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Trypanosoma cruzi-Induced Molecular Mimicry and Chagas' Disease

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Abstract Chagas' disease, caused by *Trypanosoma cruzi,* has been considered a paradigm of infection-induced autoimmune disease. Thus, the scarcity of parasites in the chronic phase of the disease contrasts with the severe cardiac pathology observed in approximately 30% of chronic patients and suggested a role for autoimmunity as

the origin of the pathology. Antigen-specific and antigen-non-specific mechanisms have been described by which *T. cruzi* infection might activate T and B cells, leading to autoimmunity. Among the first mechanisms, molecular mimicry has been claimed as the most important mechanism leading to autoimmunity and pathology in the chronic phase of this disease. In this regard, various *T. cruzi* antigens, such as B13, cruzipain and Cha, cross-react with host antigens at the B or T cell level and their role in pathogenesis has been widely studied. Immunization with those antigens and/or passive transfer of autoreactive T lymphocytes in mice lead to clinical disturbances similar to those found in Chagas' disease patients. On the other hand, the parasite is becoming increasingly detected in chronically infected hosts and may also be the cause of pathology either directly or through parasite-specific mediated inflammatory responses. Thus, the issue of autoimmunity versus parasite persistence as the cause of Chagas' disease pathology is hotly debated among many researchers in the field. We critically review here the evidence in favor of and against autoimmunity through molecular mimicry as responsible for Chagas' disease pathology from clinical, pathological and immunological perspectives.

Abbreviations

Ag(s)	Antigen(s)
IFN	Interferon
CTL	Cytotoxic T lymphocyte
IL	Interleukin
TNF	Tumor necrosis factor
mAb	Monoclonal antibody
iNOS	Inducible nitric oxide synthase
DTH	Delayed-type hypersensitivity
TCR	T cell receptor
ECM	Extracellular matrix
MHC	Major histocompatibility complex
Mhc	Myosin heavy chain
CMhc	Cardiac myosin heavy chain
SMhc	Skeletal myosin heavy chain
APC(s)	Antigen-presenting cell(s)
ICAM	Intercellular adhesion molecule
CCC	Chronic chagasic cardiompathy
VCAM	Vascular cell adhesion molecule

1 Chagas' Disease

1.1 General Aspects and Life Cycle

Chagas' disease (Chagas 1909) is a debilitating multisystemic disorder which affects several million people (approximately 18 million individuals are in-

fected with *Trypanosoma. cruzi*, with 120 million at risk) in Central and South America (Moncayo 1999; Prata 2001; Tanowitz et al. 1992) and is considered a paradigm of infection-mediated autoimmune disease. It is caused by the flagellated protozoan parasite *Trypanosoma cruzi*, with a complex life cycle involving several stages in both vertebrates and insect vectors. *T. cruzi* has three main different morphologies: epimastigote, which replicates in the blood-sucking triatomine insect vector; trypomastigote, which infects the vertebrate host's cells; and amastigote, which replicates intracellularly in the host's cells (Burleigh and Andrews 1998; Tanowitz et al. 1992).

Transmission of *T. cruzi* to humans occurs when feces released by the bug while it takes a blood meal, containing infective metacyclic trypomastigote forms of the parasite penetrate, into the bloodstream, where the metacyclic forms infect a wide variety of host phagocytic and non-phagocytic cells. Once inside the cells, the metacyclic forms escape from endocytic vacuoles to the cytoplasm, where they transform into amastigotes, which multiply intracellularly (see Fig. 1 for details).

Individuals residing in rural areas of Latin America are at highest risk of infection, because the bugs live in these dwellings and feed on the inhabitants at night. The World Health Organisation has conducted several programs for the elimination of the insect vector, with great results on the incidence of new infections (Moncayo 1999). On the other hand, transfusion-acquired Chagas' disease is becoming a significant health problem in countries other than Central and South America, especially those receiving high numbers of immigrants from that region (Kirchhoff 1989; Wendel 1998).

1.2 Clinical Findings

Two phases, acute and chronic, can be differentiated in Chagas' disease (Kirchhoff 1993; Prata 2001; Tanowitz et al. 1992). In the acute phase, encompassing a few weeks after infection, a local inflammatory lesion appears at the site of infection, where the metacyclic trypomastigotes infect and undergo their first rounds of multiplication. After parasite dissemination through the body, circulating blood trypomastigotes are easily observed in blood (parasitemia) and a small number of patients develop symptoms of cardiac insufficiency, reflecting an underlying severe myocarditis, leading, in some instances, to heart failure responsible for the few deaths in acute Chagas' disease (Dias et al. 1956; Prata 1994). Meningoencephalitis may also occur, especially in some immunosuppressed patients (Hoff et al. 1978). However, the acute phase mostly remains undiagnosed without severe clinical symptoms. In contrast, the severe pathology and the most common manifestations of this disease develop

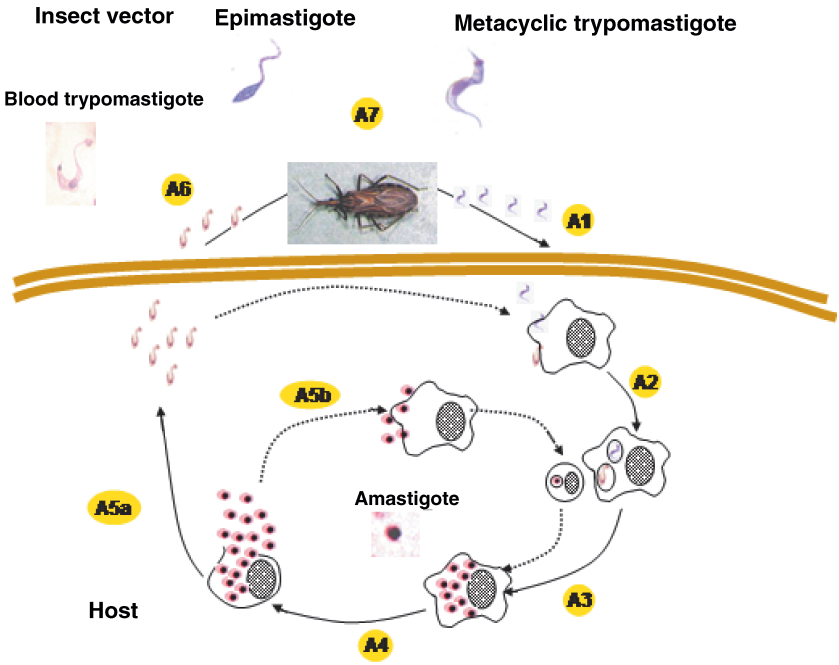

Fig. 1 Infective cycle of *Trypanosoma cruzi*. Transmission of *T. cruzi* to humans occurs when feces released by the bug while it takes a blood meal and containing infective metacyclic trypomastigote forms of the parasite (*A1*) penetrate into the bloodstream, where they infect a wide variety of host phagocytic and non-phagocytic cells (*A2*). Once inside the cells, the metacyclic forms escape from endocytic vacuoles to the cytoplasm, where they transform into amastigotes, which multiply intracellularly (*A3*). At some point, the amastigotes break off from the cell (*A4*) and differentiate into non-replicative flagellated blood trypomastigotes which in turn can penetrate and infect adjacent susceptible cells or spread to infect cells and tissues at distant locations of the body (*A5a*). Amastigotes can also directly infect phagocytic cells (*A5b*). Muscle cells, including those of the heart, are amongst the most heavily infected. Circulating trypomastigotes may be taken up by a new triatomine bug during a blood meal (*A6*). Inside the vector's intestine, ingested blood trypomastigotes differentiate into replicative epimastigotes, which, as they move to the mid and lower gut, transform into non-replicative but infective metacyclic trypomastigotes

many years (10 to 30) after the initial infection with *T. cruzi* in the so-called chronic phase, although only in 30%–40% of the infected people (Kirchhoff 1993; Prata 2001; Tanowitz et al. 1992). During the chronic phase, circulating parasites cannot be observed by inspection of blood but progressive tissue damage occurs involving the esophagus, colon and heart (Prata 2001; Tanowitz

et al. 1992). Treatment with benznidazol or nifurtimox is effective during the acute phase of infection, but no treatment exists for the chronic phase (Prata 2001; Tanowitz et al. 1992). To date, an effective immunotherapy or vaccine is still lacking.

