Cardiac $M_2$ muscarinic cholinoreceptor activation by human chagasic autoantibodies: association with bradycardia

J C Goin, E S Borda, S Auger, R Storino, L Sterin-Borda

Abstract

Objective—To assess whether exposure of cardiac muscarinic acetylcholine receptors (mAChR) to activating chagasic anti-myocardial immunoglobulins results in bradycardia and other dysautonomic symptoms associated with the regulation of heart rate.

Methods—Trypanosoma cruzi infected patients with bradycardia and other abnormalities in tests of the autonomic nervous system were studied and compared with normal subjects. Antipeptide antibodies in serum were demonstrated by an enzyme linked immunosorbent assay using a synthetic 24-mer-peptide corresponding antigenically to the second extracellular loop of the human heart $M_2$ mAChR. The functional effect of affinity purified antipeptide IgG from chagasic patients on spontaneous beating frequency and cAMP production of isolated normal rat atria was studied.

Results—There was a strong association between the finding of antipeptide antibodies in chagasic patients and the presence of basal bradycardia and an altered Valsalva manoeuvre (basal bradycardia: $\chi^2 = 37.5$, $p < 0.00001$; Valsalva manoeuvre: $\chi^2 = 70.0$, $p < 0.00001$). The antipeptide autoantibodies also showed agonist activity, decreasing the rate of contraction and cAMP production. The effects on rat atria resembled the effects of the authentic agonist and those of the total polyclonal chagasic IgG, being selectively blunted by atropine and AF-DX 116, and neutralised by the synthetic peptide corresponding in amino acid sequence to the second extracellular loop of the human $M_2$ mAChR.

Conclusions—There is an association between circulating antipeptide autoantibodies in chagasic patients and the presence of bradycardia and other dysautonomic symptoms. Thus these autoantibodies are a marker of autoimmune cardiac autonomic dysfunction. The results support the hypothesis that autoimmune mechanisms play a role in the pathogenesis of chagasic cardiomyopathy.

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Autoimmunity has been implicated in chagasic heart disease because of the scarcity of parasites in the myocardium, together with the paradox that in the chronic stage of the disease, while parasite numbers decline to virtual insignificance, the symptoms become most apparent. There is also evidence that morbidity in Chagas disease results from the misdirected effects of the humoral and cellular immune responses in infected patients induced by loss of self tolerance. One of the factors that disturbs self tolerance is the presence of cross reacting antigens shared by Trypanosoma cruzi and mammalian cells.

Cross reacting antihuman antibodies have been detected in human and experimental models. Among anti-myocardial antibodies we have described the presence of different populations of specific autoantibodies with adrenergic and cholinergic activity in human and murine chagasic sera, which trigger neurotransmitter receptor mediated effects. These autoantibodies have a variety of effects on normal myocardial activity, including alterations in the physiology and biochemistry of the myocardium. Moreover, cardiac $\beta$ adrenergic and muscarinic cholinergic activity can be reproduced by a monoclonal anti-T cruzi antibody derived from mice chronically infected with the K98 clone of the CAI strain.

The possibility that antineurotransmitter receptor antibodies may play a role in the pathogenesis of chagasic chronic cardiomyopathy has been proposed. Indeed chronic chagasic heart disease is a cardioneuromyopathy in which both the sympathetic and the parasympathetic nervous systems are affected. This dysautonomic syndrome is seen before the development of the symptoms of chagasic cardiomyopathy and may be explained by the slow and progressive binding of autoantibodies to $\beta$ adrenergic and muscarinic cholinergic receptors. The presence of autoantibodies against muscarinic acetylcholine receptors (mAChR) in the sera of chagasic patients with dysautonomic syndrome has been demonstrated and it has been shown that the second extracellular loop of human $M_2$ mAChR appears to be the main immunogenetic region of the receptor interacting with chagasic human antibodies.

