

Interaction of human chagasic IgG with human colon muscarinic acetylcholine receptor: molecular and functional evidence

L Sterin-Borda, J C Goin, C R Bilder, G Iantorno, A C Hernando, E Borda

Abstract

Background and aims—Gastrointestinal disorders is one of the clinical manifestations of chronic Chagas' disease. The pathogenesis seems to be associated with autonomic dysfunction. Here, we consider the muscarinic cholinergic mediated alteration in distal colon function in chagasic megacolon.

Patients—Patients were divided into four groups: group I, chronic chagasic patients with megacolon; group II, chronic chagasic patients without megacolon; group III, non-chagasic patients with megacolon; and group IV, normal healthy volunteers (control).

Methods—Binding assay and immunoblot of cholinergic receptors from human and rat colon and enzyme immunoassay (ELISA) using a synthetic 24mer peptide corresponding to the second extracellular loop of human M₂ muscarinic acetylcholine receptors (mAChR) were used to detect the presence of serum antibodies. The effect of antibodies on basal tone and 3',5'-cyclic monophosphate (cAMP) production of human and rat distal colon strips were also tested.

Results—Group I but not the other groups had circulating antibodies capable of interacting with human colon activating M₂ mAChR, as they competed with binding of specific radioligand to mAChR and interacted with the second extracellular loop of human M₂ mAChR. Moreover, affinity purified anti-M₂ peptide IgG from group I, in common with monoclonal antihuman M₂ mAChR, recognised bands with a molecular weight corresponding to colon mAChR. This antibody also displayed an agonist-like activity, increasing basal tone and decreasing cAMP accumulation. Both effects were blunted by AF-DX 116 and neutralised by the synthetic peptide.

Conclusions—In chagasic patients with megacolon there are antibodies that can recognise and activate M₂ mAChR. The implications of these autoantibodies in the pathogenesis of chagasic megacolon is discussed.

(Gut 2001;49:699–705)

Keywords: chagasic megacolon; acetylcholine receptor; antibodies; colon contractility

Chagas' disease is a parasitic disease that represents one of the most serious public health problems in South America. The clinical picture of this disease is dominated by cardiac and digestive disorders.

The digestive manifestations mainly involve megaesophagus and megacolon.¹ The abnormalities of the autonomic enteric nervous system seem to be an essential element in the pathogenesis of chagasic megacolon. These abnormalities include degeneration and reduction in the number of myenteric plexus that coordinate the motor activity of different segments from the oesophagus to the rectum.² These lesions occur throughout the digestive tract but the oesophagus and distal portion of the colon are the parts most affected because of the physiology of these segments.³ Furthermore, both regions have a sphincter at their end that must relax by a reflex mechanism. Chagasic megacolon has been described as a dysautonomic related disorder in which excitatory neural influences on colon motility seem to be unopposed by the impaired inhibitory neural influences that govern smooth muscle relaxation.^{4–6}

In fact, abnormalities of the distal colon^{7–9} as well as impairment of the relaxation response of the internal sphincter to rectal distension have been described in chagasic patients.^{6–8} Moreover, the motor responsiveness of the distal colon to cholinergic agents⁷ and to several pharmacological and physiological stimuli is altered. This defect may play a pathophysiological role in colonic disease.^{9, 10}

There is evidence that the enteric nervous system exerts local control of gastrointestinal function by multiple mechanisms. Excitatory motor neurones connected to the excitatory parasympathetic pathways is one of the determinants of colonic motility. These motor neurones release acetylcholine and it acts postjunctionally between motor neurones and muscarinic acetylcholine intestinal receptors (mAChRs) of muscle.^{11–13} The coexistence of more than one mAChR subtype with different contractile roles has been demonstrated in the colon.¹⁴

Most studies on cholinergic response of chagasic megacolon have focused on the integrity of preganglionic and postganglionic innervation without further consideration of eventual alterations in the effector muscarinic response.¹⁵ Muscarinic cholinergic mediated modulation of colonic function in chagasic megacolon remains to be clarified.

We showed previously the presence of serum autoantibodies against mAChR in patients with chronic Chagas' disease.^{16, 17} These antibodies

Abbreviations used in this paper: mAChR, muscarinic acetylcholine receptor; PGE₁, prostaglandin E₁; cAMP, 3',5'-cyclic monophosphate; NMS, N-methylscopolamine; KRB, Krebs-Ringer bicarbonate.

Pharmacology Unit,
School of Medicine
and Dentistry,
University of Buenos
Aires, Argentina and
National Research
Council, Buenos Aires,
Argentina

L Sterin-Borda
J C Goin
E Borda

Motility Unit,
Gastroenterology
Hospital, "Dr C
Bonarino Udaondo",
Buenos Aires,
Argentina

C R Bilder
G Iantorno
A C Hernando

Correspondence to:
Dr L Sterin-Borda,
Pharmacology Unit, School
of Dentistry, University of
Buenos Aires, Marcelo T de
Alvear 2142, 4^o floor "B",
1122 Buenos Aires,
Argentina.
leo@farmaco.odon.uba.ar

Accepted for publication
12 March 2001

displayed an "agonist-like" activity modifying intracellular events associated with M_2 mAChR activation.¹⁷ These anti- M_2 mAChR antibodies were associated with the presence of cardiac dysautonomia and/or achalasia in patients with chronic Chagas' disease.¹⁶⁻¹⁸

The aim of this study was to investigate the presence of anti- M_2 mAChR antibodies in patients with chronic Chagas' disease and megacolon. We have confirmed that such antibodies arise as a result of the chagasic state and that they interact with human colon activating M_2 mAChR, indicating a role for the second extracellular loop of human M_2 mAChR as the main target for human autoantibody mediated biological effects.

