RESUMEN

Trypanosoma cruzi es el agente causal de la enfermedad de Chagas, un paradigma de enfermedad autoimmune inducida por infección. La escasez de parásitos en la fase crónica de la enfermedad contrasta con la severa patología cardíaca observada en aproximadamente un 30% de los pacientes crónicos, y sugiere un papel para la autoinmunidad en el origen de la patología. Dependiendo de la respuesta inmunitaria contra el parásito, se han descrito mecanismos antígeno específicos y antígeno no-específicos que pueden activar células T y B y causar autoinmunidad. Entre los primeros, el mimetismo molecular (similitud de secuencias entre antígenos del agente infeccioso y del hospedador) se considera el mecanismo más importante que puede conducir a autoinmunidad y patología en la fase crónica de esta enfermedad. Con el uso de técnicas más sensibles, el parásito se detecta cada vez más fácilmente en el hospedador infectado, el cual puede padecer patología bien directamente o a través de la respuesta inflamatoria específica contra el parásito. Por ello, el tema de la autoinmunidad versus persistencia del parásito como causa de la patología en la enfermedad de Chagas es extensamente debatido entre los investigadores del área. Nuevos argumentos a favor y en contra de cada hipótesis han sido publicados recientemente. En el presente trabajo pasamos revista a ambas hipótesis de forma crítica y desde perspectivas clínicas, patológicas e inmunológicas.

PALABRAS CLAVE: Trypanosoma cruzi / Enfermedad de Chagas / Autoinmunidad / Mimetismo molecular.

ABSTRACT

Trypanosoma cruzi is the pathogenic agent responsible for the Chagas’ disease, a paradigm of infection–induced autoimmune disease. 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 suggests a role for autoimmunity as the origin of the pathology. Depending on the anti-parasite immune response, 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 ones, molecular mimicry (sequence similarity between infectious agents and self-proteins) has been claimed as the most important mechanism leading to autoimmunity and pathology in the chronic phase of the disease. The use of more sensitive techniques has led to the easy detection of the parasite in the infected host, who can undergo 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 of the field. Lately, there have been numerous reports offering arguments in favour of one or another hypothesis. We critically review here both hypotheses from clinical, pathological and immunological perspectives.

KEY WORDS: Trypanosoma cruzi / Chagas’ disease / Autoimmunity / Molecular mimicry.
TABLE I. Mechanisms for activation of T and B cells in autoimmunity

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TABLE II. Criteria required for demonstration of the involvement of molecular mimicry in an autoimmune disease

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.

ROLE OF INFECTIOUS DISEASES IN AUTOIMMUNITY

The idea that infection plays an important role in initiating autoimmune disease dates back more than a century and is one of the most enduring paradigms in the annals of autoimmunity. To understand how microbial infections might cause autoimmune diseases it must be taken into account that immunocompetent individuals harbour potentially autoreactive T and B lymphocytes, that are normally tolerant to self antigens. These cells remain innocuous unless somehow activated. The infection is thought to be the trigger of that activation. For example, in some animal models such as experimental autoimmune encephalomyelitis (EAE) or the non-obese diabetic (NOD) mouse model for type I diabetes, only activated but not resting T cells can transfer the disease.

Two main classes of mechanisms have been described by which infectious agents might activate T and B cells and lead to autoimmunity: antigen-specific and antigen-non-specific (Table I). In the microbial antigen-specific mechanism the sequence similarity between infectious agents and self-proteins (molecular mimicry or epitope mimicry) might trigger the autoimmune response. Antigenic determinants on the infecting microorganism’s proteins are structurally similar to one or various determinants on the host proteins, but are different enough to be recognised as foreign by the immune system of the host. Many reported evidences indicate that molecular mimicry is commonplace and many sequential and structural determinants of infectious agents have been shown to stimulate crossreactivity with epitopes on host tissues. Autoreactive B and/or T cells in response to foreign antigens originated by molecular mimicry can arise from a T/B-cell cooperation mechanism, but direct experimental evidence is still scarce. Moreover, there is, as yet, no absolute formal proof that molecular mimicry is the initiating cause of human autoimmune disease and responsible for the pathology, as remarked recently. Probably, Chagas’ disease is close to that paradigm. To prove the involvement of epitope mimicry in a disease of suspected autoimmune aetiology five criteria need to be confirmed experimentally (Table II).