Our aim in this study was to determine whether the presence of circulating antibodies directed against the second extracellular loop of human $M_2$ mAChR is associated with basal bradycardia of chagasic patients. Other dysautonomic tests such as the Valsalva manoeuvre,
hyperventilation, and orthostatic blood pressure measurement were also studied. Affinity purified circulating autoantibodies against human Mₐ mAChR were found to induce bradycardia and decreased production of cAMP in isolated normal rat atria. The clinical relevance of these findings is confirmed by a strong association between circulating anti-muscarinic peptide antibodies in chagasic patients and the presence of basal bradycardia and dysautonomic symptoms, especially those related to alterations in heart rate.

Methods

Patients

We studied T. cruzi infected patients with positive serology residing in metropolitan Buenos Aires. These included asymptomatic subjects (with a normal ECG, 24 hour Holter record, echocardiogram, and chest x ray), and subjects with evidence of heart disease (with an abnormal ECG and/or an abnormal 24 hour Holter, but normal echocardiogram and chest x ray and without cardiomegaly or evidence of congestive heart failure).[41] Non-infected controls were also studied.

We divided the subjects into three groups—IA, IB, and II.

Group IA—35 asymptomatic patients and 20 cardiomyopathy patients, all of whom had basal bradycardia and autonomic nervous system dysfunction, as shown by abnormal responses to two or more of the following diagnostic tests (values are means (SEM)): reduced diastolic blood pressure (72 (3) mm Hg) compared with normal subjects (91 (5) mm Hg); poor rise in diastolic blood pressure in response to tilting test (4.2 (0.9) mm Hg v 11.6 (3) mm Hg); less bradycardia during the straining phase of the Valsalva manoeuvre (RR interval −12 (5)% v −26 (5)%), and less tachycardia during the releasing phase (RR interval 15 (9)% v 47 (39)%; hyporeactivity to the cough reflex test (+4 (2) beats v +35 (9) beats); and hyporeactivity to the hyperventilation test (22 s v 9 s). The criteria for diagnosis of dysautonomia were applied on the basis of previous reports.[15][22]

Group IB—30 asymptomatic patients with normal cardiovascular response to autonomic nervous system tests and a normal heart rate. Groups IA and IB were not receiving any drug treatment. Serological studies for Chagas disease (passive haemagglutination, enzyme linked immunosorbent assay (ELISA), and immunofluorescence) were performed in all patients.

Group II (the control group)—50 healthy volunteers with negative serology and no evidence of cardiovascular disease, chronic systemic disease, or acute viral or febrile disease. These subjects had normal ECGs and chest x rays.

The patients and controls included in this study were aged between 50 and 60 years.

Specific Peptide

A 24-mer-peptide (VRTVEDGECYIQFFSNAAVTFGTA) corresponding to the sequence of the second extracellular loop of the human muscarinic acetylcholine receptor (residues 169 to 192) was synthesised by the F-moc amino acids activated using the 1-hydroxybenzotriazole/dicyclohexylcarbodiimide (HOBT/DCC) strategy with an automatic peptide synthesiser (Applied Biosystems model 431A). The peptide was desalted, purified by high performance liquid chromatography, and subjected to amino terminal sequence analysis by automatic Edman degradation with an Applied Biosystems 470 A sequencer (Applied Biosystems Inc, Foster City, California, USA).

Purification of Human IgG

The IgG fraction of human chagasic or normal (control) patients was isolated by chromatography on DEAE-cellulose. Sera were dialysed overnight against the elution buffer (10 mM Na/K phosphate, pH 8) and then passed through DEAE-cellulose columns that were previously equilibrated with elution buffer. The eluted peaks were concentrated by ultrafiltration (Amicon ultrafiltration cell, model 8010; Beverly, Massachusetts, USA) to about 10–12 mg/ml. The degree of IgG purification was tested by SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) and its concentration was determined by radial immunodiffusion assay.