Methods

PATIENTS

All patients (n=55) aged between 31 and 75 years were living in metropolitan Buenos Aires at the time of the study, although all chagasic patients had previously resided in areas endemic for *Trypanosoma cruzi*. A diagnosis of Chagas' disease was made on the basis of three standard serological reactions against *T. cruzi*: passive haemagglutination, indirect immunofluorescence, and enzyme immunoassay. Subjects were divided into three groups: group I consisted of 15 chronic chagasic patients with megacolon (eight men and seven women); group II comprised 17 chronic chagasic patients without megacolon (10 men and seven women), and group III consisted of three non-chagasic patients with megacolon (two men and one woman; mean age 59.4 (4.8) years). Group IV (normal controls) included 20 healthy volunteers (10 men and 10 women). Patients in groups I and III had clinical and radiological findings characteristic of megacolon. Barium enema showed sigmoid dilatation and/or elongation in all patients. Abnormalities of basal motility of the distal colon as well as impairment of the relaxation response of the sphincter on rectal distension were also observed in megacolon patients. Conversely, patients in group II showed no evidence of bowel alteration. Regardless of the presence of megacolon in patients in groups I and III, none of the subjects had evidence of cardiovascular, neurological, metabolic, and/or systemic disease. Patients with acute or other chronic disorders were not included in the study. None of the patients was taking medications during and/or 72 hours before blood samples were obtained and clinical studies performed. Each subject gave fully informed consent under a protocol approved by the Gastroenterology Hospital Research Committee.

DRUGS

Atropine, carbachol, prostaglandin E_1 (PGE_1), and 3-isobutyl-1-methylxanthine were purchased from Sigma Chemical Company (St Louis, Missouri, USA). [[2-[(diethylamino) methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3b][1,4] benzodiazepine-6-one (AF-DX 116) was kindly provided by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, Connecticut, USA). [3H] adenosine 3',5'-cyclic monophosphate

([3H]cAMP 30.5 Ci/mmol) and [3H] *N*-methylscopolamine ([3H] NMS 80.4 Ci/mmol) were purchased from New England Nuclear (Boston, Massachusetts, USA).

TISSUE SAMPLES

Human resection specimens were obtained from the distal colon from patients aged 31–65 years (three men and two women) undergoing partial colectomy for chagasic megacolon. A healthy section of the colon was carefully separated from the pathological tissue under the stereomicroscope at room temperature. Male Wistar rats (200–250 g) were decapitated and bled; strips of distal colon were then taken. Both human and rat distal colon samples were washed with ice cold phosphate buffered saline and subjected to further dissection. The mucosal, submucosal, and serosal layers were carefully removed, leaving the smooth muscle layer.¹⁹ Human and rat tissue homogenates for ligand binding assay and western blot analysis were obtained as previously described.¹⁶

PURIFICATION OF HUMAN IgG

The IgG fraction from chagasic patients with megacolon or from normal subjects (control) was isolated by chromatography on diethylaminoethyl-cellulose, as previously described.¹⁷ IgG was concentrated by ultrafiltration to about 10–12 mg/ml. The degree of IgG purification was tested by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and its concentrations were determined by radial immunodiffusion assay.

COMPETITION BINDING STUDIES

Membranes from rat or human distal colon smooth muscle (2.5–3.0 mg/ml) were preincubated with different concentrations of the IgG fraction from either chagasic patients with megacolon or normal subjects for one hour at 30°C in buffer A. The membranes were then incubated with 0.6 mM [3H] NMS for two hours at 25°C in buffer A (final volume 0.5 ml). The reaction was stopped with ice cold buffer A and filtered through Whatman glass fibre filters (GF/c) under vacuum. Filters were washed three times with 4 ml of buffer A and counted in an 8 ml scintillation cocktail with 0.56% efficiency. Non-specific binding was determined in the presence of 1×10^{-6} M atropine and did not exceed 10%.

PEPTIDE

A 24mer peptide (V-R-T-V-E-D-G-E-C-Y-I-Q-F-F-S-N-A-A-V-T-F-G-T-A) and a 25mer peptide (K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-S-N-P-A-V-T-F-G-T-A-I) corresponding to the sequences of the second extracellular loop of human M_2 and M_3 mAChRs¹⁶ were synthesised (model 413; Applied Biosystem, Foster City, California, USA). The peptide was desalted, purified by high pressure liquid chromatography, and subjected to amino terminal sequence analysis by automatic Edman degradation with an Applied Biosystem (model 470 A) Sequencer.