The microbial antigen non-specific mechanism of autoimmunity has several variants. The common characteristic is that no particular microbial determinant is implicated, although the infection may be the initial event that triggers the autoimmune reaction. For example, infection might cause destruction of host cells, which results in the release of large quantities of normally sequestered proteins. Those cryptic epitopes found in intracellular proteins are not normally presented in the context of class I Major Histocompatibility Complex (MHC) and are, therefore, not normally encountered by host lymphocytes. These could then be captured by dendritic cells that trigger naïve T cells present at the invasion site or at the T cell areas of the draining lymphoid organs, leading to activation of autoreactive cells but not of cells against the infecting microorganism.

Cryptic epitopes may also initiate and maintain autoimmunity through various non-mutually exclusive mechanisms. Cryptic epitopes can be presented by non-professional antigen presenting cells (APC) such as B cells, and induce T cell activation. Autoreactive B cells initiate autoimmunity in the absence of T cells specific for the self-antigen. Alternatively, autoreactive B cells may take up a foreign antigen that cross-reacts with a self-antigen at the B cell level, but contains different T cell epitopes. Finally, activated B cells that efficiently take up and present self-antigen may prime autoreactive T cells. All these mechanisms may result in a self-sustained autoimmune response.

Alternatively, or in addition, 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 tumour necrosis factor-α (TNF-α) and chemokines, that could be able to activate autoreactive T cells by lowering...
the threshold of activation\textsuperscript{[13,14]}. These T cells may then proliferate in response to self-antigen presented on host APC. Inflammation could also alter the pattern of lymphocyte migration and activate APC’s, rendering them more effective as APC by enhancing antigen uptake and processing, as well as the cell surface expression of MHC or costimulatory molecules.

Finally, infection might provoke polyclonal lymphocyte activation via either a mitogen or a superantigen effect\textsuperscript{[15]}. 

**CHAGAS’ DISEASE**

**General aspects**

Chagas’ disease is a debilitating multisystemic disorder\textsuperscript{[16]} that affects several million people (approximately 18 million individuals are infected with *Trypanosoma cruzi*, with 120 million at risk) in Central and South America\textsuperscript{[17-19]} and is considered a paradigm of infection-mediated autoimmune disease. It is caused by the flagellated protozoan parasite *T. cruzi*, which has a complex life cycle involving several stages in both vertebrates and insect vector. *T. cruzi* has three different morphologies: 1) epimastigote, which replicates in the blood-sucking triatomine insect vector; 2) trypomastigote, which infects cells of vertebrate hosts; and 3) amastigote, which replicates intracellularly in cells of the host\textsuperscript{[17,20]}. Approximately 30% of infected people develop symptoms of the disease in their lifetime, which include cardiomyopathy, neuropathies, and dilatation of colon or oesophagus\textsuperscript{[17,18]}.

Transmission of *T. cruzi* to humans occurs when infective metacyclic trypomastigote forms of the parasite (A1), present in the faeces released by the bug while it takes a blood meal penetrate in the bloodstream. They infect a wide variety of phagocytic and non-phagocytic cells (A2) of the host. Once inside the cells, the metacyclic forms escape from endocytic vacuoles to the cytoplasm where they transform into amastigotes and multiply intracellularly (A3). At some point, the amastigotes break off from the cell (A4), differentiate into non-replicative flagellated blood trypomastigotes that in turn penetrate and infect either adjacent or distant susceptible cells and tissues 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. New triatomine bugs may take up circulating trypomastigotes 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 they transform into non-replicative but infective metacyclic trypomastigotes.

**Figure 1. Infective cycle of *T. cruzi*.** Transmission of *T. cruzi* to humans occurs when infective metacyclic trypomastigote forms of the parasite (A1), present in the faeces released by the bug while it takes a blood meal penetrate in the bloodstream. They infect a wide variety of phagocytic and non-phagocytic cells (A2) of the host. Once inside the cells, the metacyclic forms escape from endocytic vacuoles to the cytoplasm where they transform into amastigotes and multiply intracellularly (A3). At some point, the amastigotes break off from the cell (A4), differentiate into non-replicative flagellated blood trypomastigotes that in turn penetrate and infect either adjacent or distant susceptible cells and tissues 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. New triatomine bugs may take up circulating trypomastigotes 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 they transform into non-replicative but infective metacyclic trypomastigotes.
areas are not affected by these programs and constitute the reservoir for the parasite. Transfusion with infected blood and congenital transmission also account for some new infections. Therefore, transfusion-acquired Chagas’ disease is becoming a significant health problem in countries other than Central and South America, especially those having large numbers of immigrants from that region[21,22].