1.3 Immune Response

The immune response against this parasite is complex and far from being clearly established. Both humoral and cellular immune responses are involved in controlling *T. cruzi*, which is not surprising because of the complexity of the parasite's life cycle. Thus, although B cell-deficient mice succumb to infection (Kumar and Tarleton 1998), the protective immune response seems to depend on $CD8^+$ T cells that produce interferon (IFN)-γ. $CD8^+$ T cells can control the infection through cytotoxic T lymphocyte (CTL)-induced perforin/granzyme-mediated killing of infected cells and/or FAS-mediated apoptosis (Kumar and Tarleton 1998). However, there are reports indicating that $CD8^+$ T cells cannot completely control infection because they become unresponsive (Martin and Tarleton 2004). Cytokines play a key role in regulating both the induction and type of immune response as well as parasite replication in infected hosts (Fresno et al. 1997). Macrophages, which can be infected by *T. cruzi*, also play a crucial role in the elimination of this parasite. Activation of monocytes by cytokines released by Th1 cells seems to be a key process in controlling infection in vitro as well as in vivo. Thus, Interleukin (IL)-12 produced by macrophages in response to infection mediates resistance to *T. cruzi* (Aliberti et al. 1996). Tumor necrosis factor (TNF)-α and IFN have been identified as the most important cytokines involved in the killing of intracellular *T. cruzi* through an NO-mediated-L-arginine dependent killing mechanism (Gazzinelli et al. 1992; Muñoz-Fernandez et al. 1992). This was corroborated in vivo, because anti-IFN-γ monoclonal antibody (mAb) administration results in a drastic increase in parasitemia and mortality (Silva et al. 1992; Torrico et al. 1991). Moreover, mice deficient for IFN-γ receptor and inducible nitric oxide synthase (iNOS) had an increased susceptibility to infection and parasitemia (Holscher et al. 1998; Goni et al. 2002), although the role of NO has been recently disputed because some iNOS-deficient mice do not seem to be more susceptible to infection (Laucella et al. 2004). TNF-R1-$FcIgG_3$ transgenic mice are also more susceptible to *T. cruzi* infection, clearly indicating a protective role for TNF-α (Castanos-Velez et al. 1998).

2
Chronic Chagasic Cardiopathy

2.1
Pathological Findings

The most important pathology of Chagas' disease develops 10–30 years after primary infection and affects several internal organs, mainly, heart, esophagus and colon, as well as the peripheral nervous system. The heart is the organ most commonly involved; cardiopathy frequently develops, congestive heart failure being a common cause of death in these patients. Megaesophagus and/or megacolon may also develop in chronic chagasic patients, which in the most severe form can cause life-threatening malnutrition and intractable constipation. Chronic chagasic cardiopathy (CCC) is thus the most devastating manifestation of Chagas' disease. However, despite affecting about a third of the infected people the pathogenesis of CCC is still poorly understood.

CCC may be considered a progressive disease, in which myocardial inflammation and fibrosis plays a pivotal role (Carrasco Guerra et al. 1987; Higuchi et al. 1987; Pereira Barretto et al. 1986). Higher percentages of severe myocarditis, fibrosis and myocardial hypertrophy are found in CCC patients with heart failure compared to patients in the indeterminate phase and with cardiac arrhythmia. Examination of the hearts of CCC patients who have died of heart failure shows biventricular enlargement with occasional apical aneurysms. In addition, individuals with CCC often develop mural thrombi, which may cause cerebrovascular accidents. Histological examination of the heart reveals diffuse interstitial fibrosis, lymphoid infiltration and damaged myocytes, all occurring in the apparent absence of parasites. Fibrosis and chronic inflammation are also detected in the conduction system of the heart, which may account for the high incidence of arrhythmias.

2.2
Mechanisms of Pathogenesis

Despite intensive research, the etiology of Chagas' heart disease, both in humans and in experimental animal models of the disease, is not clearly understood. Although the acute and chronic phases of the disease share some similar pathological findings, it is still unclear whether similar pathogenic mechanisms operate. In this regard, infiltration by $CD4^+$ T cells seems to take place in the acute phase of the disease, whereas $CD8^+$ T cells predominate in the chronic phase (Henriques-Pons et al. 2002). Moreover, it is plausible that the pathology of the acute phase may affect the final outcome of the chronic phase.

To date, many pathogenic mechanisms have been described to explain how cardiac pathology develops. They can be mediated directly by the parasite or caused by an inflammatory/immune/autoimmune mechanism or a combination of these. These mechanisms are summarized below:

- *Primary neuronal damage* resulting in denervation of the parasympathetic autonomous system in the heart. This was one of the first pathogenic mechanisms described during the acute phase (Koberle 1961, 1970). However, subsequent studies only show slight neuronal damage in the heart, suggesting that neuronal lesions are an epiphenomenon, secondary to inflammation and fibrosis (Davila et al. 1991, 2002; Rossi 1996).
- *T. cruzi-induced damage to cardiomyocytes,* due to the cytopathic effect caused by intracellular infection with amastigotes or by the release of secreted *T. cruzi* product(s), which can be toxic for host cells and tissues (Koberle and Nador 1955). This is an obvious mechanism, but may have only some relevance in the acute phase and in heavily parasitized or immunosuppressed patients.
- *Parasite-induced microvascular changes* may lead to cardiac hypoperfusion and finally to myocyte degeneration and chronic inflammation (Factor et al. 1985; Morris et al. 1990; Petkova et al. 2001).
- *Persisting T. cruzi antigens* may act as trigger for specific $CD4^+$ or $CD8^+$ T-cell mediated responses of either the delayed-hypersensitivity (DTH) type or cytotoxic $CD8^+$ cells that lead to damage to infected cells or to bystander cells in the host tissues (Ben Younes-Chennoufi et al. 1988; Tarleton 2001; Tarleton and Zhang 1999). This mechanism may take place in both the acute and the chronic phase.
- *Autoimmunity* may occur by a variety of mechanisms (listed in Table 1). Those could be due to *T. cruzi* antigen (Ag)-specific mechanisms (molecular mimicry) or non-parasite Ag-specific effector mechanisms and are discussed in detail below.

An important point which is often ignored in this debated field is that none of the mechanisms listed above is mutually exclusive. Moreover, it seems unlikely that heart damage can be attributed to only one of these mechanisms.

2.3 Immunological Findings

In CCC, 50% macrophages, 40% T cells with a predominance of $CD8^+$ over $CD4^+$ T cells and 10% B cells comprise the inflammatory infiltrate

Table 1 Mechanisms for activation of T and B cells in autoimmune diseases

a. Microbial antigen specific:
– Molecular mimicry between parasite and host antigens triggers autoimmunity
– Bystander activation (TCR dependent)
b. Microbial antigen non-specific:
– Release of autoantigen(s) during an infection
– Bystander activation (TCR independent)
– Cryptic epitopes
– Superantigens

(Cunha-Neto et al. 2004). T cell receptor (TCR) Vβ transcripts are heterogeneous in heart biopsies from CCC patients (Cunha-Neto et al. 1994) which is a characteristic of other well-defined autoimmune diseases. The number of $CD4^+$ T cells increased in parallel to the number of $CD8^+$ T cells in acute-phase but not in chronic-phase patients with heart failure, suggesting an immunological imbalance.

