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Humoral autoimmune response to ribosomal P proteins in chronic Chagas heart disease

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SUMMARY

The C terminal region of a *Trypanosoma cruzi* ribosomal P protein, encoded by the λgt11 JL5 recombinant, defined a major antigenic determinant in chronic Chagas heart disease. Immunopurified anti-JL5 antibodies were tested for anti-human ribosome reactivity by immunoblotting. They recognized the parasite ribosomal P proteins and clearly reacted with the corresponding human P proteins. The peptide R-13, that comprises the 13 C terminal residues of the JL5 recombinant and defines the specificity shared between chronic Chagas heart disease anti-JL5 antibodies and the systemic lupus erythematosus (SLE) anti-P antibodies, was used to study the specificity and the IgG subclass distribution of the anti-R-13 response by ELISA. The R-13 autoepitope is recognized mainly by sera from chagasic patients, but not by sera from malaria patients. Moreover, there was a significant correlation between anti-R-13 antibody levels and anti-*T. cruzi* antibody titres. The anti-R-13 response was mainly restricted to the IgG1 heavy chain isotype and correlated with the anti-*T. cruzi* isotype distribution.

Keywords *Trypanosoma cruzi* Chagas heart disease molecular mimicry autoanti-ribosomal P protein antibody protozoan infections erythematosus systemic lupus

INTRODUCTION

The most severe manifestation of chronic Chagas heart disease is the development of a panmyocarditis, anatomically expressed by myocardial tissue damage without detection of intracellular parasite forms (Rosenbaum, 1964). Accordingly, the hypothesis of an autoimmune process has been proposed to explain the pathogenesis of the disease (Hudson, 1985). Patients' sera are characterized by positive anti-*Trypanosoma cruzi* antibody titres, accompanied in some cases by detectable levels of anti-self-antibodies (Szarfman *et al.*, 1982; Acosta *et al.*, 1983; Schmuñis, 1987). However, neither a clear correlation between the clinical status of the patients and the presence of circulating auto-antibodies, nor the nature of the autoantibody-triggering stimuli have been established.

Recently, a λgt11-cloned parasite peptide, JL5, was characterized that reacted predominantly with sera from patients with overt Chagas heart disease (Levin *et al.*, 1989). The cloned peptide was identified as the C terminal portion of a *T. cruzi* ribosomal P protein (Levin *et al.*, 1989). Several data indicated that JL5 could bear epitopes cross-reactive with the host ribosomal P proteins: (i) sequence analysis and comparison revealed that the JL5 cDNA-derived amino acid sequence was homologous to the C terminal portion of the human ribosomal P proteins; notably, the C terminal 11-peptide in JL5 was more than 90% homologous to the sequence that defined the ribosomal P protein epitope in systemic lupus erythematosus (SLE) (Elkon *et al.*, 1988); (ii) phage-dot array immunoassays showed that sera from chronic Chagas heart disease and SLE patients with positive anti-P activity reacted both with the JL5 recombinant (Levin *et al.*, 1989) and with a JL5-derived recombinant containing the 15 C terminal residues of JL5 (A1 recombinant) (Levin *et al.*, 1990); and (iii) ELISA measurements with a synthetic peptide comprising the 13 C terminal residues of the JL5 recombinant (R-13) defined the specificity shared between chronic Chagas heart disease anti-JL5 antibodies and the SLE anti-P antibodies (Mesri *et al.*, 1990).

This study was designed to characterize further the autoimmune nature of the anti-R-13 antibody response in chronic Chagas heart disease. Our specific aims were to determine unambiguously the autoreactive properties of the anti-JL5 antibodies; to evaluate the prevalence of the anti-R-13 antibody response among patients with different protozoan parasitosis; and to assess the humoral anti-R-13 IgG subclass distribution in patients with chronic Chagas heart disease and to compare it with that from SLE anti-P-positive patients.

