

Molecular Mimicry between the Immunodominant Ribosomal Protein P0 of *Trypanosoma cruzi* and a Functional Epitope on the Human β_1 -Adrenergic Receptor

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Summary

Sera from chagasic patients possess antibodies recognizing the carboxy-terminal part of the ribosomal P0 protein of *Trypanosoma cruzi* and the second extracellular loop of the human β_1 -adrenergic receptor. Comparison of both peptides showed that they contain a pentapeptide with very high homology (AESEE in P0 and AESDE in the human β_1 -adrenergic receptor). Using a competitive immunoassay, recognition of the peptide corresponding to the second extracellular loop (H26R) was inhibited by both P0-14i (AAAESEEDDDDDF) and P0- β (AESEE). Concomitantly, recognition of P0- β was inhibited with the H26R peptide. Recognition of P0 in Western blots was inhibited by P0-14i, P0- β , and H26R, but not by a peptide corresponding to the second extracellular loop of the human β_2 -adrenergic receptor or by an unrelated peptide. Autoantibodies affinity purified with the immobilized H26R peptide were shown to exert a positive chronotropic effect in vitro on cardiomyocytes from neonatal rats. This effect was blocked by both the specific β_1 blocker bisoprolol and the peptide P0- β . These results unambiguously prove that *T. cruzi* is able to induce a functional autoimmune response against the cardiovascular human β_1 -adrenergic receptor through a molecular mimicry mechanism.

Chronic Chagas' heart disease, resulting from infection with the protozoan *Trypanosoma cruzi*, is a slowly evolving inflammatory cardiomyopathy that may lead to severe cardiac dilatation, congestive heart failure, and death (1). The histological picture includes degeneration of cardiomyocytes, coexisting with fibrosis and mononuclear cell infiltration, apparently occurring in the absence of parasites. These features suggest that an autoimmune component could be involved in the pathogenesis of the disease (2).

The occurrence of an autoimmune process leading to chronic myocardial injury may result from a parasite-induced deregulation of the immune system, leading to loss of tolerance for self-antigens or to a *T. cruzi*-induced cross-reactive immune response to self-antigens (molecular mimicry).

Although there is increasing evidence of antibodies and T cells cross-reactive for *T. cruzi* and mammalian antigens, only a few of the latter have been identified (3–7). To our knowledge, only two cross-reactive B cell epitopes have been identified and mapped: a dodecapeptide derived from the sequence of a *T. cruzi* flagellum-associated surface protein

generate antibodies that cross-react with a 48-kD nervous tissue antigen (8), and the 13 carboxy-terminal amino acids of the low molecular weight *T. cruzi* ribosomal P proteins generate antibodies that cross-react with their human counterparts (9–11).

Another ribosomal P protein of the parasite, named P0 with a molecular mass of 38 kD, is also antigenic in human *T. cruzi* infection (12, 13). This protein contains long, negatively charged stretches, which have been shown to be associated with autoantigens in SLE (14) and to give rise to polyreactive autoantibodies after experimental *T. cruzi* infection (15). It was recently proposed that the autoantibodies against the β_1 -adrenergic receptor (16) and the M2 acetylcholine receptor (17), detected in patients with idiopathic dilated cardiomyopathy, could be directed against a negatively charged sequence present on the putative second extracellular loop of these receptors (18). On the other hand, since functionally active autoantibodies against β -adrenergic receptors (19, 20) and M2 muscarinic acetylcholine receptors of the heart (21) have been observed in patients with Chagas' dis-

ease, the epitopes recognized by these autoantibodies should be localized in the same receptor domain. Therefore, a peptide corresponding to the second extracellular loop of the human β_1 -adrenergic receptor was used to screen for the presence of antireceptor autoantibodies and to affinity purify such antibodies from sera of patients with Chagas' disease for functional characterization.

In view of the extremely high similarity between a pentapeptidic sequence in the P0 ribosomal protein from *T. cruzi* and the human β_1 -adrenergic receptor (Fig. 1), using immunochemical and cell physiological techniques, the hypothesis was tested for possible cross-reactivity between the anti-P0 antibodies and the functionally active anti- β_1 adrenergic receptor autoantibodies.