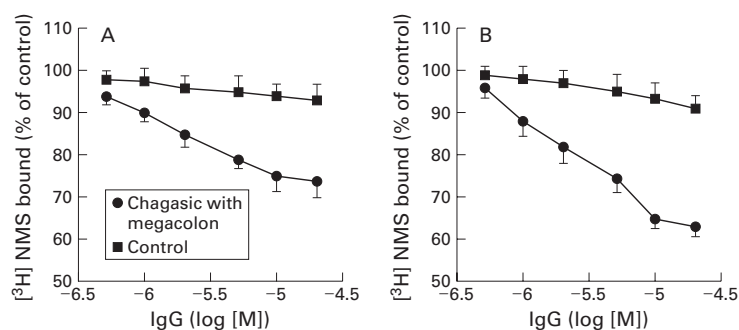


Figure 1 Concentration dependent inhibition of [^3H] *N*-methylscopolamine (NMS) binding on colonic smooth muscle membranes. Rat (A) and human (B) colon smooth muscle homogenates were incubated with serum IgG from either chagasic patients with megacolon or normal individuals in the presence of [^3H] NMS. Control binding of 100% refers to the binding of the muscarinic radioligand to membranes treated with no IgG. Data are mean (SEM) of triplicate determinations of five IgGs from different chagasic or normal subjects.

PURIFICATION OF ANTIPEPTIDE ANTIBODIES

The IgG fractions from five chagasic patients with megacolon with the highest serum anti- M_2 mAChR titres were independently subjected to affinity chromatography on the synthesised peptide covalently linked to AffiGel 15 gel (Bio Rad, Richmond, California, USA) and purified as previously described.¹⁸ The IgG concentration of anti-peptide antibodies was determined by radial immunodiffusion assay, and immunological reactivity against the muscarinic receptor peptide was evaluated by enzyme immunoassay.

ENZYME IMMUNOASSAY

Peptide solution (50 μl of 20 $\mu\text{g}/\text{ml}$) in 0.1 M Na_2CO_3 buffer, pH 9.6, was used to coat microtitre plates (NUNC, Kastrup, Denmark) at 4°C overnight, as previously described.¹⁸ Briefly, after blocking the wells, diluted sera or IgG from chagasic or control patients were added in triplicate and allowed to react with the peptide for two hours at 37°C. After thoroughly washing, 100 μl of 1:6000 biotinylated goat antihuman IgG antibodies (Sigma) were added and incubated for one hour at 37°C. Then, a 1:6000 dilution of ExtrAvidin/alkaline phosphatase (Sigma) was added. After extensive washings, p-nitrophenylphosphate (1 mg/ml) was added as substrate and the reaction was stopped after 30 minutes. The plates were read at 405 nm.

Table 1 Effect of chagasic antibodies on [^3H] *N*-methylscopolamine (NMS) binding on rat and human distal colon

| | [^3H] NMS binding | | | |
|----------------|------------------------------|-------------|-------------|-------------|
| | Rat | | Human | |
| | Bmax | Kd | Bmax | Kd |
| None (control) | 20.3 (1.2) | 1.43 (0.22) | 32.0 (2.3) | 1.36 (0.19) |
| Group I | 14.8 (0.9)* | 1.45 (0.28) | 20.6 (1.8)* | 1.40 (0.20) |
| Group II | 21.4 (1.4) | 1.40 (0.24) | 33.2 (1.9) | 1.38 (0.20) |
| Group III | 22.5 (1.4) | 1.44 (0.27) | 34.3 (3.0) | 1.41 (0.18) |
| Group IV | 21.2 (1.3) | 1.38 (0.21) | 31.8 (2.6) | 1.38 (0.17) |

Colonic smooth muscle homogenates (0.9 mg) were or were not incubated with 1×10^{-5} M IgG from chagasic patients with megacolon or IgG from normal subjects in the presence of increasing concentrations of [^3H] NMS. The number of binding sites (Bmax) expressed as fmol/mg protein and the equilibrium dissociation constant (Kd) expressed as nM were calculated from linear regression analysis. Mean (SEM) of triplicate determinations of five IgGs from different groups. Group I, chagasic patients with megacolon; group II, chagasic patients without megacolon; group III, non-chagasic patients with megacolon; group IV, normal subjects.

*Significantly different from normal IgG, $p < 0.001$.

WESTERN BLOT

Membranes from rat or human distal colon smooth muscle were subjected to electrophoresis on 8.5% sodium dodecyl sulphate-polyacrylamide gels. Proteins were transferred to nitrocellulose sheets and probed with different IgGs from chagasic patients with megacolon or normal individuals, as previously described.¹⁷ We also tested a monospecific mouse antihuman M_2 mAChR IgG as a specific marker for the receptor.