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SUBJECTS AND METHODS

Human sera

The chagasic sera were obtained from 44 individuals with chronic Chagas heart disease who were evaluated as previously described (Levin *et al.*, 1989). Sera from 50 SLE patients were provided by G. Dighiero (Unité d'Immunohématologie et Immunopathologie, Institut Pasteur, Paris), and A. M. Di Lonardo (Servicio de Inmunologia, Hospital Durand, Buenos Aires, Argentina). The sera from African patients were provided by P. Druilhe (Unité de Parasitologie Bio-Médicale, Institut Pasteur, Paris) and included sera from 20 malaria patients, sera from 20 patients with African trypanosomiasis, and sera from 24 individuals infected with *Leishmania sp.* Sera from 35 healthy subjects were used as controls.

Western blots of ribosomal and recombinant proteins

Ribosomes from different cell sources were prepared as described by Elkon *et al.* (1986). Lysogenic *Escherichia coli* RY 1089 for the JL5, A1, human ribosomal P1 and P2 recombinant phage were cultured, induced, and purified as previously described (Levin *et al.*, 1989). Monospecific anti-JL5 antibodies were affinity purified from chagasic serum samples using the protocol designed by Hall *et al.* (1984). The A1 recombinant phage encoding the 15 C terminal amino acids of JL5 was used to produce the A1 fusion protein used in inhibition experiments. Briefly, the A1 recombinant protein was immobilized on nitrocellulose filters that were incubated for 1 h at room temperature with the affinity-purified anti-JL5 antibodies. A1-depleted anti-JL5 antibodies were used as probes in Western blots. Immunoblotting of human and parasite ribosomal proteins and recombinant peptides was performed as described by Levin *et al.* (1989). Antibody detection was performed with the Vectastain ABC kit (Burlingame, CA).

ELISA measurements

The R-13 (EEEDDDMGFGLFD) and R-18 (AAAAEEEEDDDMGFGLFD) synthetic peptides, derived from the JL5 peptide sequence (ASAPTAAAAASGGAAA-PAAAAEEEEDDDMGFGLFD), were synthesized by Neosystem (Strasbourg, France). Microtitre wells were coated overnight at 4°C with 1 μg of the R-13 peptide in 0·1 M carbonate buffer, pH 9·6, or with 1·25 μg of *T. cruzi* antigen prepared as described elsewhere (Spinella *et al.*, 1989). The following steps of the ELISA protocol were performed as described by Mesri *et al.* (1990). Two-fold serial dilutions of the sera were made to ensure that at the chosen dilution, 1/400, the reactivities were on the linear portion of the curve. Samples were tested at least in triplicate and the results were averaged. Values >3 times the mean of 35 normal control sera were considered positive. For inhibition experiments, sera at 1/1000 dilution were pre-incubated for 2 h with different amounts of peptide.

Assessment of immunoglobulin subclasses

Microtitre wells coated as mentioned above were incubated with a 1/100 dilution of each serum. Thereafter, wells were incubated for 1 h with a 1/1000 dilution of the corresponding anti-human subclass monoclonal antibody (Oxoid, UK). They were revealed using a 1/1000 dilution of peroxidase-conjugated goat anti-mouse IgG (Biosys, Compiègne, France).

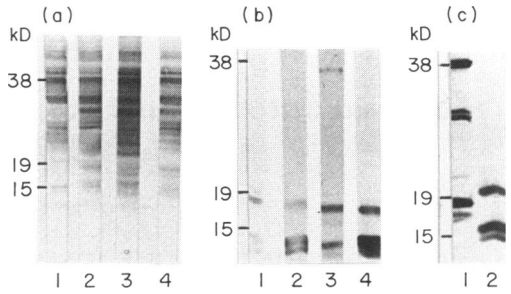

Fig. 1. Western blot analysis of human serum binding to human and *T. cruzi* ribosomal proteins. HeLa cell and parasite isolated ribosomes were electrophoresed on 15% gels and blotted. Each slot contained approximately 30 μg of total proteins. (a) Immunoreactivity of different chagasic sera with *T. cruzi* ribosomes (lanes 1–4). All serum dilutions were 1/500; (b) immunoreactivity of anti-JL5 antibodies immunoselected from the chagasic sera with *T. cruzi* ribosomes (lanes 1–4); (c) immunoreactivity of a SLE serum with HeLa (lane 1) and *T. cruzi* (lane 2) ribosomes. Serum dilution was 1/200.