**Immune response**

Cytokines play a key role in regulating both immune response and parasite replication in infected hosts[23]. 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, IL-12 produced by macrophages in response to infection mediates resistance to *T. cruzi*[24]. TNF-α and IFN-γ have been identified as the most important cytokines involved in the killing of intracellular *T. cruzi* through a NO-mediated L-arginine-dependent killing mechanism[25,26]. This was corroborated in vivo, since anti IFN-γ monoclonal antibody administration results in a drastic increase in parasitaemia and mortality[27,28]. TNF-R1-FcgIgG3 transgenic mice are also more susceptible to *T. cruzi* infection clearly indicating a protective role for TNF-α[29]. Accordingly, *T. cruzi* infection of inducible NO synthase deficient mice results in an increased parasitaemia[30].

On the other hand, several alterations of the immune response have been described in this disease. Thus, the acute infection by *T. cruzi* is associated with severe immunosuppression, measured as the loss of proliferative cell responses to mitogens and antigens, which is thought to facilitate dissemination and establishment of the parasite in host tissues. Various cell types have been ascribed to act as suppressor cells and therefore mediate immunosuppression. Some reports have pointed out to T cells, including γ/δ T cells[31] as well as to adherent cells[32] or immature myeloid cells[33]. For example, the depletion of adherent cells partially restored T cell proliferation of spleen cells[33,34]. Besides, inhibition of IL-2 synthesis[33] and reduced cell surface expression of IL-2R by splenocyte cultures of infected mice[30], as well as in human peripheral blood cells[36], has been accounted to explain this unresponsiveness. By contrast, elevated levels of IFN-γ and TNF-α are produced by infected and activated spleen cell cultures[33,37,38]. In addition, infection also alters the shaping of the central and peripheral T-cell repertoire[39].

Apoptosis of CD4+ T cells has been also described in acute *T. cruzi* infection[31], and T cells obtained from infected mice have an enhanced T cell receptor (TCR) activation-induced cell apoptosis that may explain this unresponsiveness[40]. Increased NO secretion[41] has been accounted as one mechanism responsible for immunosuppression and recently, CD11+Gr1+ myeloid cells have been identified as responsible for this NO-mediated immunosuppression[42]. Other soluble substances, including suppressive cytokines such as transforming growth factor (TGF)-β, IL-4 or IL-10, and prostaglandins, released upon contact with parasite-derived antigens, are also thought to be involved[43,44]. Finally, elevated levels of IFN-γ and TNF-α are produced by spleen cell cultures from infected mice[37], which in turn induce high levels of NO[40].

**PATHOGENESIS OF CHAGAS’ DISEASE**

**Pathological features**

Two phases, acute and chronic, can be differentiated in Chagas’ disease[17,18,50]. In the acute phase, few weeks after infection, a local inflammatory lesion (chagoma or Romaña’s sign) 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 (parasitaemia) and a small number of patients develop symptoms of cardiac insufficiency, reflecting an underlying severe myocarditis. This leads in some cases to heart failure, which is responsible for the few deaths seen in acute Chagas’ disease[51,52]. Meningoencephalitis may also occur, especially in some immunosuppressed patients[53]. However, the acute phase mostly remains undiagnosed without clinically severe symptoms. On the contrary, the most severe pathology and common manifestations of the disease develop many years (10 to 30) after the initial infection with *T. cruzi* in the so called chronic phase, although only in around 30-40% of the infected people[17,18,50]. During the chronic phase circulating parasites cannot be observed by inspection of blood but progressive tissue damage occurs in the esophagus, colon and heart[17,18].

In the chronic phase, the heart is the most commonly affected organ. Cardiomyopathy frequently develops, being congestive heart failure a common cause of death in these
Mechanisms of pathogenesis

After several decades of research, the aetiology of Chagas’ heart disease, both in humans and in experimental infection models, is not precisely understood yet. The acute and chronic phases of the disease share some similar pathological findings (see above), but their pathogenesis may differ. Up 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 by a combination of them. Those mechanisms are summarised below:

a) Primary neuronal damage and denervation of the parasympathetic autonomous system in the heart. This may lead to the development of chronic phase lesions and was one of the first pathogenic mechanisms described during the acute phase\(^{(37,58)}\). However, subsequent studies show only slight neuronal damage in the heart, suggesting that neuronal lesions are an epiphenomenon, secondary to inflammation and fibrosis\(^{(58,61)}\).

b) Secreted \textit{T. cruzi} product(s). They can be toxic for host cells and tissues\(^{(62)}\).