Cytokines and chemokines produced in response to the parasite may upregulate vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, increased on endothelial cells of patients, which recruit $VLA\text{-}4^+LFA\text{-}1^+CD8^+$ T lymphocytes (dos Santos et al. 2001). In this regard, a role for cell adhesion molecules and integrin receptors, extracellular matrix (ECM) components , matrix metalloproteinases and chemokines has been proposed in the differential recruitment and migration in infected hosts of *T. cruzi*-elicited $CD8^+$ and inflammatory cells into the heart and other susceptible host tissues (Marino et al. 2003a, 2003b). It is worth noting that ECM components may absorb parasite Ags and cytokines which could contribute to the establishment and perpetuation of inflammation. Moreover, we have found that *T. cruzi* requires β1 integrins to gain access to the cell (Fernandez et al. 1993). The inflammatory response, which is probably recurrent, undergoing periods of more accentuated exacerbation, is most likely responsible for progressive neuronal damage, microcirculation alterations, heart matrix deformations and consequent organ failure.

CCC patients have increased expression of major histocompatibility complex (MHC) molecules. Thus, class I MHC are upregulated in the sarcolemma of myocytes in the myocardium (Higuchi Mde et al. 2003) and there is also evidence for an over-expression of class II MHC in endothelial cells (Benvenuti et al. 2000; Laucella et al. 1999; Reis et al. 1993). This may favor the presentation of cryptic epitopes to infiltrating T cells.

3
Autoimmunity and Infection

Two main classes of mechanisms have been described by which infectious agents might activate T and B cells, leading to autoimmunity: Ag-specific and Ag-non-specific (Table 1). The Ag-specific mechanisms mostly state that sequence similarity between infectious agents and self-proteins (molecular mimicry or epitope mimicry) is responsible for the triggering of the autoimmune response (Oldstone 1989; Penninger and Bachmaier 2000; Rose 2001; Rose and Mackay 2000; Wucherpfennig 2001). Autoreactive B and/or T cells, in response to foreign Ags originated by molecular mimicry, can arise from a T/B cell cooperation mechanism, but experimental direct evidence is still scarce (Oldstone 1989; Rose and Mackay 2000). However, there are as yet no absolute formal proofs demonstrating that molecular mimicry is the initiating event of human autoimmune disease and responsible for the pathology, as noted recently (Benoist and Mathis 2001; Fourneau et al. 2004). Probably, Chagas' disease is close to that paradigm. There is some consensus that in order to prove the involvement of epitope mimicry in a disease of suspected autoimmune etiology five criteria must be demonstrated experimentally (Benoist and Mathis 2001; Kierszenbaum 1986) (see Table 2).

The microbial Ag-non-specific theory has several variations. The common characteristic is that no particular microbial determinant is implicated, although the infection may be the initial event which triggers the autoimmune reaction. For example, infection might cause host cell destruction, which results in the release of large quantities of normally sequestered Ags. Those cryptic epitopes found in intracellular proteins are not normally presented in the context of Class I MHC and are therefore not normally encountered by host lymphocytes. These Ags could then be captured by dendritic cells that migrate to T cell areas of the lymphoid organs, where they trigger naïve T cells, or presented at the invasion site, leading to activation of autoreactive cells (but

Table 2 Criteria required for demonstration of the involvement of molecular mimicry in a disease of suspected autoimmune etiology

1. Association of the disease with a particular microorganism
2. Identification of the culprit microorganism epitope that elicits the cross-reactive response
3. T or B cell populations against that epitope should be expanded in the infection
4. Elimination of the cross-reactive epitope from the microorganism should result in non-pathogenic infection
5. Autoreactive T cells should be able to transfer the disease

not against the infecting microorganism). In addition, cryptic epitopes may initiate and maintain autoimmunity through various non-mutually exclusive mechanisms (Lanzavecchia 1995). Those cryptic epitopes can be presented by non-professional Ag-presenting cells (APCs, such as B cells) and induce T cell activation. Autoreactive B cells initiate autoimmunity in the absence of T cells specific for the self-Ag. Alternatively, autoreactive B cells may take up a foreign Ag that cross-reacts with a self-Ag at the B cell level but contains different T cell epitopes. Finally, activated B cells, which efficiently take up and present self-Ag, may prime autoreactive T cells. All these mechanisms may result in a self-sustained autoimmune response.

Microbial infection may result in bystander activation, which may take place in the setting of a proinflammatory milieu. Thus microbial infection induces the release of proinflammatory cytokines such as TNF and chemokines which could be able to activate autoreactive T cells by lowering the threshold of activation (Kim and Teh 2001; Vakkila et al. 2001). These T cells may then proliferate in response to self-Ags presented on host APCs. Inflammation could also alter lymphocyte migration patterns and activate APCs, rendering them more effective as APCs by enhancing Ag uptake and processing, cell surface expression of major MHC molecules, or costimulatory molecules. Finally, infection might provoke polyclonal lymphocyte activation via either a mitogen or a super-Ag effect (Stauffer et al. 2001).

4 Autoimmunity in *T. cruzi* Infection

The finding of a T cell-rich inflammatory mononuclear cell infiltrate and the scarcity of parasites in heart lesions questioned the direct participation of *T. cruzi* in CCC and suggested the possible involvement of autoimmunity, although this remains a hotly debated issue (Engman and Leon 2002; Kierszenbaum 1986, 1999; Levin 1996; Soares et al. 2001; Tarleton 2001, 2003). Several early studies on Chagas' disease already emphasized the scarcity of parasites in histological sections in the chronic phase of the disease (Andrade and Andrade 1955; Mazza 1949). Since then, much research in the field has focused on the possibility that autoimmune responses set off by molecular mimicry and/or bystander activation contribute to tissue damage. Those mechanisms were initially reported many years ago (Acosta and Santos-Buch 1985; Cossio et al. 1984, 1974a, 1974b; McCormick and Rowland 1989; Santos-Buch and Teixeira 1974; Takle and Hudson 1989; Wood et al. 1982) and they were supported by a large body of circumstantial evidence thereafter and have been extensively and sequentially reviewed (Eisen and Kahn 1991; Engman

and Leon 2002; Kierszenbaum 1986, 1999; Leon and Engman 2001; Soares et al. 2001). Although the presence of "anti-self" immune responses in *T. cruzi* infections has been unquestionably demonstrated, the case of the mediation of cross-reactive antibodies or T cells in pathology is still far from settled. Taking into account the variety of the mechanisms of induction of autoimmunity shown in Table 1 the relevant question is, Which mechanisms can be applied to *T. cruzi* infection?

On the other hand, mounting evidence is challenging this view. Thus, with the use of more sensitive techniques, parasite Ags or parasite DNA has been detected during the chronic phase, attributing all the damage either to an inflammatory response against the parasite or to the parasite replication itself (reviewed in Tarleton 2001, 2003; Tarleton and Zhang 1999). It should be emphasized that to date there is no unequivocal demonstration that either autoimmunity or parasite-specific immunity is pathogenic.