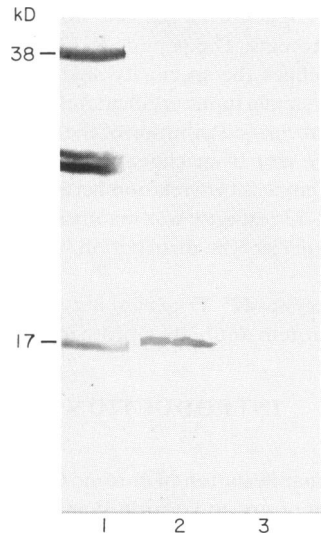

Fig. 2. Cross-reactivity of anti-JL5 antibodies with human ribosomal proteins. HeLa cell ribosomes were electrophoresed on 15% gels and blotted. Each slot contained approximately 30 μg of total proteins. HeLa cell ribosomal blots were probed with: lane 1, an SLE serum, diluted 1/200; lane 2, anti-JL5 antibodies immunoselected from a chagasic serum; and lane 3, anti-JL5 antibodies preincubated with the A1 recombinant fusion protein (A1 amino acid sequence: AEEEEDD DMGFGLFD).

Standard purified human IgG1, IgG2, IgG3 and IgG4 were provided by J. L. Preud'Homme and P. Aucouturier (URA CNRS, Poitiers, France), and were used to control the specificity of the anti-human IgG subclass reaction.

RESULTS

Anti-JL5 antibodies identified parasite ribosomal P proteins

Sera from patients with chronic Chagas heart disease reacted with several parasite ribosomal antigens (Fig. 1a). Notably,

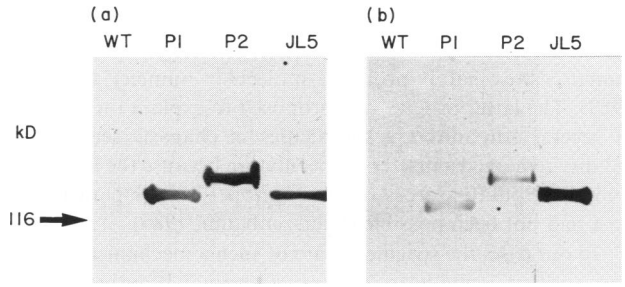

Fig. 3. Cross-reactivity of anti-JL5 antibodies with recombinant human ribosomal P1 and P2 proteins. Approximately 2 μg of each purified recombinant protein was electrophoresed on 7,5% gels and blotted. (a) Recombinants probed with an SLE serum diluted 1/200; (b) recombinants probed with anti-JL5 antibodies immunoselected from a chronic Chagas heart disease serum. WT, recombinant protein from a wild type λgt11 Y1089 lysogen; P1, recombinant protein from the human ribosomal P1 Y1089 lysogen; P2, recombinant protein from the human ribosomal P2 Y1089 lysogen; JL5, recombinant protein from the *T. cruzi* JL5 Y1089 lysogen. Arrow (116 kD) indicates position of the β-galactosidase marker.