c) \textit{T. cruzi}-induced damage of cardiomyocytes. This is due to the cytotoxic effect of the intracellular infection with amastigotes. It is an obvious mechanism, which may have some relevance only in the acute phase and in heavily parasitised or immunosuppressed patients.

d) Parasite-induced microvascular changes. They may lead to cardiac hypoperfusion, myocyte degeneration and finally to chronic inflammation\(^{(63-65)}\).

e) Persisting \textit{T. cruzi} antigens. They may act as a trigger for specific T-cell mediated responses such as delayed-type hypersensitivity (DTH) or cytotoxic CD8\(^+\) cells that lead to damage of infected cells or bystander cells in the host tissues\(^{(66-68)}\). The latter may take place in both the acute and the chronic phases. 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 of \textit{T. cruzi}-elicited CD8\(^+\) and inflammatory cells into the heart and other susceptible tissues of the host\(^{(69,70)}\). ECM components may absorb parasite antigens and cytokines that could contribute to the establishment and perpetuation of inflammation. Moreover, \textit{T. cruzi} requires \(\beta 1\) integrins to gain access to the cell\(^{(71)}\). An increased expression of ICAM-1, VCAM-1, and ECM components has been detected in heart and endothelial cells of patients with CCC\(^{(72)}\), which can be secondary to increased inflammation. Cytokines and chemokines produced in response to the parasite may also modulate VCAM-1 and ICAM-1 adhesion molecules on endothelial cells of target tissues, which recruit VLA-4\(^+\)LFA-1\(^+\)CD8\(^+\) T lymphocytes, the predominant subset present in inflamed heart\(^{(72)}\).

CCC patients also have increased expression of MHC molecules. Class I MHC molecules are up-regulated in the sarcolemma of cardiomyocytes and there is also evidence for an over-expression of class II MHC on endothelial cells\(^{(74-76)}\). This may favour the presentation of cryptic epitopes.

In addition, the number of CD4\(^+\) T cells increases parallel to the number of CD8\(^+\) T cells in the acute phase but not in the chronic phase suggesting an immunological imbalance. In the chronic phase, patients with heart failure present a predominance of CD8\(^+\) T cells with an altered CD4\(^+\)/CD8\(^+\) T cell ratio. The inflammatory response, which is probably recurrent and undergoes periods of more accentuated exacerbation, is most likely responsible for progressive neuronal damage, microcirculation alterations, heart matrix deformations and consequent organ failure.

f) Polyclonal B cell activation. A generalised activation of B cells has been shown in animal models of infection, but the exact mechanism for it is not clear. This may disrupt normal immune regulatory mechanisms and can lead to autoimmunity\(^{(77)}\).

g) Autoimmunity. It may occur either by \textit{T. cruzi}-specific mechanisms (molecular mimicry) or by non parasite–specific effector mechanisms, eventually leading to the development of pathology (see Table I).
An important issue that is often ignored in this debated field is that none of the mechanisms listed above is mutually exclusive. In fact, it seems unlikely that inflammation occurs through only one of these mechanisms.

**AUTOIMMUNITY/ PARASITE PERSISTENCE DILEMMA**

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(11). This remains, however, a hotly debated issue(11,67,78-82). Several early studies on Chagas’ disease already emphasised the scarcity of parasites in histological sections in the chronic phase of the disease(63,84). Since then, much research has focused on the possibility that autoimmune responses set off by molecular mimicry and/or bystander activation contributes to tissue damage. Those mechanisms were initially reported many years ago(85-92) and are supported by a large body of circumstantial evidence thereafter(11,79-81,93,94). All those studies have contributed to the popularity of this hypothesis among researchers in the field.

However, mounting evidence are challenging this view. The use of more sensitive techniques has allowed detection of parasite antigens or DNA during the chronic phase. Therefore, all the damage could be attributed either to an inflammatory response against the parasite or to the parasite replication itself(67,68,78). It should be emphasized that not a single report published to date has unequivocally demonstrated that either autoimmunity or parasite-specific immunity are pathogenic.

Of the mechanisms of induction of autoimmunity shown in table I, which ones can be found in *T. cruzi* infection? The detection of circulating anti-*T. cruzi* antibodies that cross-react with host tissue antigens is a common finding in human and animal models of chagasic infection. There are numerous reports of *T. cruzi* antigens cross-reactive with heart and neural tissues of the host(79,81,95). However, with few exceptions, the autoantibodies or autoreactive T cells against those antigens do not seem to be the leading cause of autoimmune pathogenesis. The most relevant autoantigens involved in pathology are described below.