5
Molecular Mimicry

The detection of circulating anti-*T. cruzi* antibodies that cross-react with host heart and neural Ags is a common finding in chagasic humans and animal models of infection (reviewed in Engman and Leon 2002; Kierszenbaum 1999, 2003) but, with few exceptions, none of the autoantibodies seems to be the leading cause of autoimmune pathogenesis. In *T. cruzi* infection many examples of molecular mimicry at the level of T cells or antibodies have been described (recently reviewed in Cunha-Neto et al. 2004). However, few of these have been extensively studied and/or defined at the molecular level (see Table 3). We will focus our review only on those examples.

5.1
Mimetic B Cell Epitopes

5.1.1
Myosin

Probably the most studied cross-reactive autoantigen in Chagas' disease is myosin. Several *T. cruzi* Ags have been shown to cross-react with myosin (cardiac or skeletal muscle) and have been implicated in pathogenesis through molecular mimicry. Cunha-Neto and collaborators have described cardiac myosin heavy chain (CMhc) as a major Ag of heart-specific autoimmunity and suggested the possible relevance of myosin recognition in human CCC (Cunha-Neto et al. 1995; Kalil and Cunha-Neto 1996). Antibodies to CMhc

Table 3 Molecular mimicry described during *T. cruzi* infection. Defined and partially defined cross-reactive epitopes

	Cross-reactive antigens	Cross-reactive epitopes	Molecular definition	Reference
Host *T. cruzi*	Cardiac myosin heavy chain (CMhc) B13	AAALDK ::: :: AAAGDK	Ab T cells	Abel et al. 1997
Host *T. cruzi*	23-kDa ribosomal protein Ribosomal protein (R13)	EESD(D/E)DMGFGLFD :: : : :::::::: EEED D DMGFGLFD	Ab	Levitus et al. 1991
Host *T. cruzi*	β1 Adrenergic receptor Ribosomal protein PO	AESDE ::: : AESEE	Ab	Ferrari et al. 1995
Host *T. cruzi*	β1 Adrenergic receptor[a] M2 muscarinic receptor[a] Ribosomal protein (R13)	--ED-D-GF-LFD[a] --EDDDMGF-LFD[a] EEEDDDMGFGLFD	Ab	Mahler et al. 2004
Host *T. cruzi*	47-kDa neuron protein FL-160	TPQRKTTEDRPQ	Ab	Van Voorhis et al. 1991
Host *T. cruzi*	Cha antigen Shed acute-phase antigen (SAPA)	SLVTCPAQGSLQSSPSMEI : . :: : :.:: . STPSTPADSSAHSTPSTPV	T cells	Girones et al. 2001b
Host *T. cruzi*	Cha antigen TENU2845/36 kDa	MRQLDTNVER .:::: ::. LRQLDF-VEE	Ab	Girones et al. 2001a, 2001b

[a]Residues of the R13 epitope that when interacting with purified antibodies trigger stimulation of the denoted receptor.

were found in symptomatic as well as asymptomatic patients. Affinity-purified anti-CMhc antibodies specifically recognized two polypeptides of 140,000 and 116,000 Da in *T. cruzi* trypomastigotes. At the molecular level, cross-reactivity was shown to exist between the amino acid sequence AAALDK of CMhc and the AAAGDK sequence from a recombinant *T. cruzi* peptide named B13 (Table 3). All sera from patients with CCC disease, but only 14% of sera from asymptomatic chagasic patients, recognized B13 (Gruber and Zingales 1993). These results have been often disputed, although no contradictory results have been published (see Kierszenbaum 2003). However, there were some discrepancies between the 100% reactivity of the sera from chronic patients

with overt heart disease with CMhc and only 61% reactivity in those with *T. cruzi,* a finding difficult to reconcile with molecular mimicry.

Cruzipain, a well-defined and highly abundant *T. cruzi* Ag, is involved in CCC pathogenesis by various direct and indirect mechanisms. The latter are also related to cross-reactivity with myosin, although not with CMhc but with skeletal muscle myosin heavy chain (SMhc). Thus purified anti-cruzipain antibodies raised in cruzipain-immunized mice cross-react with SMhc (Giordanengo et al. 2000a, 2000b) and, more importantly were associated with heart conduction disturbances in those animals. Moreover, ultrastructural findings revealed severe alterations of cardiomyocytes and IgG deposit on heart tissue of immunized mice. Giordanengo et al. investigated whether antibodies induced by cruzipain transferred from immunized mothers to their offspring could alter the heart function in the pups. All IgG isotypes against cruzipain derived from transplacental crossing were detected in pups' sera. Electrocardiographic studies performed in the offspring born to immunized mothers revealed conduction abnormalities (Giordanengo et al. 2000b). These results provide strong evidence for a pathogenic role of the humoral autoimmune response induced by a purified *T. cruzi* Ag in the development of experimental Chagas' disease. More recently, Sterin-Borda et al. have reported that immunization with cruzipain also induces autoantibodies against muscarinic acetylcholine receptors which can be implicated in pathology (Sterin-Borda et al. 2003). However, in both cases described above the molecular identification of cross-reactive epitopes of cruzipain and host proteins is still lacking.

On the other hand, *T. cruzi*- infected A/J mice (a strain of mice highly susceptible to *T. cruzi* infection) generated anti-myosin IgG, both in the acute phase and the chronic phase of infection (Leon et al. 2001). Moreover, heart lesions resembling those seen in *T. cruzi*-infected mice can be induced by immunization with purified myosin. However, not all mouse strains are equally susceptible to myocytolysis after *T. cruzi* infection (Leon and Engman 2001). Interestingly, in C57BL/6 mice, the levels of anti-myosin IgG found after *T. cruzi* infection were small or undetectable and no myocarditis was observed in the acute phase (Leon et al. 2001). Moreover, the C57BL/6 mouse strain has been claimed not to develop cardiac autoimmunity after immunization with myosin (Neu et al. 1987). These results suggest that generation of anti-myosin antibodies by *T. cruzi* infection or myosin immunization depends on the genetic background of the host and that there is a clear relationship between anti-myosin IgG and heart damage. However, from these results it is unclear whether anti-myosin IgG is the cause or the effect of heart damage. Accordingly, we have seen that C57BL/6 mice infected with *T. cruzi* did not develop clinically relevant myocarditis in the acute phase. However, at 120 days after infection, C57BL/6 mice developed a milder myocarditis compared to mice

deficient for iNOS gene with the same genetic background (Girones et al. 2004). Because C57BL/6 developed lower parasitemias than iNOS knockout mice in the acute phase, this may be taken as an indication that the presence or absence of myocarditis may depend on the initial level of control of parasite replication rather than on the genetic background of the host.

In contrast to the above, other reports suggested that anti-myosin antibodies are not involved in the pathogenesis. For example, immunization with myosin in immunosuppressed mice did not induce autoantibodies but still caused myocarditis (Neu et al. 1990). How myosin can trigger myocarditis in these immunosuppressed mice is difficult to envisage. Moreover, passive transfer of a high-titer anti-myosin antibody preparation failed to induce myocarditis (Neu et al. 1990). Because the fine specificity of the different anti-myosin Igs has not been addressed in most of those studies, they are difficult to compare. Myosin is a very large molecule and it is possible that the myosin determinant(s) recognized by the different sera are not identical.