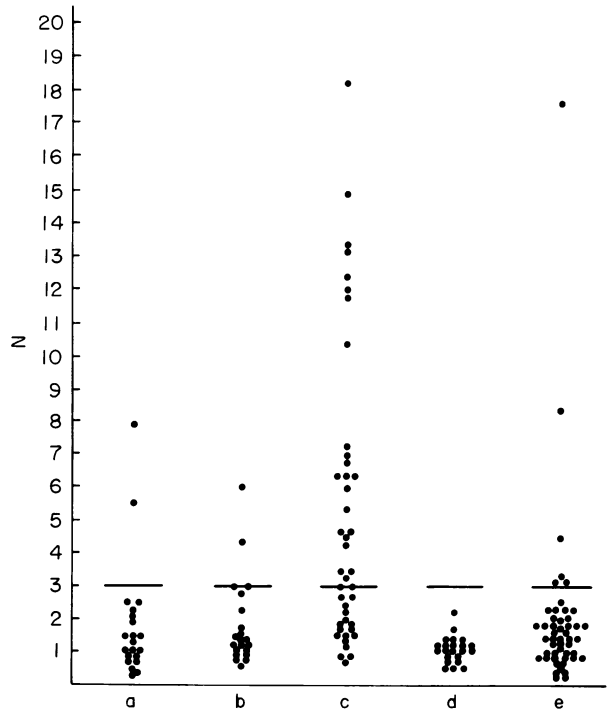

Fig. 4. Prevalence of high anti-R-13 antibody levels in chronic Chagas heart disease. Anti-R13 antibody levels were measured by ELISA using the synthetic peptide R-13 (amino acid sequence: EEEDDDMGFGLFD) as antigen. Horizontal lines indicate three times the mean OD value obtained for 35 normal control sera. (a) *Leishmania sp.*-infected individuals ($n=24$); (b) *Trypanosoma brucei* infected individuals ($n=20$); (c) chronic Chagas heart disease patients ($n=44$); (d) *Plasmodium falciparum*-infected individuals ($n=20$); (e) SLE patients ($n=50$).

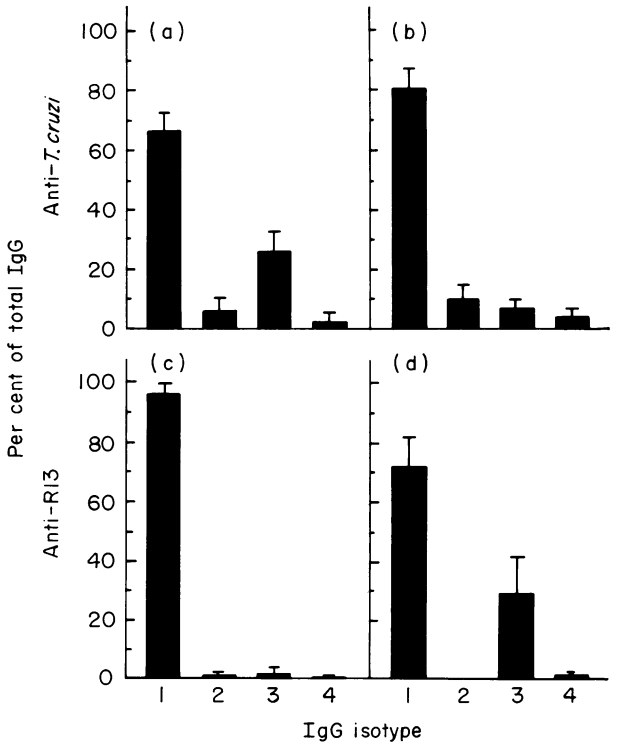

Fig. 5. IgG subclass distribution of the anti-*T. cruzi* and the anti-R-13 antibody response in sera from 25 chronic Chagas heart disease patients. (a) and (b), IgG isotype distribution of the anti-*T. cruzi* antibody response; (c) and (d), IgG isotype distribution of the anti-R-13 antibody response. Bars indicate the mean ($\pm$s.d.) percentages of total IgG subclasses. (a) and (c) represent the pattern observed in most of the sera tested (84% and 92% of the 25 sera, respectively).

anti-JL5 antibodies immunoselected from chronic Chagas heart disease sera only reacted with three or four *T. cruzi* ribosomal proteins (Fig. 1b). To assess the identity of these antigens, an SLE serum positive for anti-P antibodies was used as a ribosomal P protein marker to probe the *T. cruzi* ribosome Western blots (Fig. 1c, lane 2). The results in Fig. 1 confirmed that the parasite ribosomal proteins defined by anti-JL5 and anti-P antibodies were similar, and revealed that the P protein family in *T. cruzi* is composed by three low molecular weight components of 19 kD, a polypeptide doublet of 15 and 14 kD, and the 38-kD polypeptide.