**Autoantigens**

Cardiac myosin is one of the favourite, and most debated, autoantigens that have been implicated in the aetiology of CCC. A well-studied *T. cruzi* candidate implicated in pathogenesis through molecular mimicry is B13 protein that crossreacts with human cardiac myosin. Cunha-Neto and his collaborators have described myosin heavy chain as a major antigen of heart-specific autoimmunity and suggested the possible relevance of myosin recognition in human CCC(96,97). Antibodies to myosin were found in both asymptomatic and chronic chagasic patients: 14/23 (61%) symptomatic and 1/14 (7%) asymptomatic patients. Affinity-purified anti-myosin antibodies specifically recognised two bands of 140,000 and 116,000 daltons in extracts from *T. cruzi* trypomastigotes. Sera from all patients with CCC disease recognised a recombinant *T. cruzi* peptide, named B13, but only 14% of the anti-myosin positive sera from asymptomatic chagasic patients(98). At the molecular level, crossreactivity was shown to exist between the amino acid sequences AAALDK and AAAGDK from cardiac myosin and B13, respectively.

Results from the Engman’s group showed that *T. cruzi*-infected A/J mice (a strain highly susceptible to *T. cruzi* infection) generated anti-myosin IgG, both in the acute and chronic phases of the infection(99). They also found that immunisation with purified myosin caused some heart lesions resembling those seen in *T. cruzi*-infected mice. However, not all mouse strains are equally susceptible to myocytolysis after *T. cruzi* infection(94). Interestingly, in C57BL/6 mice, a strain more resistant to development of myocarditis, the levels of anti-myosin IgG found after *T. cruzi* infection were either small or undetectable(99). This mouse strain has been claimed not to develop cardiac autoimmunity after immunisation with myosin(100). However, we have detected some signs of inflammation in *T. cruzi* infected C57BL/6 mice, although in a much lesser extent than in iNOS knockout mice (Fig. 2). Since C57BL/6 develop lower parasitaemias, this may indicate that the presence of myocarditis may depend on the initial level of control of parasite replication in the acute phase.

Other reports suggested that anti-myosin antibodies are not involved in the pathogenesis. For example immunisation with myosin in immunosuppressed mice did not induce the production of antibodies but still caused myocarditis. However, how myocarditis can be triggered by myosin in those immunosuppressed mice is difficult to envisage. Moreover, passive transfer of a high-titre anti-myosin antibody preparation failed to induce myocarditis(101). Since the fine specificity of the different anti-myosin IgGs has not been addressed in most of those studies, it is difficult to compare them. It is possible that the myosin determinant(s) recognised by the different sera are not identical.

C57BL/6 mice infected with a highly virulent *T. cruzi* strain developed severe cardiomyopathy and produced autoantibodies that recognised antigens from a mouse heart extract(102). However, unlike Cunha-Neto et al., the authors of this study observed no cross-reactivity between mouse
anti-heart antibodies and T. cruzi epimastigote antigens. Furthermore, sera from mice hyperimmunised with myosin failed to recognise any antigen on T. cruzi epimastigote extracts, and sera from mice hyperimmunized with T. cruzi did not detect any muscle tissue antigen (102). Since these experiments were not carried out with circulating forms in the host (trypomastigotes or amastigotes), the results are inconclusive, and molecular mimicry between heart and T. cruzi antigens can not be completely ruled out. In contrast with the above, purified anti cruzipain antibodies made in mice cross-react with myosin heavy chain (103). Of note, anti-myosin antibodies are also significantly induced in patients having heart disease not related to T. cruzi infection such as viral myocarditis, myocardial infarction, coronary artery bypass and heart valve surgery, among others (104-106). Thus, rather than cross-reactive T. cruzi antigens, myocyte damage caused either by parasite replication in the heart or by inflammation may release self-antigens leading to the induction of anti-heart antibodies. It would not seem unreasonable, therefore, to infer that the initial insult resulting from T. cruzi infection could cause a rise in the level of anti-myosin immunity in Chagas’ heart disease.