Purification of Antipeptide Antibodies by Affinity Chromatography

The IgG fractions of 12 dysautonomic chagasic patients were independently subjected to affinity chromatography on the synthesised peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, California, USA). The IgG fraction was loaded on the affinity column equilibrated with phosphate buffered saline (PBS) and the non-antipeptide fraction was first eluted with the same buffer. Specific antipeptide autoantibodies were then eluted with 3 M KSCN and 1 M NaCl, followed by immediate extensive dialysis against PBS. The IgG concentration of specific antimuscarinic receptor peptide antibodies was determined by radial immunodiffusion assay and their immunological reactivity against the muscarinic receptor peptide was evaluated by ELISA.

Enzyme Immunoassay

Fifty microlitres of peptide solution (20 µg/ml) in 0.1 M Na₂CO₃ buffer, pH 9.6, were used to coat Costar microtitre plates at 4°C overnight. After blocking the wells with 2% bovine serum albumin in PBS for one hour at 37°C, 100 µl of different dilutions of sera or purified IgG from chagasic or normal patients were allowed to react with peptide for two hours at 37°C. After a single thorough washing with 0.05% Tween in PBS, 100 µl of 1:6000 biotinylated goat antimouse IgG antibodies (Sigma, St Louis, Missouri, USA) were added and incubated for one hour at 37°C. Then a 1:6000 dilution of avidin–alkaline phosphatase (Sigma) was added for an additional 30 minutes at 37°C. After extensive washings, p-nitrophenylphosphate (1 mg/ml) was added as substrate and the reaction was stopped at 30 minutes. Optical density values were measured with an ELISA reader. In some experiments, the
peptide (5 × 10⁻⁷ M) was included in the incubation volume with different sera or IgG dilutions as a test for the specificity of the reaction between antibodies and the coating peptide.

CONTRACTILE STUDIES
Male rats weighing around 200 g were decapitated and bled. Their chests were opened and their hearts were quickly excised. The atria were immediately dissected and mounted on a polygraph as described previously.²⁰ One end of each atrium was attached to a stationary glass holder, immersed in a tissue chamber filled with a modified Krebs–Ringer bicarbonate (KRB) solution, and gassed with a mixture of 95% O₂/5% CO₂ as reported previously.²³ The other end of each atrium was connected to a force transducer (Statham UC-3 Gold Cell; San Diego, California, USA). Throughout the experiments, the preparations were subjected to a constant resting tension of 750 mg by means of a micrometric device attached to the transducer, the output of which was amplified and recorded by a direct, ink writing oscillograph. The bath solution was gassed with a mixture of 5% CO₂ in oxygen and kept at a constant temperature of 30°C and pH 7.4. Chronotropism was determined on spontaneous beating atria and was analysed in terms of atrial rate (beats/min). Control values (equal to 100%) refer to the rate before the addition of different immunoglobulins or carbachol. The absolute values of rate at the end of equilibrium (30 minutes) ranged between 140 and 150 beats/min.

CYCLIC AMP ASSAY
Rat atria were preincubated in the presence of M₁ peptide, atropine, AF-DX 116, or pertussis toxin in KRB solution for 15 minutes. Samples were then incubated for a further 15 minutes with chagasic or normal IgG or antipeptide antibodies. After incubation, tissues were homogenised in 2 ml absolute ethanol and centrifuged at 6000 × g for 15 minutes. Supernatants were collected and the pellets were rehomogenised with EtOH/water (2:1). Supernatants from both centrifugations were evaporated to dryness and residues resuspended in 5 mM Tris-HCl, pH 7.4, containing 8 mM theophylline, 0.45 mM EDTA, and 6 mM mercaptoethanol. Cyclic AMP determination was developed by the competitive protein binding assay described by Brown et al.²¹ using [³H]-cAMP as tracer.

DRUGS
Carbachol, atropine, and theophylline were purchased from Sigma and AF-DX 116 was kindly provided by Boehringer Ingelheim Pharmaceuticals Inc (Ridgefield, Connecticut, USA). Stock solutions of the drugs were dissolved in distilled water and were freshly prepared.

STATISTICAL ANALYSIS
Prevalence values from different groups were compared by χ² tests with Yates correction. For comparison among mean values all data were first examined by analysis of variance. Differences between means were determined by the Student-Neuman-Keuls test. Differences were considered significant at a ρ value equal to or less than 0.05.

