OVERVIEW OF MOLECULAR MECHANISMS IN CHAGASIC CARDIONEUROMYOPATHY AND ACHALASIA

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Abstract
Evidence accumulated by our investigations over the years give adequate proof for the existence of circulating antibodies in Chagas disease which bind to ß adrenergic and muscarinic cholinergic receptor of myocardium. The interaction of agonist-like antibodies with neurotransmitter receptors, triggers in the cells intracellular signal transductions that alter the physiological behaviour of the target organs. These events convert the normal cells into pathologically active cells. The interaction of antibodies with heart ß adrenergic and cholinergic receptors triggers physiologic, morphologic, enzymatic and molecular alterations, leading to tissue damage. The analysis of the prevalence and distribution of these antibodies reveals a strong association with cardiac and esophageal autonomic dysfunction in seropositive patients in comparison with those without alteration of the heart and esophasg autonomic disorders: therefore, the presence of these antibodies may partially explain the cardiomyoneuropathy and achalasia of Chagas disease, in which the sympathetic and parasympathetic systems are affected. The deposit of autoantibodies behaving like an agonist on neurotransmitter receptors, induces desensitization and/or down regulation of the receptors. This in turn, could lead to a progressive blockade of neurotransmitter receptors, with sympathetic and parasympathetic denervation, a phenomenon that has been described during the course of Chagas cardioneuropath and achalasia. The clinical relevance of these findings is the demonstration, using biomolecules, of a strong association between the existence of circulating autoantibodies against peptides corresponding to the second extracellular loop of the human heart beta, adrenercoceptor and M₂ cholinceptor in chagasic patients, and the presence of dysautonomic symptoms, making these autoantibodies a proper early marker of heart and digestive autonomic dysfunction.

Key words: Chagas disease, neurotransmitter receptors, autoantibodies, dysautonomia, cardioneuromyopathy, synthetic peptides, achalasia

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Chagas disease, one of the most common determinants of congestive heart failure and sudden death in the world, is caused by a parasite, Trypanosoma cruzi (T. cruzi) which is widely distributed in South and Central America. However, chagasic cardiomyopathy is an extraordinarily complex process with a poorly understood pathophysiology. The paradoxical severe involvement of the heart in the absence of any intracellular form of the parasite, has prompted many investigators to propose autoimmune mechanisms involved in the pathogenesis of this cardiomyopathy.

Why does the myocardium fail during the evolution of chronic Chagas’ disease? Borrowing from basic science, Bristow postulated two categories of molecular specific mechanisms that may be abnormal in the failing human heart (Fig. 1): Intrinsic function; which comprises the mechanism responsible for contraction and relaxation of the heart in the basal or resting state, in the absence of neural or hormonal influence and Modulated function; which comprises the mechanism responsible for the ability of the heart to rapidly increase or decrease its performance (by two-fold to ten-fold) in response to various physiological or physical stimuli. This adaptative response of the heart is modulated by endogenous bioactive compounds, including neurotransmitters, cytokines, autocrine and paracrine hormones. Both abnormal mechanisms lead to myocardial dysfunction. Continuous chronic use of these compensatory mechanisms to...
support the failing heart, contribute to cellular necrosis and apoptosis. When cell loss occurs, a remodelling process takes place, which is one of the major determinants of hypertrophy or diluted cardiomyopathy.

There is no current consensus as to which altered intrinsic function abnormalities are present in primary or secondary dilated cardiomyopathies in human beings. By contrast, a consensus has been reached on several specific abnormalities in the stimulation component of modulated function. Most of these changes include β-adrenergic and muscarinic cholinergic signal transduction. The ability of the autonomic nervous system to modulate the systolic function is substantially altered in the failing heart because of multiple changes at the level of receptors, G proteins and adenylate and guanylate cyclases.