CYCLIC AMP ASSAY

Human and rat distal colon smooth muscle strips were obtained as described above and incubated as previously reported.¹⁸ Normal or chagasic IgG fractions were added 15 minutes before the end of the reaction and PGE_1 (1×10^{-6} M) was added in the last five minutes as an adenylate cyclase activator. The specificity of the antibody- M_2 mAChR interaction was assessed by addition of 1×10^{-6} M atropine or AF-DX 116 to the incubation medium. cAMP determination was performed by competitive protein kinase binding assay, as described by Gomez and colleagues,¹⁹ using [^3H] cAMP as tracer.

CONTRACTILITY STUDIES

Human and rat distal colon smooth muscle strips were obtained as described above and placed in Petri dishes filled with Krebs-Ringer bicarbonate (KRB) solution containing (mM): Na^+ 145; K^+ 6.02; Ca^{2+} 2.2; Mg^{2+} 1.33; Cl^- 126; HCO_3^- 25.3; SO_4^{2-} 1.33; HPO_4^{2-} 1.20; and glucose 5.5. The strips were mounted on an organ bath containing KRB solution at 37°C gassed with 5% CO_2 in oxygen at pH 7.4.¹⁸ One end of each tissue was anchored to a stationary glass holder and the other was connected to a force displacement transducer (Stathan UC-3 Gold Cell) coupled to an ink writing oscillograph (Grass RPS 7C). A constant resting tension of 500 mg was applied to preparations, and strips were allowed to equilibrate for 30 minutes. Each preparation was exposed to different concentrations of IgG antibodies from chagasic patients with megacolon or normal control subjects over 15 minutes. Inhibition experiments were performed by incubating tissues with 5×10^{-6} M AF-DX 116 or pertussis toxin for 30 minutes before addition of the antibodies.

STATISTICAL ANALYSIS

Prevalence values between groups were compared by the χ^2 test. For multiple comparisons among mean values, data were first examined by analysis of variance, and differences between means were evaluated by the Student-Newman-Keuls test. All statistical significances were justified at $\alpha = 0.05$.

Results

CHAGASIC IgG BINDING TO COLON mAChR

The interaction between serum IgG from chagasic patients with megacolon mAChRs and colon smooth muscle membranes is shown in fig 1. IgG from chagasic patients with megacolon (group I) but not from normal subjects (group III) inhibited specific binding of

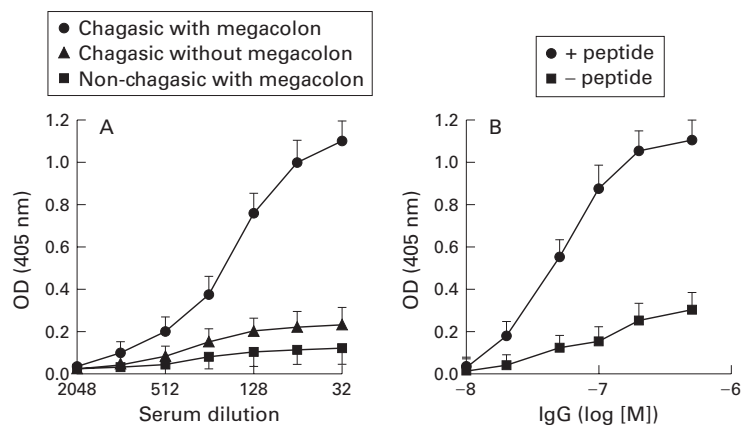


Figure 2 Immunoreactivity of anti-muscarinic acetylcholine receptor (mAChR) antibodies from chagasic sera against the second extracellular loop of the human M_2 mAChR tested by ELISA. Microtitre wells were coated with 1 μ g of M_2 peptide and an enzyme immunoassay was performed (see materials and methods). (A) Mean (SEM) optical density (OD) values, representing triplicate assays from 15 chagasic patients with megacolon, 17 chagasic patients without megacolon, and three non-chagasic patients with megacolon. (B) Mean (SEM) optical density (OD) values, representing triplicate determinations of five affinity purified anti-peptide IgGs from different chagasic patients with megacolon in the presence (+) or absence (-) of 1×10^{-5} M peptide.

cholinergic radioligand [3 H] NMS to rat (fig 1A) and human (fig 1B) colon membrane preparations in a concentration dependent manner.

In saturation studies and Scatchard analysis (table 1), an irreversible interaction was established. Thus when rat and human colon preparations reacted with chagasic IgG, a decrease in the number of binding sites (Bmax) without a significant change in the equilibrium dissociation constant (Kd) was observed. IgGs from groups II, III, and IV gave negative results.

DETECTION OF SERUM AUTOANTIBODIES AGAINST HUMAN M_2 mAChR

To determine the molecular interaction between autoantibodies from chagasic patients with megacolon and human M_2 mAChR, we carried out an enzyme immunoassay using the synthetic 24mer peptide corresponding in amino acid sequence to the second extracellular loop of human M_2 mAChR.

As can be seen in fig 2A, immunoreactivity of sera from chagasic patients with megacolon (group I) was significantly higher than in sera from chagasic patients without megacolon (group II) ($p < 0.0005$) and from non-chagasic patients with megacolon (group III) ($p < 0.0001$). Values for optical density in group I were always higher than 3 SDs compared with those from normal individuals (group IV).