Autoreactive nature of anti-JL5 antibodies

To determine the autoreactive nature of antibodies directed against the C terminal region of the *T. cruzi* ribosomal P proteins (JL5), two series of Western blots were performed. First, anti-JL5 immunoselected antibodies were used to probe human ribosomal proteins. Figure 2 (lane 1) shows the reaction of HeLa ribosomal proteins with SLE anti-P antibodies, and (lane 2) the reaction of chronic Chagas heart disease anti-JL5 antibodies with the same proteins. The latter reaction was completely blocked when the anti-JL5 antibodies were pre-incubated with the A1 recombinant peptide (Fig. 2, lane 3). Second, in order to prove further that the chronic Chagas heart disease anti-JL5 antibodies were able to crossreact with the host ribosomal P proteins, purified fusion proteins from bacteria lysogenized by λgt11 recombinants coding for the human

ribosomal P1 and P2 proteins were tested with immunoselected anti-JL5 antibodies. Figure 3b shows their reaction with the human P1 and P2-β-galactosidase fusion proteins. The reaction of an anti-P-positive SLE serum with the recombinants is shown in Fig. 3a. These results and those in Fig. 2 were in agreement with previous experiments, and indicated that the ribosomal P epitope shared by parasite and host was limited to the highly conserved C terminal residues (Mesri *et al.*, 1990).

Anti-R-13 responses in different protozoan infections

In order to evaluate the relevance of the two additional amino acids present in A1 but not in R-13, ELISA measurements were performed using the synthetic peptide R-18 as antigen. The comparison of anti-R-13 and the anti-R-18 antibody titres showed a highly significant correlation ($r=0{\cdot}96$, $P<0{\cdot}001$), indicating that the only epitope contained within the 18 C terminal amino acids was defined by R-13. This fact was confirmed by ELISA inhibition experiments in which R-13 inhibited completely the serum binding to R-18 (data not shown). Subsequently, an ELISA was employed to assess the prevalence of the anti-R-13 autoantibody response in sera from 108 patients with various protozoan parasitosis, 50 SLE sera and 35 normal control sera (Fig. 4). Positive anti-R-13 antibody levels were detected in 57% of the 44 patients with chronic Chagas heart disease studied. In this group, the anti-R-13 values were compared with the corresponding anti-*T. cruzi* titres measured by ELISA. A weak but significant correlation ($r=0{\cdot}6$; $P<0{\cdot}001$) was observed between the two assays. Of the sera from patients with leishmaniasis and African trypanosomiasis, 8% and 20% respectively, were positive. In contrast, all malaria serum samples were negative. The frequency of anti-R-13 positives among the SLE sera was of 12% confirming previous reports on the frequency of anti-P antibodies in SLE (Bonfa & Elkon, 1986).

Anti-R-13 IgG subclass distribution

The IgG subclasses displayed by anti-R-13 and anti-*T. cruzi* antibodies of 25 anti-R-13-positive chagasic sera were examined (Fig. 5).

For 21 sera tested (84%), the IgG1 and IgG3 isotypes accounted for the total anti-parasite activity (Fig. 5a), whereas for the remaining four (16%), the anti-*T. cruzi* reactivity was mainly IgG1 (Fig. 5b). The IgG1 isotype accounted for the anti-R-13 activity of 23 sera (92%) (Fig. 5c). Only two out of 25 individuals showed a mixed IgG1 and IgG3 anti-R-13 isotype distribution (Fig. 5d). The chagasic sera with the latter anti-R-13 isotype profile presented an anti-*T. cruzi* isotype profile similar to the one shown in Fig. 5a. The anti-R-13 response of SLE anti-P-positive sera was equally represented by the IgG1 and IgG2 subclasses (data not shown). Similar isotype profiles were reported for the anti-ribosomal P autoantibody response in SLE sera (Bonfa, Llovet & Elkon, 1988).