A criticism often raised against cross-reactive T. cruzi proteins is that, despite evidence of immune responses against both the parasite and the putative self-antigens, there is no direct evidence demonstrating that they can induce autoimmunity. However, there are increasing numbers of reports addressing this criticism. Thus, autoimmune response in mice immunised with cruzipain was associated to heart conduction disturbances. In addition, ultrastructural findings revealed severe alterations of cardiomyocytes and IgG deposition on heart tissue of immunised mice. We investigated whether antibodies induced by cruzipain transferred from immunised mothers to their offsprings 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 offsprings born to immunised mothers revealed conduction abnormalities. These results provide strong evidence for a pathogenic role of autoimmune response induced by a purified T. cruzi antigen in the development of experimental Chagas’ disease (103). Moreover, antibodies to other cross-reactive antigens such as anti β-adrenergic (107) and anti muscarinic receptor (108,109) that cross-react with T. cruzi ribosomal proteins may also cause cardiac pathology. Recently, it has been described that cruzipain also induces autoantibodies against muscarinic acetylcholine receptors which can be implicated in pathology (110). All those purified autoantibodies may alter cardiac beating in the mouse heart and explain some of the pathological findings of CCC. This not necessarily proves molecular mimicry, since the bystander activation mechanism for the generation of anti-self responses cannot be ruled out.

Along those lines, some authors believe that other mechanisms different from molecular mimicry can explain autoreactivity (9,79). They think that mimicry is less likely to be true than antigen release due to myocardial damage leading to expansion of normally tolerant myosin-reactive T cells, particularly since myosin autoimmunity is seen in myocarditis associated with other insults. Besides, B cell anti myosin response seems to be the main cause of pathology in other heart infections, induced by Coxsackie B3 (111) or by bacteria (112). In this regard, it is worth mentioning that peptides of cardiac myosin, a cytoplasmic protein, are associated with MHC class II molecules on APC even in normal mouse myocardium (113) and that MHC class II molecules are increased in the heart of T. cruzi-infected patients and animals (see
above). Alternatively, it is possible that some pathogens may share the ability to destroy the heart but they may have different cross-reactive epitopes with heart proteins (myosin). Thus, it is possible that the trigger is the combination of pathogen and pathogen damage, although the fine specificity of the autoreactive response will be different in each case.

While the presence of «anti-self»- immune responses in \textit{T. cruzi} infections has been unquestionably demonstrated, evidence for the mediation of cross-reactive antibodies or T cells in pathology is still lacking.

\textbf{Autoreactive T cells}

Perhaps the most compelling evidence supporting a role for autoantigen-specific autoimmunity in the pathogenesis of the disease derives from T cell mediated immunity. We have shown that reactivity against a dominant autoantigen (named Cha) in human and mice \textit{T. cruzi} infections is the result of molecular mimicry between clearly distinct T and B cell Cha epitopes and highly immunogenic parasite antigens, which triggers strong T and B cell-dependent responses\textsuperscript{114}. More interestingly, adoptive transfer of autoreactive T cells isolated from spleen of chronically infected mice induced heart infiltrates in recipient mice and triggered autobody production in the absence of the parasite. In contrast, we were not able to induce pathology by immunising with the Cha crossreactive antigen. Although our results suggested that Cha might be involved in pathology, this by no means indicates that Cha would be the only autoantigen involved in autoimmune pathology of Chagas’ disease.

In the same direction, Ribeiro-Dos-Santos et al have described that a CD4\textsuperscript{+} T cell line obtained from a chronic chagasic mouse induces carditis in immunised mice and rejection of normal hearts in the absence of \textit{T. cruzi}. Therefore, in some cases the presence of the parasite is not necessary to produce pathology if activated autoreactive T cells are transferred. The requirement of the parasite to cause rejection in mice transferred with T cells from infected mice has also been widely debated\textsuperscript{97,115-117}. Chronically infected mice cause rejection of syngeneic transplanted hearts either in the absence\textsuperscript{116} or in the presence of the parasite\textsuperscript{111}. These differences may be due to different mice and parasite strains used, and when the presence of the parasite is required for rejection, inflammation and not \textit{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.

It has been shown that immunological tolerance to heart antigens induced in mice by heart antigen administration prior to their infection by \textit{T. cruzi} resulted in less intense cardiopathy than control non-tolerised animals\textsuperscript{118}. However, 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\textsuperscript{119}. Thus, it is probable that autoreactivity itself is not sufficient to induce tissue inflammation, and that a proinflammatory environment, induced by infection\textsuperscript{120}, is needed.