Materials and Methods

Sera of Patients. Sera were obtained from chronically infected chagasic individuals ($n = 36$) from the same endemic region, located in northwest Argentina. These individuals were evaluated at the Servicio de Cardiología, Ramos Mejía Hospital (Buenos Aires, Argentina) by clinical examination, resting electrocardiography, ajmaline tests, and M and B node echocardiography. The *T. cruzi* infection was assessed by complement fixation, passive hemagglutination, and immunofluorescence. Sera of a group of healthy blood donors ($n = 8$) were included as controls. The sera sampled from 15 individuals infected with *Leishmania* species included 6 samples from patients with kala-azar and 9 samples from patients with sylvatic leishmaniasis. These sera reacted in an immunofluorescence test with *Leishmania donovani* promastigotes. They were provided by Dr. M. E. Camargo (Laboratory Fleury, Sao Paulo, Brazil) and by A. Luquetti (Laboratorio Pesquisa de Chagas, Goiania, Brazil). The IgG fractions of two patients with idiopathic dilated cardiomyopathy, which induced a positive chronotropic effect in neonatal rat cardiomyocytes, were used.

Peptides. Peptides were synthesized by the solid-phase method developed by Merrifield (22) using a 430A automated peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). The peptides were purified by HPLC and verified by amino-terminal sequencing. The sequences of the peptides used are summarized in Fig. 1.

PEPTIDES

PEPTIDES DERIVED FROM *T. CRUZI*

PO-14I AAASEEEDDDDDF
PO- β AESEE

Peptides derived from the human β -adrenoceptors

H26R (β_1) HWWRAESDEARRCYNPKCCDFVTNR
H26Q (β_2) HWYRATHQEA I NCYANETCCDFFTNQ

Unrelated peptide derived from tobacco mosaic virus coat protein

TMVP RNR I IEVENQ

Figure 1. Sequence of the peptides used. PO-14i corresponds to sequence 56–68 of C-P0 (12). H26R corresponds to sequence 197–222 of the human β_1 -adrenergic receptor (38), and H26Q corresponds to sequence 172–197 of the human β_2 -adrenergic receptor (39). TMVP corresponds to sequence 90–99 of the coat protein of tobacco mosaic virus.

Enzyme Immunoassay. 50 μ l of peptide (10 μ g/ml) in 100 mM Na_2CO_3 , pH 11, was coated for 1 h on microtiter plates (Nunc, Kastrup, Denmark). The wells were then saturated with PMT, which is PBS (10 mM phosphate, 140 mM NaCl, pH 7.4) supplemented with 3% (wt/vol) skim milk and 0.1% Tween 20 (Merck, Darmstadt, Germany). 50 μ l of sera or IgG fractions, diluted from 1:50 to 1:200, was added to the saturated microtiter plates at 4°C overnight. After washing the wells three times with PBS plus 1% Tween 20, an affinity-purified biotinylated rabbit anti-human IgG (H+L) was allowed to react for 1 h. After three more washings, the bound biotinylated antibody was detected by incubating the plates for 1 h with streptavidin-peroxidase (1 μ g/ml) solution in PMT. This was followed by three washings in PBS and the addition of substrate 2.5 mM H_2O_2 , 2 mM 2,2'-azino-di(ethyl-benz-thiazoline) sulfonic acid (Sigma Chemical Co., St. Louis, MO). Optical densities were read after 30 min at 405 nm in a microplate reader.

Competitive Immunoassay. Before using the sera in the enzyme immunoassay as previously described, serum samples diluted at 1:200 were incubated with increasing concentrations of peptides for 1 h at room temperature.

Purification of Recombinant β -Galactosidase Fusion Protein C-P0. *Escherichia coli* RY-1089 lysogenic for the λ gt11 wild-type and λ gt11 recombinant C-P0 phage were cultured from single colonies at 32°C, shifted to 42°C for 25 min, and induced with 10 mM isopropyl β -D-thiogalactopyranoside for 2 h (23). The bacteria were pelleted and lysed, and β -galactosidase wild-type or fusion protein was purified as described previously (12, 24). Briefly, the fusion protein was purified with a ProtoSorb lacZ column (Promega Corp., Madison, WI) following the instructions of the manufacturer. The purity was assessed by SDS-PAGE and Western blot analysis.

Western Blots. Purified proteins were analyzed by denaturing SDS-PAGE, using 5% stacking and 7.5% running gels (25). Western blot analysis was performed according to a standard method (26). The nitrocellulose transfers were blocked with 5% dried skim milk powder in Tris-buffered saline, pH 7.4, supplemented with 0.1% Tween 20, probed with sera (dilution 1:200), and adsorbed with 20% *E. coli* lysate. For peptide inhibition experiments, peptides were added at 30 μ M. Antibody detection was performed with the Vectastain ABC kit (Vector Labs, Inc., Burlingame, CA).