Then, IgG fractions from chagasic patients with megacolon were subjected to affinity chromatography purification on the M_2 synthetic peptide (anti- M_2 peptide IgG) and their immunoreactivity against the M_2 peptide was evaluated. As shown in fig 2B, the anti- M_2 peptide IgG increased optical density values in a concentration dependent manner. The specificity of these anti- M_2 peptide IgGs was assessed by the ability of the M_2 peptide (10-fold concentration) to inhibit the interaction.

It is important to note that when a synthetic peptide corresponding in amino acid sequence to the second extracellular loop of human M_3 mAChR was used as a coating antigen for

Table 2 Immunoreactivity of circulating IgG antibodies against the second extracellular loop of the M_2 and M_3 muscarinic acetylcholine receptor (mAChR) tested by ELISA

| Sera | OD (405 nm) synthetic peptide | |
|--------------------|-------------------------------|-------------|
| | M_2 mAChR | M_3 mAChR |
| None | 0.12 (0.02) | 0.18 (0.02) |
| Group I | 1.17 (0.1)* | 0.20 (0.03) |
| Group II | 0.15 (0.05) | 0.17 (0.03) |
| Group III | 0.18 (0.06) | 0.23 (0.04) |
| Group IV (control) | 0.16 (0.03) | 0.20 (0.02) |

Microtitre wells were coated with 1 μ g of M_2 or M_3 mAChR peptides and enzyme immunoassay was carried out with sera from different groups.

Values are mean (SEM) of serum sample (1/32 dilution) from 13 chagasic patients with megacolon (group I), 13 chagasic patients without megacolon (group II), three non-chagasic patients with megacolon (group III), and 15 normal subjects (group IV). OD, optical density.

*Significantly different from normal control, $p < 0.0001$.

ELISA, negative results were obtained with circulating IgG antibody from all groups (table 2).

To further investigate the interaction between anti- M_2 peptide IgG and colon M_2 mAChRs, we carried out immunoblotting experiments on rat and human colon smooth muscle preparations and tested the ability of anti- M_2 peptide antibody to recognise the whole receptor. Figure 3 shows that total IgG from chagasic patients with megacolon (group I) but not IgG from normal individuals (group III) recognised the whole mAChR on rat and human homogenates in a similar manner to a monoclonal anti- M_2 mAChR IgG. As expected, anti- M_2 peptide IgG also revealed bands of M_2 mAChRs coincident with those labelled by the monoclonal IgG.

Table 3 shows the distribution of anti-peptide autoantibodies in chagasic patients with (group I) or without (group II) megacolon. It can be

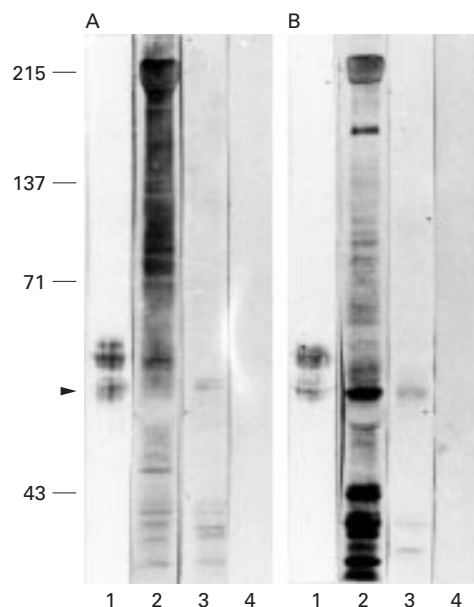


Figure 3 Immunoblotting of M_2 muscarinic acetylcholine receptor (mAChR) on rat (A) and human (B) colon smooth muscle homogenates. Lane 2: IgG fraction from chagasic patients with megacolon; lane 3: affinity purified anti-peptide IgG from chagasic patients with megacolon; lane 1: monospecific mouse anti-human M_2 mAChR IgG; and lane 4: serum IgG fraction from normal patients.

Table 3 Age and anti-M₂ muscarinic acetylcholine receptor (mAChR) antibody distribution in chagasic patients with (group I) or without (group II) megacolon and in normal controls (group IV)

| Serum reactivity against M ₂ mAChR peptide | Group I | | Group II | | Group IV | |
|---|---------|-------------|----------|--------------|----------|------------|
| | n | Age (y) | n | Age (y) | n | Age (y) |
| Positive | 13 | 55.9 (3.1)* | 4 | 37.0 (5.0)** | 0 | — |
| Negative | 2 | 74.0 (1.0) | 13 | 63.9 (3.6) | 20 | 61.4 (2.9) |
| Total | 15 | 58.3 (3.2) | 17 | 57.6 (3.8) | 20 | 61.4 (2.9) |

Values are mean (SEM).

*p<0.05, **p<0.002 versus seronegative patients from the same group.

