections, or even the success of the treatment. Concerning this last point, it is noteworthy the lack of market availability of new anti-T. cruzi drugs over the past 20 years. It is clear that, although an autoimmune response to heart antigens participate in the pathogenesis of CChC, the persistence of parasite or parasite antigens (Andrade et al. 2000) may be required for triggering or sustaining it. The development of heart failure or dysfunction may also depend on multiple events occurring in different phases of infection, such as neuronal destruction and activity of autoantibodies to cardiac beta-adrenergic and muscarinic membrane receptors, in addition to myocardial inflammation.

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