\textbf{Parasite persistence}

Tarleton has sequentially reviewed, ardently and somewhat biased, all the arguments in favour of the parasite persistence hypothesis to explain the patogenesis of chronic Chagas’ disease in general and of CCC in particular\textsuperscript{16,78}. Arguments in favour that the disease is linked to parasite presence are supported by the fact that treatments that decrease the parasite burden in the acute phase are associated to a decrease in the clinical symptoms\textsuperscript{121}. Effective chemotherapy could also enhance anti-\textit{T. cruzi} immunity in mice\textsuperscript{122}. In humans, the link between persistence of \textit{T. cruzi} and clinical disease is also supported by the tissue-specific detection of parasite DNA in the heart, but not in the oesophageal tissue, of individuals with cardiac disease; and vicedesera\textsuperscript{123,124}. Moreover, several data support that enhancing the efficiency of the anti-parasite response by immunotherapy, gene-deletion, or vaccination, results in a decreased severity of the chronic phase, and not exacerbation of the disease as predicted by the autoimmune hypothesis\textsuperscript{78}. Very recent data in humans show a very high frequency of parasite-specific IFN-\gamma-producing CD8\textsuperscript{+} T cells among chronic 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 the chronic stage of the disease\textsuperscript{125}. Thus, the stronger the immune response, the better the outcome. Conversely, it has been observed that immunosuppressive treatments correlate with exacerbation of the infection and disease\textsuperscript{126}. Apparently this supports the parasite persistence hypothesis in opposition to autoimmunity, strongly arguing in favour of the participation of an effective antiparasite response in preventing the disease\textsuperscript{78}. However, those reports suggest that all the damage is parasite-mediated and could be also taken as an argument against supporters of parasite-induced immune response as a mediator of cardiac damage\textsuperscript{72}.

We have found that autoreactivity in the chronic phase is also linked to the parasitaemia since the antibody titre and reactive T cells against the Cha autoantigen are lower in C57BL/6 (non-susceptible) than in BALB/c (susceptible) mice\textsuperscript{114}. Moreover, potentially pathogenic anti-Cha autoantibodies also decreased with the treatment of patients. The titre of anti-Cha and anti-parasite antibodies decreased
TABLE III. Myocarditis and antibody responses in chagasic patients increase with symptomatology and decrease with treatment

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<th>Chagasic patients</th>
<th>Anti-Cha antibodies</th>
<th>Anti- \textit{T. cruzi} antibodies</th>
<th>Myocarditis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptomatic</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Asymptomatic untreated</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Asymptomatic treated</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

The presence or absence of myocarditis was given by clinical histories of patients. Antibody response was quantitated as reported elsewhere (127): +, OD 450 nm < 0.3; ++, OD 450 nm 0.3-1.0; ++++, OD 450 nm < 1.0.

in parallel with treatment and increased with symptomatology(127) (Table III).

The presence of \textit{T. cruzi} in the chronic phase of the disease was already observed in early descriptions(128) and has been documented later by other authors(129,130). With more sensitive techniques such as polymerase chain reaction (PCR), parasite DNA is commonly detected in chronic patients(72). Recent studies using modern immunohistochemistry have demonstrated higher frequencies of \textit{T. cruzi} antigens. \textit{T. cruzi} antigens were detected in 100% of hearts from chronic chagasic patients that died from heart failure when several samples of the myocardium were analysed(131,132). Many previous failures to detect parasite antigens from biopsy material of 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(72). Using a mouse strain that develops chagasic cardiomyopathy when infected with a highly virulent \textit{T. cruzi} strain, amastigotes were detected in myocytes throughout the chronic phase, although their numbers strongly decreased and were scarce already in the early chronic phase(133). 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(134). However, a significant association between the presence of \textit{T. cruzi} antigens in the heart and severe or moderate inflammation was observed both in humans(72) and animal models of the disease(130). Thus, a low number of parasites correlated with intense myocarditis and whole myocardial fibres containing parasites did not elicit inflammation(72). Besides, some of the work cited to support parasite persistence could be used against it. For instance Buckner et al said cardiac tissue had relatively dense acute parasitism (although 100-fold less than in the acute phase) but showed minimal inflammation in the chronic stage. In contrast, peripheral nerve tissue had few parasites but was heavily inflammed in the chronic stage. At the most, inflammation in the nerve occurred in the presence of very few \textit{T. cruzi} parasites, and it possibly occurred in the presence of none(135). This suggests two possibilities: exuberant host reactions to the few remaining parasites, either immune-mediated or not, or autoimmune-induced inflammation. Parasite antigens probably work as a trigger response against the myocardial fibres. It is possible that some lesions lack parasites or parasite antigens because of the effective clearance of parasites from this site by an effective anti-parasite immune response, thus preventing the observation of an exact correlation. However, as mentioned before, this is difficult to reconcile with the fact that a strong anti-parasite immune response results in decreased symptoms(125).