Results
BASAL CARDIAC RATE ON CHAGASIC PATIENTS
T. cruzi infected patients had a lower heart rate than normal subjects. Table 1 shows the distribution of heart rates in selected seropositive patients. It can be seen that bradycardia was strongly associated with T. cruzi infection regardless of whether they had cardiomyopathy. No significant differences in patients’ ages among the different groups were observed. Thus heart rate variation as a function of age and duration of disease was avoided.

DETECTION OF ANTIPEPTIDE ANTIBODIES IN CHAGASIC PATIENTS
The presence of autoantibodies against the second extracellular loop of human heart M₁ mAChR was explored in chagasic and normal subjects using the 24-mer peptide as antigen. Table 2 shows the distribution of anti-M₁ mAChR autoantibodies in seropositive patients with normal heart rate or with basal bradycardia (less than 55 beats/min). It can be seen that the frequency of antipeptide autoantibodies detected by ELISA was significantly higher in seropositive patients with basal bradycardia than in seropositive patients with normal heart rate. No differences between asymptomatic (non-cardiac) and cardiac patients of group IA were detected. All normal sera were negative in the study system.

Table 3 shows that when the distribution of antipeptide antibodies in the same group of chagasic patients was correlated with altered responses to the different dysautonomic tests, the sensitivity and specificity for basal bradycardia was lower than for the Valsalva manoeuvre.
Table 3  Detection of serum anti-M₂ mAChR antibodies in chagasic patients subjected to different tests for dysautonomia

<table>
<thead>
<tr>
<th>Tests</th>
<th>Basal heart rate</th>
<th>Tilting</th>
<th>Coughing</th>
<th>Hyperventilation</th>
<th>Valsalva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7/30</td>
<td>18/50</td>
<td>12/35</td>
<td>20/55</td>
<td>3/40</td>
</tr>
<tr>
<td>Altered: Sensitivity (%)</td>
<td>89.1</td>
<td>62.8</td>
<td>64.0</td>
<td>83.3</td>
<td>100.0</td>
</tr>
<tr>
<td>Altered: Specificity (%)</td>
<td>76.7</td>
<td>64.0</td>
<td>65.7</td>
<td>63.6</td>
<td>92.5</td>
</tr>
<tr>
<td>Altered: Youden index</td>
<td>65.8</td>
<td>26.8</td>
<td>29.7</td>
<td>46.9</td>
<td>92.5</td>
</tr>
</tbody>
</table>

Chagasic patients (group I, n = 55; group II, n = 30) were subjected to a variety of tests for detection of cardiovascular dysautonomia. The presence of circulating anti-M₂ mAChR antibodies in every patient was determined at 1/50 serum dilution by ELISA as described in Methods.

Prevalence of antibodies in patients with normal or altered basal heart rate was determined by tilting, coughing, hyperventilation, and Valsalva tests.

Table 4  Changes in atrial rate and cAMP production by antipeptide chagasic autoantibodies

<table>
<thead>
<tr>
<th>Additions</th>
<th>Atrial rate (beats/min)</th>
<th>cAMP (pmol/mg wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>143 (11)</td>
<td>3.8 (0.3)</td>
</tr>
<tr>
<td>Antipeptide IgG + atropine</td>
<td>95 (4)*</td>
<td>0.9 (0.1)*</td>
</tr>
<tr>
<td>Antipeptide IgG + pertussis toxin</td>
<td>138 (9)</td>
<td>3.1 (0.3)</td>
</tr>
<tr>
<td>Antipeptide IgG + AF-DX 116</td>
<td>145 (10)</td>
<td>3.7 (0.5)</td>
</tr>
<tr>
<td>Antipeptide IgG + AF-DX 116</td>
<td>140 (9)</td>
<td>3.4 (0.4)</td>
</tr>
<tr>
<td>Antipeptide IgG + pertussis toxin</td>
<td>142 (8)</td>
<td>2.9 (0.3)</td>
</tr>
<tr>
<td>Normal IgG</td>
<td>146 (10)</td>
<td>3.6 (0.2)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>93 (8)*</td>
<td>0.7 (0.1)*</td>
</tr>
</tbody>
</table>