Therefore chronic Chagas heart disease belongs to the category of cardiac dysfunction in which the modulated function is primarily affected. Chagasic cardiomyopathy is a cardioneuromyopathy in which the chronic activation at the level of cardiac neurotransmitter receptors and their cellular signaling, induces cardiac failure. We have described antibodies with reactivity against cardiac neurotransmitter receptors. As a consequence of this recognition they trigger signal transduction which alters normal myocardial function inducing physiological, biochemical and pharmacological alteration of the heart. Moreover, we have described myocardial dysfunction induced by mononuclear cell infiltration caused by release of cytokines and biologically active lipid metabolites. These factors (ILs, IFNs, SRS-A, PGE) may alter normal myocardial function either directly or indirectly via sympathetic and/or parasympathetic activation.

Therefore, both the autoantibodies and the cell infiltration are able to alter the autonomic nervous system modulation of systolic function in chronic chagasic myocardium failure. Furthermore, we have reported that the myocardial β-adrenergic action of chagasic autoantibodies was highly enhanced by human peripheral lymphocytes.

It is important to note that chagasic heart disease has a neurogenic nature. It has been demonstrated that it is caused by a poor regulation of the autonomic control of heart activity. In fact, the denervation of both parasympathetic and sympathetic systems of the heart was proven to be higher in the former. It was confirmed that Chagas' heart disease is a cardiac neuromyopathy in which sympathetic and parasympathetic systems are affected. This dysautonomia is characterized by a slow and progressive blockade of the neurotransmitter receptors in patients who are asymptomatic with normal electrocardiogram and chest X ray.

Based on a strong association between clinical symptoms and the presence of antibodies interacting with neurotransmitter receptors, we postulated that those antibodies could play a role in the progressive dysautonomic syndrome described in Chagas’ cardiomyopathy. Analyzing the prevalence of these antibodies, we observed differences in their distribution in patients in the

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**Fig. 2.** Central role of neurotransmitter autoantibodies and cytokines in production of adverse biological effects that lead to progressive myocardial dysfunction in Chagas disease.
indeterminate stage, depending on the presence or absence of dysautonomic syndrome. A strong association of the titer of these antibodies in chagasic patients with dysautonomic dysfunction was observed. Thus, the frequency of chagasic IgG with autonomic activity was higher on sera from seropositive patients with dysautonomia than on those from seropositive patients without dysautonomia27.

Studies from our laboratory have demonstrated that chagasic sera can react in vitro with the plasma membrane of living heart cells inducing morphologic and functional changes and modifying their ß adrenergic and cholinergic receptor activity27, 28. Immunofluorescent and ultrastructural immunohistochemical studies of myocardial cells contracting in the presence of chagasic sera, showed widely distributed sarcolemmal deposits of immunoglobulins and C3. Transmission electron microscopy demonstrated sarcolemmal damage27. Fixation of the antibody to the sarcolemma occurs concomitantly with alteration of physiologic and pharmacologic function of the isolated myocardium. These effects were prevented by ß adrenergic and cholinergic blocker agents6, 29. The deposit of an antibody on the myocardial neurotransmitter receptors, which behaves like an agonist could induce desensitization and/or down regulation of the receptor. This, in turn, could lead to a progressive blockade of myocardium neurotransmitter receptors, with sympathetic and parasympathetic denervation, a phenomenon that has been described in the course of Chagas cardioneuromyopathy26.

In fact, chagasic IgG behaving as a ß1 agonist, binds and stimulates the myocardium ß1 adrenoceptors. Then, receptor-mediated activation of guanine nucleotide protein (Gs) occurs. This in turn increases levels of intracellular cAMP production6, 30 and intracellular calcium concentration31-33; both systems could be the mediators of the positive inotropy and chronotropy induced by chagasic IgG. Furthermore, the cAMP-dependent protein kinase acting sinergistically with the calcium-dependent protein kinase through different phosphorylase kinases increases intracellular calcium concentration. Such an increase, inhibits Na+/K+/ATPase activity and stimulates Ca2+/ ATPase activity34. Another regulatory factor involved in the effect of chagasic IgG associated to ß1 adrenergic stimulation of myocardium is the ionic distribution by Na+/K+/ATPase activity. An inhibition of Na+/K+/ATPase activity would theoretically result in a decrease in intracellular K+ concentration and an increase in intracellular Na+ concentration; which may increase the cytoplasmatic Ca2+ 34. The inhibition of the enzyme leads to a greater increase in intracellular calcium concentration and decreases intracellular potassium concentration, altering contractility, and the conduction and generation of action potential. Moreover, the autoimmune progressive and irreversible inhibition of the enzyme, may also be involved in the morphological alterations produced by sodium and water retention34.