Some authors believe that other mechanisms than molecular mimicry can explain myosin autoreactivity (see Benoist and Mathis 2001; Engman and Leon 2002; Kierszenbaum 2003). They feel that mimicry is less likely to be occurring than Ag release due to myocardial damage leading to expansion of normally tolerant myosin-reactive T cells, particularly because myosin autoimmunity is seen in myocarditis associated with other insults. Thus anti-myosin antibodies are induced in patients with heart disease unrelated to *T. cruzi* infection such as viral myocarditis, myocardial infarction, coronary artery bypass and heart valve surgery, among others (de Scheerder et al. 1989; Fedoseyeva et al. 1999; Nomura et al. 1994). B cell anti-myosin response seems to be mainly responsible for pathology in other heart infections, induced by Coxsackie B3 viral infection (Rose and Hill 1996) or by bacteria (Cunningham 2004). In this regard, it is worth mentioning that peptides of CMhc, a cytoplasmic protein, are associated with MHC class II molecules on APCs even in normal mouse myocardium (Smith and Allen 1992) and MHC class II molecules are increased in the heart of *T. cruzi*-infected patients and animals. Cardiomyocyte damage caused either by parasite replication in the heart or by inflammation may release self-Ags, leading to the induction of anti-heart antibodies rather than anti-cross-reactive *T. cruzi* Ags. Thus it could be likely that the initial heart tissue destruction resulting from infection could induce anti-myosin immunity in Chagas' heart disease, being thus the effect and not the cause of the pathology. Alternatively, is possible that although several pathogens may share the ability to destroy the heart they may have different cross-reactive epitopes with heart proteins (myosin). Thus it is possible that the trigger is the combination of pathogen and damage together, although the fine specificity of the autoreactive response against myosin will be different for each heart pathogen.

In summary, before suggesting a possible role for anti-myosin immunity in Chagas' heart disease, some questions need to be fully addressed: (1) Is the damage during *T. cruzi* infection different from heart tissue injury of a different etiology? (2) Do anti-myosin antibodies truly contribute to chagasic pathology? (3) If anti-myosin antibodies appeared after the occurrence of tissue damage, would they aggravate the pathology by mediating the destruction of intact cardiomyocytes?

5.1.2 Ribosomal Proteins

Another set of autoantigens which have been involved in CCC pathology are ribosomal proteins. Anti-ribosomal P protein antibodies were detected in the serum of chagasic patients and their titer associated with the degree of myocarditis, suggesting a correlation between the appearance of these antibodies and heart pathology (Levin et al. 1990, 1989; Skeiky et al. 1992). By screening a *T. cruzi* expression cDNA library with such sera, some DNA clones were identified. One of the clones, termed JL5, codified for a *T. cruzi* ribosomal protein, TcP2L, and showed sequence homology with human P ribosomal proteins. The homology was between the EDDDMGFGLFD region of Tc2PL and the SD(D/E)DMGFGLFD sequence present in the C-terminal region of human P ribosomal protein (R13 epitope) which was responsible for the cross-reactivity in chagasic serum (Table 3). However, reactivity with ribosomal proteins is also found in some patients with systemic lupus erythematosus (SLE); approximately 15% of SLE patients have autoantibodies to a shared epitope (H13) located in the C-terminal regions of the ribosomal proteins, P0, P1, and P2 (Elkon et al. 1986). However, antibodies against ribosomal proteins from CCC and SLE patients show differential recognition. Thus sera from patients with chronic Chagas' heart disease have been shown to contain relatively high levels of anti-R13 but low levels of anti-H13 antibody (Lopez Bergami et al. 1997), whereas both titers are comparable in SLE sera (Kaplan et al. 1997, 1993). Despite this positive correlation between molecular mimicry and pathology, some discrepancies exist. First, attempts to link anti-R13 reactivity by ELISA in the sera with the symptomatology in chronic or asymptomatic patients failed to find a significant correlation. Thus 60% and 49%, of chronic and asymptomatic sera, respectively, displayed reactivity with R13 but varied significantly depending on the geographical origin of the patients (Aznar et al. 1995). Moreover, no correlation between anti-R13 reactivity and cardiomyopathy was found in a group of 14 patients from whom endomyocardial biopsies and blood samples were taken at the same time. Furthermore, mice immunized with TcP2L developed antibodies against

the cross-reactive epitope, as well as many others, in contrast with the fine specificity of antibodies obtained from infected mice (Sepulveda et al. 2000).

On the other hand, some evidence indicates that those anti-P ribosomal antibodies could be pathogenic. Thus purified IgG, reactive with the C-terminus epitope of *T. cruzi* ribosomal P protein, caused a chronotropic alteration in primary rat cardiomyocytes through selective stimulation of β1-adrenergic receptors (Elies et al. 1996; Ferrari et al. 1995). However, in this case, the relevant cross-reactive epitope included the AESDE amino acid sequence from the second extracellular loop of the human β1-adrenergic receptor, which is homologous to the internal AESEE sequence of TcP0 (Table 3) (Ferrari et al. 1995). Moreover, passive transfer of a mAb against R13, which cross-reacts with the human β1-adrenergic receptor, had a chronotropic effect on cultured rat cardiomyocytes (Mahler et al. 2001). Mice immunized with P0 *T. cruzi* ribosomal protein develop electrocardiographic alterations late after immunization, when the titer of antibodies is extremely high, similar to those in chagasic animals but not identical to the complex response of chronic *T. cruzi* infection (Lopez Bergami et al. 2001). In contrast, those hyperimmunized with TcP2L died at an earlier time and did not show heart inflammation.

Sera from chagasic patients also contain IgG antibodies which immunoprecipitated human M2 muscarinic cholinergic receptor molecules and which were able to activate them, having an agonist effect on cardiomyocytes and causing partial desensitization (Leiros et al. 1997). The original stimulus for the formation of these antibodies was not ascertained and whether they could cause heart dysfunctions of the types seen in chagasic patients remains an open question.

5.1.3 Cha

We have described an autoantigen, Cha, a mammalian transcription factor which is recognized by almost all chagasic sera and by sera from *T. cruzi*-infected mice (Girones et al. 2001b). This Ag was isolated by screening of a library with seven CCC sera and has two regions of homology with *T. cruzi*, one with in an expressed sequence tag of the parasite (TENU2845) and another with SAPA, the Ag shed in the acute phase of *T. cruzi* infections (Table 3) (Cazzulo and Frasch 1992; Pollevick et al. 1993). Interestingly, we found that the two epitopes, named R1 and R3, are recognized by T and B cells, respectively, both having significant sequence homology. Very interestingly, there is a strong association of anti-Cha (R3) antibodies and pathology. Thus the titer of the sera from chagasic patients against R3 increases with symptomatology and decreases with treatment (Girones et al. 2001a). However, we have not

determined yet whether anti-R3 antibodies have any effect on pathology of the disease. Future experiments will focus on this. Our hypothesis is that these antibodies arise during infection by cooperation of Cha-specific B cells with T cells of different specificity (Fig. 2).