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

Demonstration of hypotheses

As mentioned above, the hypothesis of molecular mimicry is very popular in the \textit{T. cruzi} field. Several criteria(9), put originally forth in the \textit{T. cruzi} field(11) need to be met to consider a disease as caused by molecular mimicry (Table II). In \textit{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 antigen, infection with genetically deficient parasites lacking the inducing antigen, 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 this respect, the presence of autoreactive T cells against cardiac myosin/B13 proteins in heart lesions
has been described, but its contribution to the pathology has not been demonstrated\(^9\).

The autoimmune hypothesis for Chagas’ disease is based on the fact that parasites are almost undetectable in the chronic phase of the disease and because of that it postulates that autoimmunity would be the result of an autoimmune aggression. 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 antigens. Nevertheless, evidence for the mediation of cross-reactive antibodies or T cells in pathology is still lacking. Moreover, most of the data come from the 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 pathologic effect of autoreactive T or B cells would be to immunise mice with cross-reactive antigens to see if this induces pathology. However, immunisation with an autoantigen (injected together with adjuvants and via routes different from that of the natural infection) may not reflect the way the autoantigen is presented during natural infection and may elicit either hyperimmune responses, or tolerance, or regulatory T cells that may suppress autoimmunity. An alternative approach is the transfer of putative autoreactive T cells from chronically infected mice or T cells specific for a given autoantigen. Either way may answer some of these questions and determine which of the candidates are really relevant for pathology. Altered peptide ligands may also be useful for treatment of the disease.

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. A demonstration of this hypothesis is also difficult to perform, since one ought to separate the components of the immune response, self and anti-self during infection. If there were a unique cross-reactive antigen, gene deletion of cross-reactive antigens in the parasite should have an impact (either positive or negative) on the pathology. This would theoretically solve the dilemma. However, things are not so easy, since cardiac damage and humoral and cellular cardiac autoimmunity have been recently reported in mice during the acute phase when parasitaemia is high\(^9\). Furthermore, the coexistence of self and non-self antigens would enhance the immune response against both, always triggered by the parasite.

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?, and 2) why does the parasite burden not always correlate with disease severity?

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**Figure 3.** Diagram representing the different mechanisms of induction of autoimmune disease by \(T. cruzi\). \(T. cruzi\) can induce T and B cell anti-parasite responses, which can induce autoimmune disease through molecular mimicry with extracellular antigens or epitopes in antigens normally presented by APC. \(T. cruzi\) can also induce cytokines that mediate some cardiac damage and liberation of autoantigens recognised by autoreactive T cells and autoantibodies that further damage the cardiac tissue via bystander activation. Simultaneously, \(T. cruzi\) can induce release of self-antigens, usually intracellular, which contain cryptic epitopes that can be presented by APC. Overexpression of intracellular antigens induced by \(T. cruzi\) can also end up with presentation of cryptic epitopes by APC. 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-antigen specific manner, known as 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.

**Coexistence of parasite persistence and autoimmunity**

We think that since cardiac myosin autoimmunity develops in the acute phase, where there is lysis of cardiac myocytes and easily detectable parasites, it is very likely that both processes, bystander damage and molecular mimicry coexist till the chronic phase. Then, damage goes via effector cells which recognise crossreactive \(T. cruzi\)/autoantigen through molecular mimicry.

Thus, we propose that parasite is the trigger that activates some T cells (autoantigen/crossreactive parasite antigen). Once they are activated, they secrete inflammatory cytokines that mediate some cardiac damage. This liberates autoantigen that is also recognised by some other autoreactive T cells and autoantibodies that further damage the cardiac tissue via bystander activation. This is like a vicious circle triggered by parasite antigens but fuelled by crossreactive 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 fibre antigens, resulting in severe lymphocytic myocarditis (Fig. 3).
Thus, parasites are necessary to trigger autoantibodies and autoreactive T cells and may be even necessary to maintain them in the chronic phase. Nevertheless, this does not prove that crossreactivity of those autoantibodies and autoreactive T cells with self-components, which either are expressed at the cell surface or presented by APC upon infection, have no effect at all in the pathology. Altogether, we believe that active *T. cruzi* infection is necessary to trigger the autoimmune process, most likely through autoreactive T cells, that once induced can transfer the cardiac pathology. In summary, we believe that the debate on *T. cruzi* myocarditis closely resembles the one generated by coxackie virus(1,20). After all, the right answer may lie between both hypotheses, pathogens (parasites, virus) are the trigger but autoreactive T and B cells are the actual effector cells.

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