In addition to the occurrence of ß-adrenergic antibodies35, adequate proof has been presented supporting the existence of another antibody activity that reacts with the muscarinic cholinergic receptor of myocardium28, 36, 37.

In an attempt to elucidate the nature of the parasympathetic mechanism involved, we characterized the participation of muscarinic cholinergic system in the effect of chagasic IgG, analyzing the action of the antibody on the binding of the specific muscarinic cholinergic receptor radioligand to cardiac membrane. We observed a concentration-dependent inhibition of the specific radioligand to cardiac membrane. Chagasic IgG behaves as an irreversible inhibitor of radioligand binding decreasing the available binding sites without affecting the receptor affinity27, 28, 36, 37. This behaviour was also demonstrated for cardiac ß1 adrenoceptor7 and for lymphocyte ß2 adrenergic and muscarinic cholinergic receptors38, 39. As a consequence of the interaction of chagasic IgG with the muscarinic cholinergic receptor of myocardium, intracellular signal transduction that reflects the biological effect of the antibody occurs. Among the intracellular events triggered by chagasic IgG interacting with myocardial cholinergic receptors we described: a) a decrease in atria contractility; b) a decrease in cAMP formation; c) an increase in cGMP and d) activation of phosphoinositide turnover. Chagasic IgG stimulation of phosphoinositide turnover appears to be mediated by phospholipase C. The stimulation of nitric oxide synthase (NOS) is also included among the signal transduction pathways activated by chagasic IgG acting on muscarinic cholinergic receptors. The mechanism involved in muscarinic cholinergic receptor-dependent activation of NOS appears to occur secondarily to an increase in intracellular calcium and activation of calcium/calmodulin-dependent NOS and by the stimulation of protein kinase C (PKC), which in turn leads to activation of NOS with increased production of nitric oxide (NO). NO formation induced activation of soluble guanylate cyclase activity increasing cGMP production. All of this signalling cascade would account for the alteration in contractility observed with chagasic antibody28, 36, 37, 40-43. It is possible that chronic interaction of chagasic IgG on myocardial muscarinic cholinergic receptors inducing release of NO leads to cell dysfunction and tissue damage, as described for some NO-dependent toxic effects when NO is immunologically generated.

A molecular interaction between chagasic IgG and muscarinic cholinergic receptors was demonstrated by the fact that: 1) chagasic sera but not normal sera quantitatively precipitated the muscarinic cholinergic receptor of the heart; 2) by cardiac membrane protein solubilization and electrophoretic fractionation, chagasic sera recognized a protein fraction of 78-80 kDa, similar
to the molecular weight of the atria muscarinic cholinergic receptor\textsuperscript{36, 37} and 3), chagasic but not normal IgG are able to immunoprecipitate the human M\textsubscript{2} muscarinic cholinergic receptor and to induce internalization of those receptors in M\textsubscript{2} CHO cells in a concentration and time-dependent manner. Chagasic antibodies induced the phosphorylation of M\textsubscript{2} muscarinic cholinergic receptors from Sf9 cells\textsuperscript{44}. These results point to a role for these autoantibodies in rapid receptor desensitization events, given the possibility that these antibodies could impair cardiac function by desensitization of receptors \textit{in vivo}.

It is important to note that either the binding assay or the intracellular signal transduction events induced by chagasic IgG, are specific since they were exerted by the F(ab\textsuperscript{'})\textsubscript{2} fraction.