Prevalence values of anti-M₂ mAChR antibodies between groups I and II were compared by χ^2 test ($\chi^2=10.35$, p<0.001).

seen that the frequency of anti-M₂ peptide antibodies detected by ELISA was significantly higher in seropositive patients with megacolon, which accounts for the strong association between the existence of serum anti-M₂ mAChR antibodies and the presence of megacolon in chagasic patients. Also, we can see in table 3 that there were no significant differences in patient age between the two groups. However, mean age of the four patients with positive anti-M₂ mAChR antibody titres from group II was significantly lower than the mean age of the 13 seropositive patients from group I. In contrast, two patients with negative anti-M₂ mAChR antibody titres from group I were significantly older than the mean ages of the 13 seropositive patients in the same group.

FUNCTIONAL EFFECT OF HUMAN CHAGASIC AUTOANTIBODIES

To determine if serum anti-M₂ mAChRs from patients with chagasic disease with megacolon exert biological actions on the distal colon smooth muscle through M₂ mAChR activation, we tested changes in the contractile behaviour associated with modifications in cAMP production in strips of rat and human colon exposed to such antibodies. As shown in fig 4, addition of anti-M₂ peptide IgG from chagasic patients with megacolon (group I) resulted in an increase in tone of both rat and human distal colon preparations in a concentration depended manner. These effects were neutralised after preincubation of the antibodies with the peptide (1 \times 10⁻⁵ M). Moreover, the affinity purified anti-M₂ peptide IgG was more potent

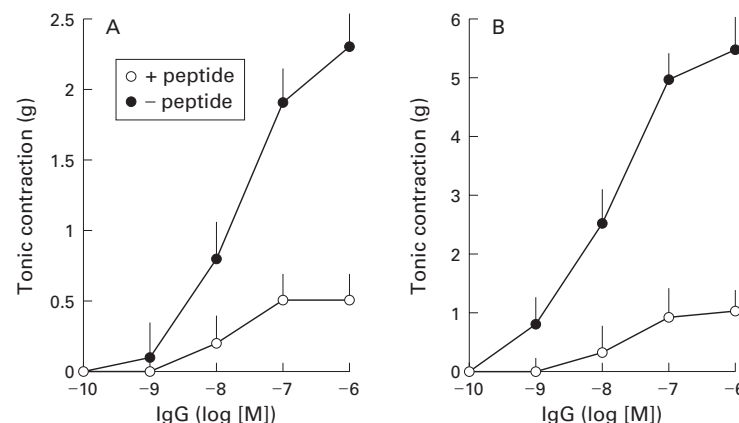


Figure 4 Concentration dependent effect of the anti-M₂ peptide IgG fraction from chagasic patients with megacolon on rat (A) and human (B) colon tonic contractions. Isolated distal colonic strips were incubated with affinity purified antipeptide antibodies in the presence (+) or absence (-) of 1 \times 10⁻⁵ M peptide and tonic contractions were recorded. Values are mean (SEM) of five antipeptide IgG fractions in each group.

Table 4 Changes in tone induced by anti-M₂ muscarinic acetylcholine receptor (mAChR) peptide IgG on colonic smooth muscle

| Condition | Tonic contraction (g) | |
|---|-----------------------|-------------|
| | Rat | Human |
| Chagasic IgG | 2.0 (0.3) | 5.2 (0.4) |
| Anti-M ₂ peptide IgG | 2.1 (0.2) | 5.4 (0.3) |
| Anti-M ₂ peptide IgG+AF-DX 116 | 0.5 (0.06)* | 0.8 (0.05)* |
| Anti-M ₂ peptide IgG+PTX | 0.7 (0.08)* | 0.9 (0.06)* |
| Normal IgG | 0.02 (0.01) | 0.05 (0.03) |

Contractile tone was measured after incubation of tissues for 15 minutes with 1 \times 10⁻⁶ M anti-M₂ peptide IgG or 1 \times 10⁻⁵ M chagasic or normal IgG. Inhibition experiments were performed by incubating tissues with 5 \times 10⁻⁶ M pertussis toxin (PTX) or AF-DX 116 over 30 minutes before addition of anti-M₂ peptide IgG.

Values are mean (SEM) of anti-M₂ peptide IgG from six different chagasic patients with megacolon or normal individuals in each group.

*Significantly different from anti-M₂ peptide IgG, p<0.0001.

than the total IgG from the same patients. Table 4 shows that with 1 \times 10⁻⁶ M of anti-M₂ peptide IgG, tonic contraction was the same as that obtained with 1 \times 10⁻⁵ M total IgG. Furthermore, anti-M₂ peptide IgG elicited contracture was inhibited by the M₂ mAChR selective antagonist AF-DX 116 and by pertussis toxin. These results demonstrate that chagasic IgG elicits a cellular response by interacting with the M₂ mAChR coupled to a pertussis toxin sensitive G protein of the Gi family that mediates inhibition of adenylate cyclase activity. Figure 5 shows original