Values are mean (SEM) and represent the effect of 12 chagasic antipeptide IgG experiments from patients in group IA. Results show the maximum effects on frequency and cAMP production by antipeptide IgG (1 × 10⁻⁷ M) compared with normal IgG (5 × 10⁻⁷ M) and carbachol (5 × 10⁻⁷ M). Inhibition studies were performed by incubating rat atria either with peptide, atropine, AF-DX 116 (5 × 10⁻⁷ M) or pertussis toxin (1 µg/ml).

**Discussion**

As already reported, chagasic IgG contains autoantibodies against the second extracellular loop of the human M₂ muscarinic cholinoceptors, identified using a synthetic 24-mer peptide in immunoblotting and enzyme immunoassay experiments. Both monoclonal antihuman M₂ mAChR and chagasic IgG were associated with a band of molecular weight corresponding to the cardiac mAChR. The 24-mer peptide inhibited the binding of chagasic antibodies. The molecular interaction of chagasic antibodies with purified human M₂ mAChR has been shown by immunoprecipitation, as with anti-M₂ mAChR antibodies.

In this study we have shown that the antipeptide antibodies obtained from chagasic patients with bradycardia could not only interact with the second extracellular loop of the human M₂ mAChR but also displayed agonist-like activity, decreasing contraction frequency and attenuating cAMP production when these were tested in parallel in spontaneously beating normal rat atria. Both these biological effects triggered by chagasic antipeptide antibodies were blunted by the specific peptide and by a specific M₂ mAChR antagonist.
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muscarinic cholinergic antagonist, and they resembled the effects of the authentic agonist carbachol.

The fact that the antipeptide antibodies behaved as cholinergic agonists suggests that they were able to activate heart mACHR. We showed previously that in intact mammalian CHO cells transfected with human M2 mACHR, the chagasic IgG mimicked the ability of the agonist acetylcholine to induced effects associated with agonist induced receptor desensitisation—decreased affinity for agonist binding to M2 mACHR and sequestration of the M2 mACHR from the cell surface. We have postulated that chagasic IgG can interact directly with heart M2 mACHR, inducing a dysautonomic syndrome. Nevertheless, the fact that both anti mACHR and β adrenergic receptor antibodies coexist in the sera of chagasic patients indicates that some of the clinical manifestation could be the result of autoantibodies acting as muscarinic agonists and β adrenergic partial agonists, impairing the action of endogenous noradrenaline.

The existence of autoantibodies against autonomic receptors has been reported in other cardiomyopathies, but the mechanism underlying their potential pathogenic role is still unknown. Whether autoantibodies against neurotransmitter receptors play a general role in other cardiomyopathies remains to be determined.

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A 42 year old man (not a drug abuser) diagnosed with a ruptured aneurysm of the sinus of Valsalva seven years ago was referred because of low grade fever of one week duration, fatigue, vomiting, and abdominal pain. His blood pressure was 115/45 mm Hg. A continuous machinery murmur (grade 3/6) was heard along his left sternal border. Six blood cultures grew methicillin sensitive *Staphylococcus aureus*. Transoesophageal echocardiography using a multiplane probe identified an aneurysm of the non-coronary sinus of Valsalva, which appeared as a calcified, circular structure protruding into the right atrium. The aneurysm was resected and a 2 cm long vegetation attached to the lower edge of the fistula was seen (top). Colour Doppler showed a turbulent left to right shunt through the perforation (bottom). The aneurysm was resected and the defect closed with an autologous pericardial patch. The patient had an uneventful postoperative recovery, and one year later, was alive and asymptomatic. (LA, left atrium; RA, right atrium; Ao, aorta; T, tricuspid valve; RV, right ventricle.)

JUAN CARLOS CASTILLO DOMÍNGUEZ
MANUEL ANGUITA SÁNCHEZ
ANTONIO RAMÍREZ MORENO