Circulating autoantibodies are present previous to the development of the cardiomyopathy (Fig. 3). This could be explained, by the fact that the peak of serum immunoglobulin with sympathetic and parasympathetic activity precedes the impairment of heart neurotransmitter receptors mediated activity\textsuperscript{45-47}, indicating that these antibodies may be an early marker of heart autonomic dysfunction. In this sense, we have observed in human and experimental models of chagasic disease\textsuperscript{46}, that circulating \(\beta\) adrenergic and muscarinic cholinergic antibodies increase with the time of infection. At least in chagasic mice, the increase of circulating antibodies is coincident with the increase of myocarditis index (Fig. 3).

A very important issue for the understanding of the immune pathology of chronic chagasic neuromycardopathy is the fact that using an experimental autoimmune myocarditis model very similar to chronic Chagas myocarditis on the basis of immunopathologic and histologic data we have detected circulating autoantibodies against myocardial \(\beta\) adrenergic and muscarinic cholinergic receptors\textsuperscript{45-47}. Moreover, we have detected

![Fig. 3.— Circulating \(\beta\) adrenergic (●) and muscarinic cholinergic (■) autoantibodies in the function of time post-infection of murine (A) and human (B) Chagas' disease. In experimental model, circulating antibodies increase parallel with the increase of myocarditis index (columns).](image-url)
an anti *T. cruzi* monoclonal antibody which recognizes a 150 kDa antigen of *T. cruzi* and reacts with cardiac tissue. This MAb CAK20.12 (resembling circulating antibodies of chagasic patients) reacted with purified cardiac membranes and interferes with the binding of β adrenergic and muscarinic cholinergic receptor radioligands in a non-competitive fashion. As a consequence of this interaction both neurotransmitter receptors were activated, triggering intracellular signals that lead to alterations in cardiac contractility. The fact that this MAb modulates and modifies the mechanical and biochemical activity of normal heart established an important basis for future research and for understanding how the host’s humoral immune system responds during the development of chronic chagasic myocardiopathy.

It is known that muscarinic acetylcholine receptors (mAChRs) and β adrenergic receptors belong to members of G protein-coupled receptor family and undergo desensitization upon persistent stimulation by their specific agonists. The whole process includes a phosphorylation reaction by either second messenger-dependent kinases or G protein-coupled receptor kinases (GRKs) that precedes uncoupling of receptors from G proteins and, subsequently, sequestration and down regulation of receptors can occur. As can be drawn from the above considerations, the *in vivo* situation during the course of Chagas disease can be compared to that of a persistent stimulus - circulating antibodies - acting on cardiac mAChRs and therefore the possibility that a desensitization process is set up, appears plausible. To test this hypothesis, in that study we used both purified *M. β* mAChRs (CHO cells stably transfected with *M. β* receptors) to assess the ability of chagasic IgG to bind and desensitize those receptors. We observed that chagasic antibodies induce uncoupling of receptors from G proteins and a rapid sequestration of receptors away from membrane environment.

The real clinical relevance of these findings is the demonstration of a strong association between the existence of circulating anti-peptide antibodies in chagasic patients and the presence of dysautonomic symptoms making these autoantibodies proper markers of heart autoimmune neurocardiomyopathy. We were able to correlate clinical and experimental data demonstrating that anti neurotransmitter receptor antibodies were an early marker of disease evolution.

In fact, using a synthetic peptide for immunoblotting and enzyme immunoassays, we reported that autoantibodies react against the second extracellular loop of the human heart muscarinic receptor and β, adrenergic receptor in patients with Chagas disease. Thus, chagasic IgG similarly to a monoclonal anti-human *M. β* mAChR recognizes bands with a molecular weight corresponding to the cardiac mAChR. The specificity of interaction was assessed by inhibiting the binding of anti-peptide antibodies to mAChRs by the peptide (50). These anti-peptide antibodies were able not only to interact with the second extracellular loop of the human *M. β* mAChR, but they also displayed an “agonist like” activity modifying the intracellular events associated with specific muscarinic receptor activation i.e. decreased contractility, increased cGMP and decreased cAMP production in atria. All of the biological effects on rat atria triggered by chagasic anti-peptide antibodies were blunted by atropine and resembled the effects of the authentic agonist, confirming the participation of cardiac mAChR activation. Not only did anti peptide autoantibodies behave as cholinergic agonists, but they also diminished the reaction of myocardium to exogenous carbachol, suggesting that while in an early step they were able to activate mAChR, they might ultimately bind irreversibly to those receptors.