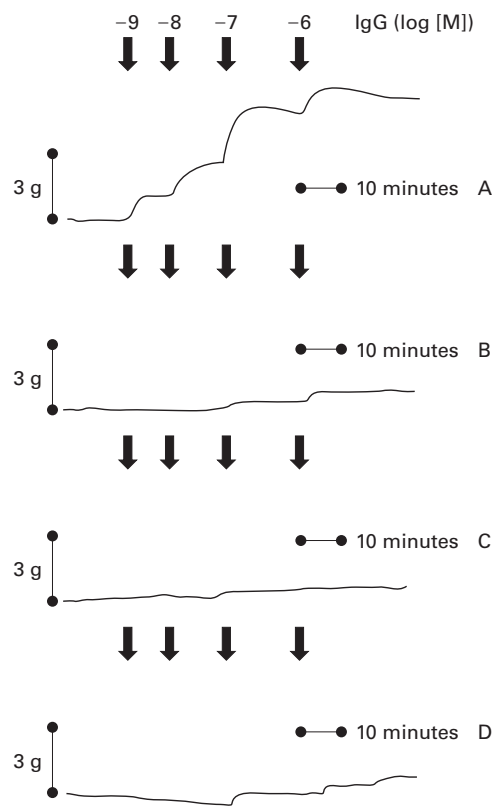


Figure 5 Original tracings showing dose dependent increases in human colon tonic contractions by anti-M₂ peptide IgG (IgG log [M]) from chagasic patients with megacolon alone (A) and after exposed tissues to 5 \times 10⁻⁶ M AF-DX 116 (B), 5 \times 10⁻⁵ M synthetic M₂ peptide (C), and 5 \times 10⁻⁶ M pertussis toxin (D) over 30 minutes before anti-M₂ peptide IgG from chagasic patients with megacolon was added.

Table 5 Effect of chagasic anti-M₂ muscarinic acetylcholine receptor (mAChR) antibodies on cAMP accumulation in rat and human distal colon

| Additions | cAMP (pmol/mg wet weight) | |
|--|---------------------------|--------------|
| | Rat | Human |
| None (basal) | 1.43 (0.09) | 1.98 (0.10) |
| PGE ₁ alone | 4.32 (0.35) | 5.60 (0.34) |
| PGE ₁ +chagasic IgG | 1.96 (0.16)* | 2.35 (0.15)* |
| PGE ₁ +normal IgG | 4.05 (0.36) | 5.42 (0.40) |
| PGE ₁ +chagasic IgG + atropine | 3.93 (0.43) | 5.30 (0.39) |
| PGE ₁ +chagasic IgG + M ₂ peptide | 3.58 (0.39) | 5.10 (0.42) |
| PGE ₁ +anti-M ₂ peptide IgG | 1.87 (0.22)* | 2.10 (0.17)* |
| PGE ₁ +anti-M ₂ peptide IgG+AF-DX 116 | 3.85 (0.41) | 4.95 (0.45) |
| PGE ₁ +anti-M ₂ peptide IgG+M ₂ peptide | 3.94 (0.40) | 5.12 (0.40) |
| PGE ₁ +anti-M ₂ peptide IgG+PTX | 4.52 (0.35) | 5.78 (0.38) |

3',5'-Cyclic monophosphate (cAMP) accumulation was measured after incubation of tissues for 15 minutes with 1×10^{-7} M total IgG or 1×10^{-6} M anti-M₂ peptide IgG from chagasic patients with megacolon or IgG from normal subjects. Then, 1×10^{-6} M prostaglandin E₁ (PGE₁) was added five minutes before the end of the reaction. Inhibitory experiments were performed by incubating tissue for 15 minutes with 5×10^{-6} M pertussis toxin (PTX), atropine, or AF-DX 116, before IgGs were added. The M₂ peptide neutralisation experiments were performed by preincubating IgG with the peptide (5×10^{-5} M) over 30 minutes at 30°C prior to tissue exposition.

Values are mean (SEM) of the IgG fraction from five different chagasic megacolon patients or normal individuals.

*Significantly different from PGE₁ alone, $p < 0.0001$.

tracings of the dose-response curve of anti-M₂ peptide IgG on human distal colon preparations from patients in group I (fig 5A) and the inhibitory action of different blockers (fig 5B–D).

Consequently, we were interested in determining modifications in cAMP production by human and rat colon preparations exposed to chagasic IgG from group I. For this purpose, colon preparations were exposed to PGE₁ which can activate adenylate cyclase. Table 5 shows that cAMP accumulation induced by PGE₁ was significantly inhibited by both total IgG (1×10^{-5} M) and the corresponding anti-M₂ peptide IgG (1×10^{-6} M) from sera of chagasic patients with megacolon; anti-M₂ was more potent than total IgG. Inhibition of PGE₁ induced effects triggered by chagasic IgG were neutralised by preincubation of the antibodies with the peptide and were also blocked by atropine, AF-DX 116, and pertussis toxin sensitive Gi protein. As a control, IgG from sera of group III was unaffected in the studied system.