Antibody Purification. The IgG fraction of the sera was prepared by dilution of the sera 1:5 in PBS and precipitation at 40% $(\text{NH}_4)_2\text{SO}_4$ saturation. The precipitate was redissolved in PBS at a 1:1 dilution.

The H26R peptide was coupled to activated CNBr-Sephacryl (Pharmacia Diagnostics AB, Uppsala, Sweden) according to the standard procedure.

The IgG fraction was incubated overnight at 4°C with 300 mg of H26R-Sephacryl. The gel was washed seven times by centrifugation for 5 min at 200 g before elution with 0.2 M glycine buffer, pH 2.8. The elution volume was directly neutralized with 400 μ l of 1 M Tris buffer, pH 8.0. The purified antibodies were then dialyzed against PBS before use.

Functional Activity of the Affinity-purified Antibodies. Cultured neonatal rat heart myocytes were used as a functional test system (27). Single cells were dissociated from the minced heart ventricle of 1–3-d-old Wistar rats with a 0.25% solution of trypsin. The myocytes were cultured as monolayers on the bottom of 45-ml Müller bottles (1.2×10^6 seeded cells in 3 ml of medium) in SM20-I medium (Max Delbrück Center, Berlin, Germany) containing 10% heat-inactivated calf serum and 2 μ M fluorodeoxyuridine (the latter prevented proliferation of nonmuscle cells). They were cultured for 4 d at 37°C in air.

The spontaneous beating frequency of myocytes was measured at 37°C on a heated stage of an inverted microscope. The changes in beating frequency were measured 1 h after the addition of the antibodies. The basal beating rate was 160 ± 20 beats/min.

Statistical Analysis. Means and standard deviations of the OD values obtained in the enzyme immunoassays were calculated by the STATVIEW program on a Macintosh computer. Positivity was defined as mean ± 2 SD of the OD at 405 nm of the sera from healthy donors ($n = 8$). Significance between the different groups was assessed by the ANOVA module of the STATVIEW program. Correlations between the responses against the different peptides were calculated by the correlation module of the same program.

Results

Enzyme Immunoassays. Since the second extracellular loop of the human β_1 -adrenergic receptor has been shown to contain a functionally autoimmune B cell epitope (16), H26R recognition by sera of chagasic patients was assayed. Table 1 summarizes the results. 13 of 36 sera from chagasic patients were found to be positive compared with 0 of 8 control sera ($P < 0.05$). This response was compared against that of the immunodominant ribosomal P0 protein from *T. cruzi* (C-P0) and the peptide derived from it and thought to cross-react with the receptor (P0- β). As already shown elsewhere (12), >80% of the chagasic sera reacted with C-P0, whereas only 47% reacted with P0- β . Although there was no correlation between the responses to H26R and C-P0 ($P = 0.282$), a significant correlation was found between the responses to H26R and P0- β ($P = 0.0147$). The intensity of the response, as shown in Fig. 2, increased from H26R to C-P0.

The specificity of the response against H26R and C-P0 was assessed by assaying sera from 15 patients with leishmaniasis. None of them yielded a positive response.

To study cross-reactivity between the parasitic antigen and the human β_1 -adrenergic receptor, competitive immunoenzyme assays were set up using H26R and P0- β . As shown in Fig. 3, recognition of H26R was inhibited with H26R as a control inhibitor and P0- β or P0-14i, peptides derived from P0 of *T. cruzi*. Alternatively, recognition of P0- β was inhibited with P0- β as the control inhibitor and also with H26R. An unrelated peptide derived from tobacco mosaic virus coat protein (TMVP)¹ developed no inhibitory ca-

Table 1. Number of Patients with Sera Positive for the Three Antigens

Patients	H26R	C-P0	P0- β
Healthy controls ($n = 8$)	0	0	0
Chagasic patients ($n = 36$)	13	30	17
Leishmaniasis patients ($n = 15$)	0	0	ND

¹ Abbreviation used in this paper: TMVP, tobacco mosaic virus coat protein.