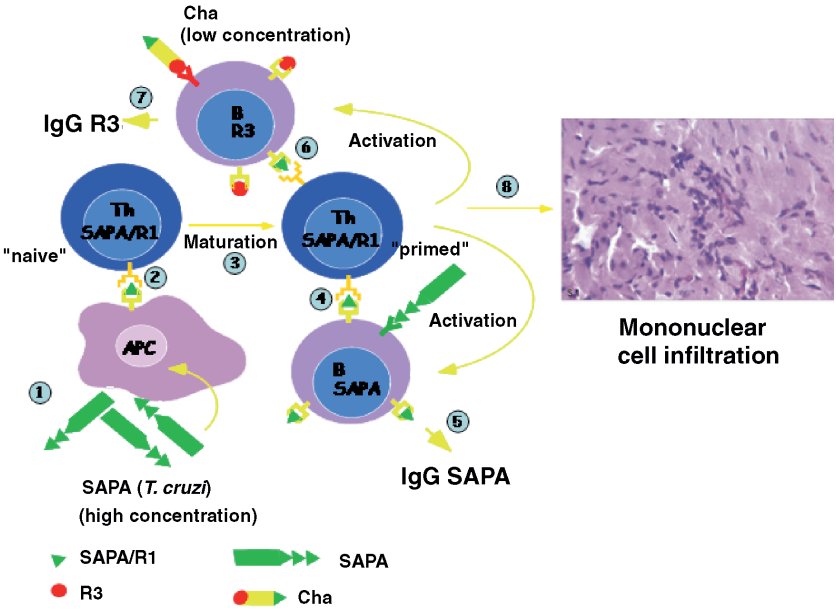

Fig. 2 Generation of anti Cha T/B cell responses. *T. cruzi* infection causes the secretion of parasite Ags to the medium and lyses host's cells, triggering release of self Ags. In particular, during *T. cruzi* infection SAPA Ag is released to the extracellular environment and is taken up by macrophages (*1*). There SAPA is processed intracellularly and presented to naïve T cells through MHC Class II molecules (*2*). These T cells undergo maturation and develop into primed effector T cells specific for SAPA and the cross-reactive epitope R1 of Cha (*3*). Interaction of SAPA/R1 T cells with B cells of the same specificity (*4*) triggers anti-SAPA antibody production (*5*), which is observed during infection. However, the Cha Ag epitopes can be presented on the surface of B cells by MHC Class II molecules by two possible mechanisms: (a) The Cha epitopes can be naturally presented on B cells and (b) the Cha epitopes can be released during infection due to lysis of infected cells. Then, SAPA/R1 T cells can interact with B cells that present the R1 cross-reactive epitope of Cha (*6*) and trigger anti-Cha(R3) antibodies of different specificity (*7*). On the other hand, SAPA/R1 T cells are able to induce inflammatory infiltrates and damage in hearts of recipient mice through cytokines and/or activation of CD8 T cells (*8*)

5.2 Autoreactive T Cells

Perhaps the best evidence supporting a role for autoantigen-specific autoimmunity in disease pathogenesis derives from studies on T cell-mediated immunity in mice. Ribeiro-Dos-Santos et al. have reported that a $CD4^+$ T cell line obtained from a chronic chagasic mouse consisting of approximately 95% $CD4^+$ T cells proliferated in response to either a crude *T. cruzi* Ag preparation or heart tissue extracts from different animal species (Ribeiro-Dos-Santos et al. 2001). In culture, this cell line arrests the beating of fetal heart cells and, more importantly, induces myocarditis in immunized mice and promotes rejection of transplanted normal hearts in the absence of *T. cruzi* (Ribeiro-Dos-Santos et al. 2001). The requirement of the parasite to cause rejection in mice transplanted with T cells from infected mice has been also widely debated (Cunha-Neto et al. 1995; dos Santos et al. 1992; Ribeiro-Dos-Santos et al. 2001; Tarleton et al. 1997). Thus rejection of syngeneic transplanted hearts in chronically infected mice has been shown to take place either in the absence (dos Santos et al. 1992) or in the presence (Tarleton et al., 1997) of the parasite. These differences may be due to the different mice and parasite strain combinations used, and when the presence of the parasite is required for rejection, inflammation and not *T. cruzi* replication may be necessary to provide the necessary adjuvant effect to trigger autoreactivity and could be the rejection-inducing agent in the implanted hearts.

Besides proposing that B cell cross-reactivity against myosin is involved in pathogenesis, Cunha-Neto et al. have also proposed that myosin cross-reactive T lymphocytes infiltrating heart tissue lesions are also involved in chronic chagasic heart tissue lesions (Cunha-Neto et al. 1996, 1995). These T cells are also activated by CMhc cross-reactive *T. cruzi* Ag B13 as in B cells (Cuhna-Neto 2000; Cunha-Neto et al. 1996) (Table 3). Thus T cells from chagasic patients with overt heart disease or asymptomatic patients responded to in vitro stimulation with B13 with increased IFN-ɣ and reduced IL-4 production, suggesting a Th1-type cytokine profile (Cunha-Neto and Kalil 2001; Cunha-Neto et al. 1998). Those authors proposed that heart damage in CCC could be secondary to the release of inflammatory cytokines and a DTH process initiated by B13. However, the assumption that pathology arises from molecular mimicry between B13 *T. cruzi* and CMhc has been challenged by other authors because T cell autoreactivity against B13 was shown to exist not only in CCC but also in asymptomatic patients and in other cardiopathies (Kierszenbaum 2003). Moreover, both the level of the response to B13 and the cytokine production profile of lymphocytes from asymptomatic chagasic

patients were similar to those of T cells from patients with overt heart disease (Cunha-Neto and Kalil 2001).

It is noteworthy that immunological tolerance to heart Ags induced in mice by heart Ag administration and anti-CD4 antibody before their infection by *T. cruzi* resulted in less intense cardiopathy than that in control non-tolerized animals (Pontes-de-Carvalho et al. 2002), which is in favor of an autoimmune pathology. This treatment affects $CD4^+$ responses and not the production of anti-myosin IgG. Although this suggests that the regime to make the mice tolerant was not as effective as expected, at least regarding the humoral response (Th2 mediated), it is becoming increasingly evident that the response involved in heart damage is Th1 mediated. Recently, Leon et al. have described (although in the acute phase) that myosin autoimmunity, while a potentially important inflammatory mechanism in acute and chronic infection, is not essential for cardiac inflammation (Leon et al. 2003), although immunization with a *T. cruzi* extract induced a DTH response against myosin (Leon et al. 2004).

We also studied the T cell response to Cha autoantigen during *T. cruzi* infection. T lymphocytes from *T. cruzi*-infected mice also proliferated to recombinant Cha. More interestingly, transfer of T cells from chronically infected mice to naïve syngeneic mice led to heart infiltration and to production of anti-Cha antibodies, detectable 60 days later (when chronic pathology arises in mice after *T. cruzi* infection) (Girones et al. 2001b). Transferred T cells were almost pure CD3 cells (99%). Consistently, transfer of T cell clones specific for SAPA/R1 cross-reactive epitopes results in heart infiltration in the absence of anti-Cha antibody production (Girones et al., in preparation). Therefore, in some cases the presence of the parasite is not necessary to produce pathology if one transfers activated autoreactive T cells. How this takes place and whether the Cha autoantigen (normally an intracytoplasmic protein) comes to be presented to T cells are under investigation in our laboratory (see Fig. 2 for a hypothetical model). The observed anti-Cha response is likely due to a cooperation of R1-Cha-specific T cells with naïve anti-R3 autoreactive B cells. We believe that Cha autoreactive T cells are responsible for the heart damage, and that Cha autoantibodies are an epiphenomenon secondary to heart tissue destruction. Our results suggest that T cells cooperate with naïve B cells in the animal after heart damage because transfer of T cell clones induces heart infiltration but no anti-Cha antibodies. Although our results suggest that Cha may be involved in pathology, this by no means indicates that Cha would be the only autoantigen involved in the pathology of Chagas' disease.