Noteworthy, affinity purified anti-peptide antibodies (but not the fraction of chagasic IgG devoid of anti-peptide antibodies eluted from affinity column) affected contractility and signal transduction to the same extent as total polyclonal chagasic IgG, supporting the assumption that these antibodies are fully responsible for mAChR-mediated effects of total chagasic IgG. The fact that the synthetic peptide corresponding in aminoacid sequence to the second extracellular loop of the human *M. β* mAChR, selectively suppressed the IFI and the biological effects of chagasic anti peptide and total IgG mediated by mAChRs, strongly suggests that the second extracellular loop can be considered essential for the biological action of these autoantibodies. So far, we have postulated that different populations of specific antibodies interacting with neurotransmitter receptors are present in the sera of *T. cruzi* infected mice and human chagasic patients having a variety of functional consequences for the myocardium. Thus, affinity purified anti-peptide chagasic antibodies could trigger cardiac mAChR-mediated biological effects resembling the effect of total chagasic IgG when its sympathetic action was abolished with propranolol. In addition, at least in contractility, non-anti-peptide antibodies obtained by direct elution from the column exerted a β adrenergic action, similar to the total chagasic IgG when cardiac parasympathetic activity was prevented by treating atria with atropine. Therefore, we favor the existence of two populations of neurotransmitter receptor - specific autoantibodies activities that co-occur in the same chagasic patient, suggesting the multiplicity of the autoimmune response in Chagas disease.

Autoantibodies against the second extracellular loop of *M. β* mAChR were found in patients with dilated cardiomyopathy but not in unrelated cardiovascular diseases; however, their involvement in the pathogenesis of idiopathic cardiomyopathy is still matter of...
controversy. Herein we demonstrate (Table 1) an association between the existence of the circulating anti-peptide M₂ mAChR autoantibodies and the presence of dysautonomic syndrome in chagasic patients (asymptomatic and cardiopathic). The presence of these anti-peptide autoantibodies detected by ELISA and biological assays is strongly associated with their ability to alter the myocardial behaviour of the heart through mAChR activation. Taken together, these observations point to the potential role of these autoantibodies in the pathogenesis of chronic chagasic cardioneuromyopathy. The seropositive patients without myocardial autonomic dysfunction with detectable anti-peptide autoantibodies, must be evaluated sequentially in order to ascertain the prognostic value of this test as an early marker of heart autonomic dysfunction.

Chagasic achalasia has been described as a dysautonomia-related dysfunction in which excitatory neuronal influence of esophageal motility appears to be unopposed by the impaired inhibitory neural influence that governs smooth muscle relaxation. On this basis, we have considered the possibility that autoantibodies against mAChRs participate in the pathogenesis of chagasic achalasia by modulating the muscarinic effector response in the lower esophagus. In effect, we have reported the presence of circulating IgG autoantibodies against mAChRs in chagasic achalasia able to increase the contractile tone and decrease cAMP accumulation of the lower esophageal sphincter. Moreover, those autoantibodies inhibit the relaxant contractile effect of the b-adrenergic agonist and the accumulation of cAMP triggered by isoproterenol. Table 2 shows the prevalence of circulating anti peptide M₂ mAChRs autoantibodies in chagasic patients with or without achalasia. It can be seen that in patients with achalasia the prevalence of autoantibodies is significantly higher than in other groups. These results suggest that the presence of mAChRs autoantibodies that exert a stimulatory effect on esophageal tone could contribute to the predominantly excitatory unbalance characteristic of chagasic achalasia.