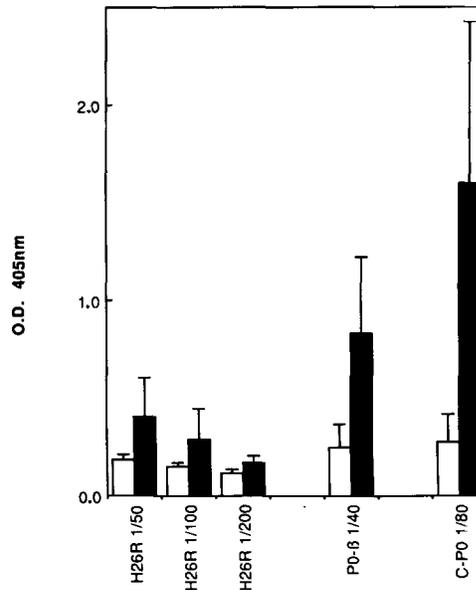


Figure 2. Enzyme immunoassays of the sera against the different peptides. Sera from healthy blood donors (white bars) and sera from positive chagasic patients (black bars) were tested against the three peptides indicated on the abscissa. Numbers correspond to the serum dilutions used.

capacity. These results show the mean and standard deviation of competition experiments with four different sera. The broad standard deviation reflects the different sensitivities of the individual sera toward the competitive peptides.

Western Blots. To ascertain whether the results obtained with the peptides were also valid for the antigenic proteins, the inhibitory effects of the peptides on recognition of ribosomal protein P0 (C-P0) of *T. cruzi* expressed in *E. coli* were investigated.

Two sera, used for the competitive enzyme immunoassays, were tested (Fig. 4). Preincubation of the antisera with the antigen (C-P0) completely abolished recognition of the electrotransferred protein, showing that the competition conditions were adequate. The peptides P0-14i, P0- β , and H26R completely inhibited the recognition of the C-P0 protein by serum 15 (Fig. 4 A), and recognition by serum 112 (Fig. 4 B) was highly decreased in the presence of these peptides. Two peptides, one corresponding to a sequence of TMVP and the other to the second extracellular loop of the β_2 -adrenergic receptor, were used as negative controls. The first peptide did not show any effect, whereas the second peptide had only a small effect on recognition of P0 by serum 15.

Functional Activity of the Anti-H26R Antibodies. Specific anti-H26R antibodies were affinity purified to assay for functional recognition of the β_1 -adrenergic using spontaneously beating neonatal rat heart myocytes. Since this system has already been used for the study of anti-receptor autoantibodies in idiopathic dilated cardiomyopathy (27) and the sequences of the second extracellular loop of rat and human β_1 -adrenergic receptors are totally similar (28), it is suitable for cross-reactive studies. To increase the specificity of the functional response, assays were performed in the presence of 1 μ M atro-

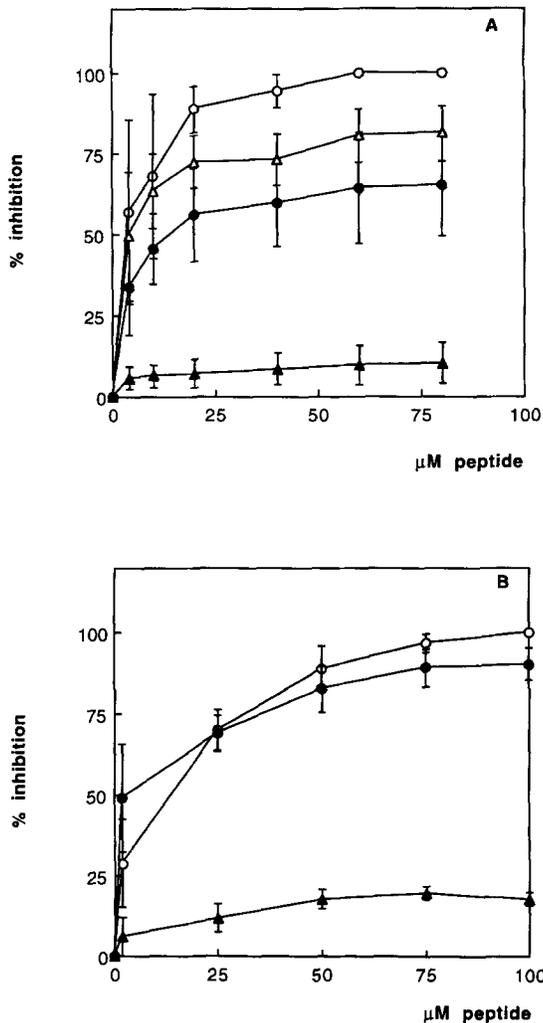


Figure 3. Competition enzyme immunoassay with the cross-reacting peptides. Results show the mean \pm SD of the percent inhibition with increasing amounts for four sera. (A) Inhibition of recognition of the H26R peptide with H26R (○), P0- β (●), P0-14I (△), and TMVP (▲). (B) Inhibition of recognition of the P0- β peptide with H26R, P0- β , and TMVP. Symbols are the same as in A.

pine to avoid any interference with autoantibodies directed against the muscarinic acetylcholine receptor, which are present in sera of chagasic patients (21).