6
Bystander Activation

As reviewed recently by von Herrath et al. (2003), bystander activation is defined as the activation of autoreactive lymphocytes that do not recognize microbial Ags. This can be mediated through cytokines and/or APCs (TCR-independent bystander activation). However, bystander activation might also require concurrent exposure to the cognate Ag. Ag-specific cells induced by molecular mimicry can be activated by a non-specific stimulus such as other

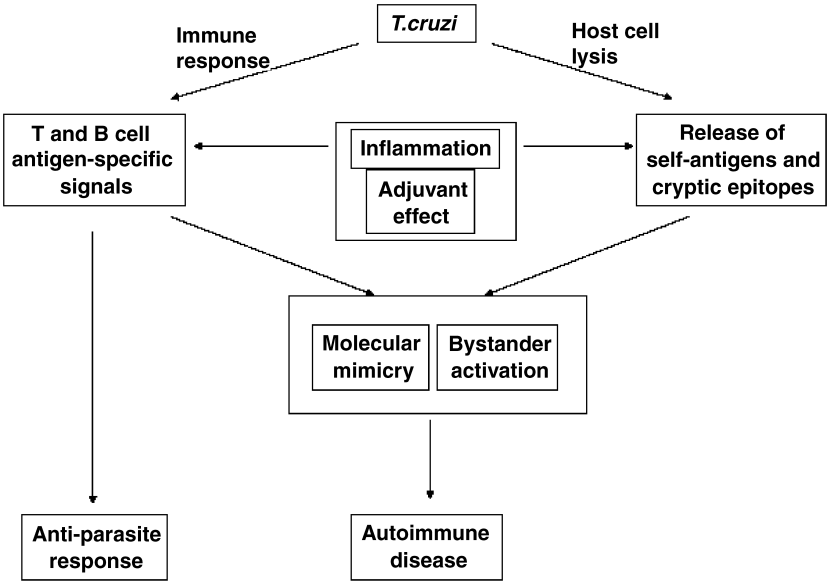

Fig. 3 Diagram of the different mechanisms of induction of pathogenicity by *T. cruzi*. *T. cruzi* induces T and B cell anti-parasite responses which, through molecular mimicry with extracellular Ags or epitopes in Ags normally presented by APCs, can lead to autoimmune disease. *T. cruzi* can lead to secretion of cytokines which mediate some cardiac damage, liberating autoantigens reconized by autoreactive T cells and autoantibodies that further damage the cardiac tissue via bystander activation. Simultaneously parasite replication can induce release of self-antigens, usually intracellular, which contain cryptic epitopes that can be presented by APCs. Also, over-expression of intracellular Ags induced by *T. cruzi* can result in presentation of cryptic epitopes by APCs. If cryptic epitopes are cross-reactive with *T. cruzi* epitopes, then autoimmune disease can arise. *T. cruzi* contains several molecules capable of stimulating the immune system in a non-Ag-specific manner, known as the adjuvant effect, which together with the release of self-antigens and exposure of cryptic epitopes can contribute to sustain a local immune activation known as bystander activation

infections or heart damage, or by adjuvants in experimental settings, to induce autoimmune disease. Regarding *T. cruzi* Ags, both types of bystander activation have been described. *T. cruzi* parasitization of host tissue induces the release of autoantigens (Talvani et al. 2000) and a proinflammatory environment rich in cytokines, nitric oxide and chemokines sufficient to activate autoreactive T cells by lowering the threshold of activation (Fedoseyeva et al. 1999) (see Fig. 3). These cells may then proliferate in response to self-Ag presented on host APC. If this were the case for myosin, aberrant Ag presentation per se would not be necessary, because peptides of cardiac myosin, a cytoplasmic protein, are found complexed with class II MHC molecules on APCs even in normal mouse myocardium (Smith and Allen 1992) and are increased in the heart of infected patients and animals. The anti-self response is initiated and tissue damage may ensue if the response is of sufficient intensity.

7
Parasite Persistence

Despite all the facts mentioned above, several researchers in this field defend the idea that *T. cruzi* persistence in the infected host is solely responsible for the damage in the chronic phase. Tarleton has reviewed all the arguments in favor of the parasite persistence hypothesis to explain the pathogenesis of chronic Chagas' disease in general and of CCC in particular (Tarleton 2001, 2003; Tarleton and Zhang, 1999). Arguments in favor of the idea that disease is linked to parasite presence are supported by the fact that treatments which decrease the parasite burden in the acute phase are associated with a decrease in clinical symptoms (Viotti et al. 1994). Enhancing the efficiency of the anti-parasite response by immunotherapy, gene deletion, or vaccination results in decreased severity of the chronic phase, not exacerbation of disease as predicted by the autoimmune hypothesis (Tarleton 2003). However, this argument cannot be used against autoimmunity because the fine specificity (cross-reactivity?) of those anti-parasite responses was not studied. Effective chemotherapy could also enhance anti-*T. cruzi* immunity in mice (Olivieri et al. 2002). In humans, the link between persistence of *T. cruzi* and clinical disease is also supported by the tissue-specific detection of parasite DNA in the hearts, but not in the esophageal tissue, of individuals with cardiac disease, and vice versa (Jones et al. 1993; Vago et al. 1996). Very recent data in humans show a higher frequency of parasite-specific IFN-γ-producing $CD8^+$ T cells among chronic Chagas' disease patients with mild clinical disease than in those with the most severe form of the disease, supporting a link between the strength and nature of the anti-parasite response and the severity of chronic-

stage disease (Laucella et al. 2004). Apparently this supports the parasite persistence hypothesis in opposition to autoimmunity, arguing strongly in favor of the participation of an effective anti-parasite response in preventing disease (Tarleton 2003). Conversely, it has been observed that immunosuppressive treatments correlate with exacerbation of the infection and disease (Ferreira and Borges 2002) although this is not always the case. Thus the use of cyclosporin A has been shown to reactivate parasitemia in several but not all of the heart-transplanted patients. In general, chagasic heart transplants are not rejected or suffer from myocarditis despite the use of immunosuppressive drugs (see Kierszenbaum 2003). These data have been taken as an argument against an autoimmune-based pathology in CCC because transplanted hearts given to patients with the most severe cases of Chagas' heart disease remained essentially undamaged for so many years. However, we need to be cautious because few studies have gone more than 10 years when in a normal infection the pathology of CCC sometimes appears 15–30 years after primary infection. Moreover, if proven true, this mostly discards autoantibodies as the main pathological cause of CCC, but not autoreactive T cells. The same treatment that suppressed alloantigen T cell reactivity may have suppressed autoreactive T cells. So a role for T cells cannot be discarded.

In addition, we have found that autoreactivity in the chronic phase is also linked to parasitemia because the antibody titer and number of reactive T cells against the Cha autoantigen are lower in C57BL/6 (non-susceptible) than in BALB/c (susceptible) mice (Girones et al. 2001b). Moreover, potentially pathogenic anti-Cha autoantibodies also decreased with chemotherapeutic treatment of Chagas' patients. The titer of anti-Cha antibodies, as well as anti-*T. cruzi* antibodies, decreased in parallel with treatment and increased with symptomatology (Girones et al. 2001a) (Table 4). Thus anti-parasite response, some anti-self responses and pathology seem to go together. This poses a word

Table 4 Myocarditis and antibody responses in chagasic patients increase with symptomatology and decrease with treatment

Chagasic patients	Anti-Cha antibodies	Anti-*T. cruzi* antibodies	Myocarditis
Symptomatic	+++	+++	+++
Asymptomatic untreated	++	++	−
Asymptomatic treated	+	+	−

The presence or absence of myocarditis was given by clinical histories of patients. Antibody response was taken from Girones et al. 2001a (OD 450 nm<0.3, $^{+}$; OD 450 nm between 0.3 and 1.0, $^{++}$; OD 450 nm<1.0, $^{+++}$).

of caution in interpreting some clinical data when not all aspects of the problem are measured. Those results may be interpreted in very different ways: (a) the parasite is the only cause of the disease and anti-parasite and anti-self responses are direct consequences of parasite replication, (b) pathology may be caused by the anti-*T. cruzi* response or (c) the parasite is the trigger of autoimmune response which is the effector mechanism.

