Molecular Mimicry between the Immunodominant Ribosomal Protein P0 of Trypanosoma cruzi and a Functional Epitope on the Human β1-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 β1-adrenergic receptor. Comparison of both peptides showed that they contain a pentapeptide with very high homology (AESEE in P0 and AESDE in the human β1-adrenergic receptor). Using a competitive immunoenzyme assay, recognition of the peptide corresponding to the second extracellular loop (H26R) was inhibited by both P0-14i (AAAESEEEDDDDDF) and P0-1 (AESEE). Comcomitantly, recognition of P0-1 was inhibited with the H26R peptide. Recognition of P0 in Western blots was inhibited by P0-14i, P0-1, and H26R, but not by a peptide corresponding to the second extracellular loop of the human β1-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 β1 blocker bisoprolol and the peptide P0-1. These results unambiguously prove that T. cruzi is able to induce a functional autoimmune response against the cardiovascular human β1-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 β1-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 β1-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 pentapeptide sequence in the P0 ribosomal protein from T. cruzi and the human β1-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-β1 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 haemagglutination, and immunofluorescence. Sera of a group of healthy blood donors (n = 8) were included as controls. The sera sampled from 15 patients 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, São Paulo, Brazil) and by A. Luqueti (Laboratorio Pesquisa de Chagas, Goiânia, 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, 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  AAEAASEEEDDDDF
PO-β    AESEE

Peptides derived from the human β-adrenergic receptors

H26R (81) HWWRAEDSEARECNPDSDKDCDFVTNR
H26Q (82) HWYRTHQAICNYANETCDDFTTNQ

Unrelated peptide derived from tobacco mosaic virus coat protein

TMVP  RNRI EVENQ

**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 β1-adrenergic receptor (38), and H26Q corresponds to sequence 172–197 of the human β2-adrenergic receptor (39). TMVP corresponds to sequence 90–99 of the coat protein of tobacco mosaic virus.
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 β1-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 β1-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)

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

<table>
<thead>
<tr>
<th>Patients</th>
<th>H26R</th>
<th>C-P0</th>
<th>P0-β</th>
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</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(n = 8)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Chagasic patients</td>
<td>13</td>
<td>30</td>
<td>17</td>
</tr>
<tr>
<td>(n = 36)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Leishmaniasis</td>
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<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>(n = 15)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

**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 β1-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 β1-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 β1-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 μM atro...
Figure 3. Competition enzyme immunoassay with the cross-reacting peptides. Results show the mean ± 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.

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 ∼1 μg. The inhibitory concentrations of peptides was 30 μM.

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 β1-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.
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 β1-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 (β1 and β2) 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 Ca2+ channels on cardiac myocytes (35).

Although idiopathic dilated cardiomyopathy is considered an autoimmune disease (36), it does not answer to the Wittebeysky 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-β1-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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References