The purified antibodies showed the same positive chronotropic response as those purified from patients with idiopathic dilated cardiomyopathy (29). After a 60-min preincubation with the antibodies, a significant increase in beating frequency of the heart myocytes was observed; this was completely blocked by the addition of the β_1 -specific blocker bisoprolol.

When the antibodies were preincubated with 14 $\mu\text{g}/\text{ml}$ P0- β before addition to the cardiomyocytes, the positive chronotropic effect of the purified antibodies was inhibited completely for the antibodies purified from patient 99 and partially for those from patient 32. Peptides alone did not have any effect on the cardiomyocytes. To ensure that the anti-

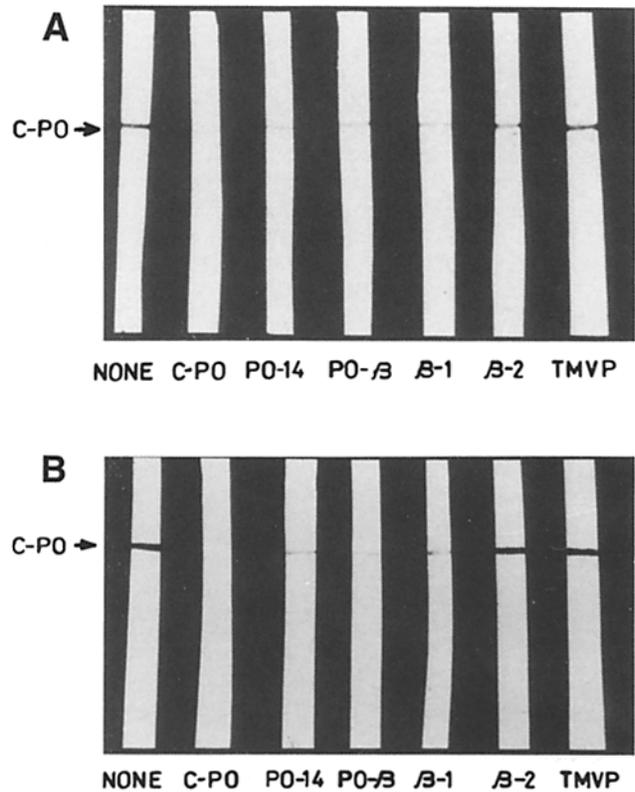


Figure 4. Immunoblots of recombinant C-P0 and inhibition with peptides. Immunoblots and inhibitions are shown for two different sera. The inhibitory proteins and peptides defined under the different lanes correspond to the structures shown in Fig. 1. The amount of protein blotted was $\sim 1 \mu\text{g}$. The inhibitory concentrations of peptides was 30 μM .

body-peptide complex had no toxic effects on the cells, the cardiomyocytes were treated with 10 μM isoproterenol, a β -adrenergic agonist. The cardiomyocytes reacted in a normal way to this stimulant.

In contrast, 20 $\mu\text{g}/\text{ml}$ peptide did not inhibit the positive chronotropic effect of IgG fractions from patients with idiopathic dilated cardiomyopathy. (Fig. 5).

Discussion

Molecular mimicry by *T. cruzi* is evidenced by the existence of cross-reactive antibodies to *T. cruzi* and mammalian cells. These autoantibodies were characterized by immunoblots on target tissues and/or immunocytochemical localization of immunologically cross-reactive material on the same tissues. The existence of autoantibodies that could interfere with the functional properties of the target cells was inferred from the physiological activity of IgG fractions isolated from patients with Chagas' disease (19, 21). The structural determination of the second extracellular loop as the antigenic region recognized by functionally active autoantibodies against the β_1 -adrenergic receptor in idiopathic dilated cardiomyopathy (16) prompted us to look for a possible sequence similarity between this domain and major antigenic proteins of *T. cruzi*.