The presence of *T. cruzi* in the chronic phase of the disease was already observed in early descriptions (Vianna 1911) and was documented subsequently by other authors (Almeida et al. 1984; Teixeira Vde et al. 1993). With more sensitive techniques such as polymerase chain reaction (PCR), the parasite (more properly parasite DNA) is commonly detected in chronic patients (reviewed in Higuchi Mde et al. 2003). Recent immunohistochemistry studies have demonstrated higher frequencies of *T. cruzi* Ags, reaching 100% of hearts from chronic chagasic patients who died due to heart failure when several samples of the myocardium were analyzed (Higuchi 1993; Palomino 2000). Many previous failures to detect parasite Ags in biopsy material from patients in the chronic phase have been attributed to the fact that it seems necessary to examine several different sections of the heart to detect the parasite in this phase of the disease (Higuchi Mde et al. 2003). Using a mouse strain which develops chagasic cardiomyopathy when infected with a highly virulent *T. cruzi* strain, amastigotes were detected in myocytes through the chronic phase, although their numbers were low and much lower than in the acute phase (Guarner et al. 2001). A general finding not always acknowledged by the supporters of the parasite persistence hypothesis is that there is no direct correlation between the sites of parasite detection and heart damage, and also no correlation between the levels of parasites (for example, as detected by PCR) and clinical findings (Monteon-Padilla et al. 2001). However, a significant association between the presence of *T. cruzi* Ags in the heart and severe or moderate inflammation was observed both in humans (Higuchi Mde et al., 2003) and in animal models of the disease (Buckner et al. 1999). However, the number of parasites was low in relation to the intensity of the myocarditis and whole myocardial fibers containing parasites did not elicit inflammation (Higuchi Mde et al. 2003). This suggests two possibilities: exuberant host reactions to the few remaining parasites, either immune mediated or not, or autoimmune-induced inflammation. Parasite Ags probably work as a trigger response against the myocardial fibers. In addition, it is plausible that some lesions lack parasites or parasite Ags because of the effective clearance of parasites from the site by an effective anti-parasite immune response, thus preventing observation of an exact correlation. However, this is difficult to reconcile with the fact that a strong anti-parasite immune response results in decreased symptoms (Laucella et al. 2004).

Thus parasites are somehow present in the chronic phase, but what one ought to know is whether relevant parasite Ags persist and are presented by APCs to T cells. No matter the Ag recognized, Ag-specific T cells must be stimulated to become effector cells (helper, cytotoxic or other). For this, the Ag needs to be presented. Although some APCs could be very efficient in presenting Ags, it is rather unlikely that there are enough parasite Ags to continuously support chronic T cell stimulation.

Recently, it has been shown that *T. cruzi* kinetoplast DNA is able to integrate into human and other mammalian cell genomes and was transmitted to the descendants (Nitz et al. 2004). This has important implications not only for the detection of parasite mentioned above (some based in kinetoplast DNA) but also for pathology, because *T. cruzi* kinetoplast Ag could be continuously presented and may continuously trigger a response to those Ags of the parasite, thus killing normal cells.

8
Coexistence of Parasite Persistence and Autoimmunity

We think that because cardiac myosin autoimmunity develops in the acute phase, when there is lysis of cardiac myocytes and easily detectable parasites, it is very likely that the two processes, bystander damage and molecular mimicry, co-exist until the chronic phase, where damage is produced via effector cells recognizing cross-reactive *T. cruzi*/autoantigen through molecular mimicry.

Thus we propose that the parasite is the trigger which activates some T cells (autoantigen/cross-reactive parasite Ag). Once they are activated, they secrete inflammatory cytokines which mediate some cardiac damage. This liberates autoantigen which is also recognized by some other autoreactive T cells and autoantibodies which further damage the cardiac tissue via bystander activation. This is like a vicious cycle triggered by parasite Ags but fueled by cross-reactive autoantigens and implies that purely parasite-specific T cells may cause very little cardiac damage. This also involves two of the proposed pathogenic mechanisms: bystander damage and molecular mimicry. *T. cruzi* might also function as an adjuvant for an immunological cross-reaction between common parasitic and myocardial fiber Ags, resulting in severe lymphocytic myocarditis (see Fig. 3).

Thus parasites are necessary to trigger autoantibodies and autoreactive T cells and may be necessary to maintain them in the chronic phase. Altogether, we believe that active *T. cruzi* infection is necessary to trigger the autoimmune

process, most likely through autoreactive T cells, which once induced can produce the cardiac pathology.

Obviously, the elucidation of the mechanisms of pathogenesis in Chagas' disease may have implications for vaccination and therapy. If autoimmunity by molecular mimicry is responsible, anti-*T. cruzi* chemotherapy would not necessarily suppress pathogenic autoimmune responses initially elicited by parasite Ags and subsequently boosted by host tissue Ags. Also, in the search for protective vaccine we should discard *T. cruzi* Ags that elicit pathogenic anti-self responses.

9 Final Remarks

As mentioned above, several criteria, put originally forth in the *T. cruzi* field by Kierszenbaum 1986 and more broadly by Benoist et al. (Benoist and Mathis, 2001) must be met to consider a disease as caused by molecular mimicry (see Table 2). In *T. cruzi* infection, the first three conditions have been clearly demonstrated, and this has allowed the identification of several candidate autoantigens. If there were a unique cross-reactive Ag, infection with genetically deficient parasites lacking the inducing Ag, or infection of knockout mice lacking the cross-reactive autoantigen, would prevent the disease. However, as multiple autoantigens seem to be involved in the pathology of Chagas' disease, such experiments are very difficult to perform, and therefore the fourth criterion has not been demonstrated yet.

The fifth criterion is considered to be the decisive test of the concept of autoimmunity.

In most publications about autoimmunity in Chagas' disease the putative causes are either autoantibodies or autoreactive T cells originated by molecular mimicry between parasite and host Ags. Nevertheless, evidence for the mediation of cross-reactive antibodies or T cells in pathology is still far from settled. Moreover, most of the data come from experimental *T. cruzi* infection, and an additional problem is the extrapolation of the results to the human model which is more difficult to study.

One way to determine the pathological effect of autoreactive T or B cells would be to immunize mice with cross-reactive Ags to see whether this induces pathology. However, immunization with an autoantigen (injected together with adjuvants and via different routes than natural infection) may not reflect the way the autoantigen is presented during natural infection and may elicit hyperimmune responses, tolerance or regulatory T cells which may suppress

autoimmunity. An alternative approach is the transfer of putative autoreactive T cells from chronically infected mice specific for a given autoantigen. Either immunization with or transfer of T cells specific for autoantigens may answer some of these questions and determine which of the candidates are really relevant for pathology. In this respect, the presence of autoreactive T cells against Cha proteins shown in our experiment with Cha autoantigen is the closest to this. Recently, an interesting observation was made by von Herrath et al. (2003), proposed that autoimmune diseases could be induced and exacerbated by many different microbial infections. Their hypothesis is that after infection there is exposure to self, foreign and environmental agents. After clearance of infection, the inflammatory response drops, but when there are additional infections the threshold for autoimmunity is reached and autoaggressive T cells expand and develop.

On the other hand, the parasite persistence hypothesis is based on the fact that *T. cruzi* persists in the chronic phase of Chagas' disease and that treatment against the parasite results in a decrease of the severity of the disease. There are also some questions that need to be fully addressed: (1) Why do lesions develop primarily in the heart and not at other sites of parasite persistence? (2) Why does parasite burden not always correlate with disease severity? A demonstration of these hypotheses is also difficult to perform, because one ought to separate the components of the immune response, self and anti-self during infection. However, we think that things are not so easy, because co-existence of self and non-self Ags would enhance the immune response against both, being always triggered by the parasite.

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