Discussion

In the present study we have provided evidence for the presence of autoantibodies against M₂ mAChR in chagasic patients with megacolon. These antibodies not only interacted with both human and rat colon mAChRs but also displayed muscarinic-like activity, modifying intracellular events associated with specific receptor activation. Also, a molecular interaction between chagasic antibodies from patients with megacolon and the second extracellular loop of human M₂ mAChR was demonstrated using a synthetic 24mer peptide in immunoblotting and enzyme immunoassays. Thus affinity purified anti-M₂ peptide IgG, in common with monoclonal antihuman M₂ mAChR antibody, recognised a band with a molecular weight corresponding to the colon mAChR. The different patterns of recognition observed with total chagasic IgG corresponded with other high molecular weight proteins recognised by additional antibodies that have been reported on heart preparations.¹⁷ The specificity of the interaction with M₂ mAChR subtype

was assessed by inhibiting immune recognition of anti-M₂ peptide antibody by the M₂ peptide. Also, IgG from chagasic patients with megacolon did not recognise the synthetic peptide with an amino acid sequence identical to the second extracellular loop of human M₂ mAChR.

In distal colon strips from humans or rats, antibodies from chagasic patients with megacolon and the corresponding anti-M₂ peptide IgG increased contractile tone and decreased cAMP production. These effects promoted by chagasic autoantibodies were inhibited by a selective M₂ mAChR antagonist, indicating the participation of M₂ mAChRs in the biological effect of the autoantibodies. Moreover, that the synthetic M₂ peptide selectively blocked the biological action of chagasic antibodies and that the anti-M₂ peptide chagasic IgG triggered an increase in tone and decrease in cAMP confirms that the second extracellular loop of the receptor is essential to the biological action of these antibodies.¹⁷ Affinity purified anti-M₂ peptide autoantibodies affected contraction, and cAMP accumulation was prevented by pertussis toxin, suggesting that the effects of these antibodies are mediated by Gi/o proteins. This result is in agreement with previous observations that showed that activation of M₂ mAChR by a specific agonist causes pertussis toxin sensitive inhibition of adenylate cyclase in colonic smooth muscle.¹¹ Such an action in smooth muscle modulates contraction by inhibiting the relaxant effect of agonist known to activate adenylate cyclase.²⁰ However, direct colonic tonic cholinergic excitatory control by M₂ mAChR subtype has been described.¹⁴

Hence our results could indicate that chagasic autoantibodies exert their biological effects through two mechanism: direct contraction and indirect contraction by prevention of the relaxant effects of cAMP generating agents. However, M₂ muscarinic presynaptic auto-receptors that regulate release of acetylcholine by means of a negative feedback mechanism²¹ triggered by chagasic autoantibodies could not be excluded; an in vivo study is needed to demonstrated this.

Human colon smooth muscle has a greater density of M₂ mAChRs than rat colon.²² Our results confirm this. The difference in mAChR expression could explain the higher response in the biological patterns (contractility and cAMP) triggered by chagasic autoantibodies in human compared with rat colon preparations.

The prevalence of circulating anti-M₂ mAChR autoantibodies in chagasic patients with megacolon was significantly higher than the prevalence of such antibodies in chagasic patients without megacolon or non-chagasic patients with megacolon. It is tempting to speculate that chagasic patients without megacolon who have anti-M₂ mAChR autoantibodies may develop symptoms and signs of megacolon in the future. This is supported by the fact that anti-M₂ mAChR autoantibody positive patients from group II were significantly younger than patients in group I and had no more than five years of evolution. These chagasic patients without megacolon should be examined periodically to evaluate the potential

prognostic value of this test. The presence of circulating anti-M₂ mAChR autoantibodies with agonist activity in chagasic patients without evidence of megacolon could be explained by the fact that during the course of the disease, peak serum concentrations of anti-neurotransmitter receptor antibodies may precede binding to muscarinic receptors from target tissue, as was shown in chagasic and autoimmune myocarditis.^{23 24}

On the other hand, it is difficult to explain why some chagasic patients with megacolon showed no circulating M₂ mAChR autoantibodies. The fact that they were significantly older than the mean age of group I with more years of evolution (30–60 years) indicates that these patients were studied during a stage of chronic chagasic disease in which autoantibodies were fixed to tissues forming an immune complex. In fact, visceral deposits of immunoglobulins in chronic Chagas' disease have been shown.²⁵

In conclusion, we have provided evidence that most patients with Chagas' disease with megacolon have circulating autoantibodies against M₂ mAChRs. These antibodies increase contractile tone and decrease cAMP production in human and rat distal colon through specific activation of M₂ mAChR. We suggest that the stimulatory effect of such autoantibodies, in common with other muscarinic agonists, can rapidly activate mAChR and thereby trigger a biological effect.^{17 18} However, these agonist effects are followed by receptor desensitisation and sequestration which impairs further muscarinic receptor activation,²⁶ giving rise to megacolon. In this way autoantibodies could contribute to rectum-sigmoid motor incoordination as well as to impairment of the internal sphincter relaxation response to rectal distension.²⁵ In contrast, if the agonist promoted contraction effect of autoantibodies predominates, microcolon could be predicted, such as is seen in aganglionosis diseases. This finding suggests that an alternative humoral factor contributes to the pathogenesis of chronic digestive damage during Chagas' disease.

The authors thank Mrs Elvita Vannucchi and Fabiana Solari for expert technical assistance. Support was provided in the form of a grant from CONICET (PIP) and University of Buenos Aires (UBACyT).

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