It is important to note that the biological activity of the M₂ muscarinic agonistic circulating autoantibodies, present in both chagasic cardioneuromyopathy and achalasia, could be neutralized by short synthetic peptides corresponding to the functional epitopes in the antigenic extracellular loops and that this could be of possible therapeutic use to prevent the development of cardiac and esophageal chagasic dysautonomia.

**Conclusion**

This report supports the hypothesis that circulating autoantibodies in chagasic patients interact with myocardial β₁ adrenergic and M₂ muscarinic cholinergic receptors, triggering intracellular signals transduction that alter the physiological behaviour of the heart and lower esophageal sphincter. Moreover, they immunoprecipitate human M₂ muscarinic cholinergic receptors, they induce phosphorylation and internalization of these receptors in transfected cells, which may lead to desensitization or down-regulation. A positive correlation between the clinical symptoms and the presence of neurotransmitter receptor interacting-antibodies could be addressed, suggesting that those chagasic autoantibodies have a role in the cardiac and esophageal dysautonomic syndrome described in Chagas disease.

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**TABLE 1.– Distribution of antibodies from chagasic sera directed against peptide corresponding to the second extracellular loop of human neurotransmitter receptors tested by Elisa and Contractility**

<table>
<thead>
<tr>
<th>Chagasic groups</th>
<th>Biological Methods</th>
<th>Serological Elisa Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df/dt %</td>
<td>Elisa %</td>
</tr>
<tr>
<td>Asymptomatic with dysautonomia</td>
<td>343/350 98%</td>
<td>340/350 97%</td>
</tr>
<tr>
<td>Asymptomatic without dysautonomia</td>
<td>104/400 26%</td>
<td>99/400 25%</td>
</tr>
<tr>
<td>Cardiopathic with dysautonomia</td>
<td>178/180 99%</td>
<td>178/180 99%</td>
</tr>
<tr>
<td>Cardiopathic without dysautonomia</td>
<td>3/150 2%</td>
<td>2/150 1%</td>
</tr>
<tr>
<td>Control (non chagasic)</td>
<td>1/500 1%</td>
<td>2/500 1%</td>
</tr>
</tbody>
</table>

Microtiter wells were coated with 1 mg peptide and enzyme immunoassay (ELISA) was carried out in the presence of sera from different chagasic groups and controls. Values of O.D. above two S.D. of those of normal individuals were taken as positive. Contractility (df/dt) was measured in isolated atria; 5x10⁻⁷ M of different sera were used during 15 min of exposition.

**TABLE 2.– Distribution of anti-peptide antibodies from sera of chagasic patients with or without achalasia**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Biological Methods</th>
<th>Serological Elisa Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tone %</td>
<td>Elisa %</td>
</tr>
<tr>
<td>Chagasic with achalasia</td>
<td>14/15 93%</td>
<td>13/15 87%</td>
</tr>
<tr>
<td>Chagasic without achalasia</td>
<td>4/16 25%</td>
<td>4/16 25%</td>
</tr>
<tr>
<td>Control (non chagasic)</td>
<td>0/20 0%</td>
<td>0/20 0%</td>
</tr>
</tbody>
</table>

Microtiter wells were coated with 1 mg peptide and enzyme immunoassay (ELISA) was carried out in the presence of sera from different chagasic groups and controls. Values of O.D. above two S.D. of those of normal individuals were taken as positive. Tone was measured on low esophageal sphincter; 5x10⁻⁷ M of different sera were used during 15 min of exposition.
In support of the clinical relevance of these findings, we have demonstrated a strong association between the existence of circulating autoantibodies against peptides corresponding to the second extracellular loop of human cardiac M2 cholinoceptor and β2 adrenoceptors, with the symptoms and signs present in chagasic cardiac and esophageal dysautonomia.

The use of biomolecules as antigens for serological assays may be useful as tools for the diagnosis, prognosis and assessment of evolution of chagasic cardioneuromyopathy and achalasia.

References

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**Carlos Fuentes**