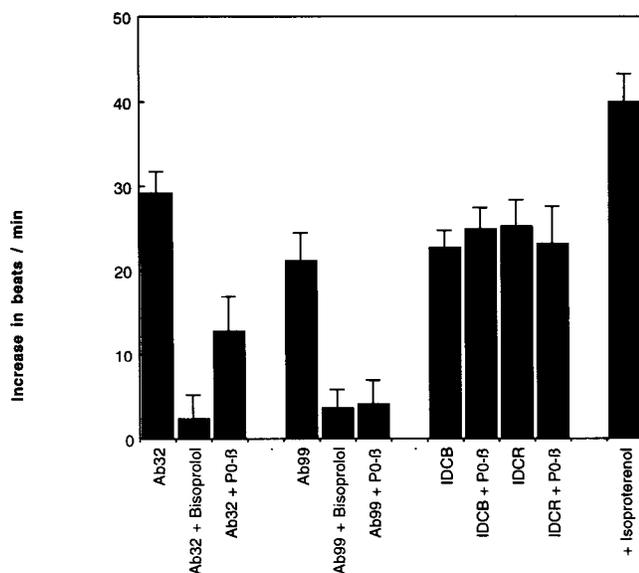


Figure 5. Agonist-like effect of affinity-purified anti-H26R autoantibodies. Autoantibodies affinity purified from two different sera were added at a 1:100 dilution to neonatal rat cardiomyocytes in culture, and the increase in beating frequency was determined. Means \pm SEM of 10 different measurements are shown. The final antibody concentration for patient 32 was 4 nM; that for patient 99 was 10 nM.

The specificity of the effect was studied by inhibition with the β_1 -specific antagonist bisoprolol (1 μ M) and the peptide P0- β (18 μ M). IgG fractions from two patients with idiopathic dilated cardiomyopathy (IDCB and IDCR) were tested in the absence and presence of P0- β . No inhibitory effect of the peptides was observed. The cells studied for peptide inhibition with the antibodies of both patients were subsequently stimulated with the β -adrenergic agonist isoproterenol (10 μ M). The results obtained in the presence of antibody from patient 99 and peptide P0- β are shown.

A pentapeptide derived from the P0 ribosomal protein of *T. cruzi* showed high similarity with a pentapeptide in the receptor loop. The results presented here show not only that this peptide is a cross-reacting B cell epitope, but also that it is important for the functional activity of the autoantibodies on the receptor.

The fact that the second extracellular loop of the human β_1 -adrenergic receptor is recognized by autoantibodies in two cardiomyopathies with different etiologies confirms our proposed hypothesis that this domain of G protein-coupled receptors can be tagged as a main immunogenic region in the sense given to the region recognized by anti-nicotinic

acetylcholine receptor autoantibodies in myasthenia gravis (30). The second extracellular loop, however, contains several epitopes that have functional meaning. This may be concluded from the fact that the pentapeptide P0- β only partially inhibited the functional effect in one of the patients and did not inhibit at all the functional autoantibodies found in idiopathic dilated cardiomyopathy (Fig. 5). These results are in agreement with the observations that in idiopathic dilated cardiomyopathy, the peptide encompassing the cross-reactive epitope did not block the functional effect of the autoantibodies, but a neighboring heptapeptide (ARRCYND) did (31). These results suggest that recognition by autoantibodies of different B cell epitopes, present on the second extracellular loop, could lead to the same stimulatory effect. They also confirm that, as may be expected from the molecular mimicry hypothesis, recognition of the cross-reactive epitope is limited to patients infected with the parasite.

The fact that autoantibodies against both β -adrenergic receptor subtypes (β_1 and β_2) have been observed in sera of patients with Chagas' disease (32) suggests that another functionally important B cell epitope may be localized at the carboxy-terminal end of the second extracellular loops, which show the highest similarity between the two receptors (Fig. 1).

The cross-reactivity between a ribosomal protein and a membrane receptor as described here may also be relevant to the existence of cross-reactive membrane-related targets on human cells for autoantibodies against ribosomal proteins in SLE (33). Indeed, it gives further support to the hypothesis that autoantibodies against intracellular targets have pathogenic importance when they cross-react with membrane proteins. It is worthwhile to note that autoantibodies against the mitochondrial ADP-ATP carrier, known to be present in idiopathic dilated cardiomyopathy (34), similarly seem to functionally recognize Ca^{2+} channels on cardiac myocytes (35).

Although idiopathic dilated cardiomyopathy is considered an autoimmune disease (36), it does not answer to the Witebsky criteria as recently revised (37). In contrast, Chagas' disease—both human and experimental—has been forwarded as a model for autoimmune disease (2). The common existence of autoantibodies directed against the same receptors in both diseases could shed light on their pathogenic importance. Since the specific anti- β_1 -adrenergic receptor response seems to be absent in leishmaniasis, another parasitic disease with an autoimmune cellular component, molecular mimicry is probably more important than a parasite-immune deregulation in the onset of the autoimmune disease.

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