DISCUSSION

Experiments were designed to characterize further the autoimmune nature of the anti-ribosomal P protein antibody response in chagasic patients, and to evaluate the possible mechanisms underlying this anti-self-response. Previous results favoured two basically different mechanisms: polyclonal B cell activation (D'Imperio Lima *et al.*, 1986), and an antigen-driven mechanism, namely immunization, during chronic infection, with *T. cruzi* ribosomal P antigens bearing molecular similarities to human ribosomal P proteins, molecular mimicry (Damian, 1989). The latter process was proposed to explain the presence of several autoantibody specificities in chagasic sera but its validity remained a matter of speculation because the identification of the putative cross-reactive epitopes in both parasite and host had not been possible (Kierszenbaum, 1985).

In our case, the specific nature of such a mechanism implies that antibodies made to *T. cruzi* ribosomal P antigens could cross-react with human cell ribosomal P proteins. In accordance with this premise, the results in Fig. 1 showed that *T. cruzi* ribosomal proteins elicited a strong humoral response in patients with chronic Chagas heart disease while whole serum samples from these patients failed to react with human ribosomal proteins. This lack of autoreactivity may be due to a relative low serum concentration of anti-ribosomal P protein antibodies in the tested sera, since immunoselected anti-JL5 antibodies reacted with the parasite and the human ribosomal P proteins. The ability of the A1 recombinant peptide to inhibit completely this anti-self-reactivity proved firmly that the cross-reactive epitopes, parasite–host, were defined by the C terminal amino acids of the *T. cruzi* ribosomal P proteins. The amino acid sequence comparison of the human ribosomal P1 and P2 proteins with the complete P-JL5 amino acid sequence, together with previously reported data, further confirmed these results (Rich & Steitz, 1987; Mesri *et al.*, 1990; Schijman *et al.*, 1990).

The observation that the autoepitope R-13 is recognized principally by sera from chagasic patients, not at all by sera from malaria patients (Fig. 4) and by only a low proportion of sera from individuals infected with other kinetoplastidae (Fig. 4) was striking, above all because ribosomal P proteins from all eukaryotic cells possess a distinctive, antigenically cross-reactive C terminal amino acid sequence (Elkon *et al.*, 1986). Of interest is the observation that anti-JL5 antibodies were not detectable in sera from patients with acute Chagas disease ($n=9$), nor in sera from Latin American individuals infected with *Leishmania sp.* ($n=30$) or *Plasmodium falciparum* ($n=20$) (manuscript submitted).

This suggests that the anti R-13 antibody response was not generated by polyclonal B cell activation, a mechanism described to occur in the mentioned protozoan parasitosis (Kobayakawa *et al.*, 1979; Galvao-Castro *et al.*, 1984; Langhorne, Kim & Asofsky, 1985). In contrast, it can be proposed that this response is a reflection of a repeated challenge to the immune system due to parasite ribosomal antigens released after recurrent parasite lysis, characteristic of chronic *T. cruzi* infections. An alternative explanation, that the anti-R-13 antibodies are the result of immunization with self-ribosomal P antigens leaked from injured tissues is conceivable but not convincing in the light of reported results that fail to detect anti-R-13 antibodies in sera from individuals with non-chagasic idiopathic dilated cardiomyopathy (Mesri *et al.*, 1990).

The analysis of the anti-R-13 IgG subclass distribution in sera from chagasic and SLE patients provides additional clues to the nature of the anti-R-13 autoantibody response. In chagasic patients it was mainly IgG1, while the anti-*T. cruzi* isotype pattern in chagasic sera was IgG1–IgG3, with a clear prevalence of the IgG1 isotype, suggesting that the anti-R-13 response may be a component of the humoral anti-*T. cruzi* response. Furthermore, this IgG1-restricted anti-R-13 isotype

profile indicated that the regulatory mechanisms involved in the Chagas disease anti-R-13 and in the SLE anti-P autoantibody response were different and argued against the hypothesis of a polyclonal B cell activation process as a source of anti-R-13 autoantibodies in Chagas disease.

In view of the fact that high anti-R-13 antibody titres were only detected in chagasic patients with severe heart complaint (Mesri *et al.*, 1990), it may be relevant to point that autoantibodies to the Ro antigen, markers of congenital heart block in SLE, also present an IgG1-restricted isotype profile (Bonfa *et al.*, 1988).

Our data constitute the first firm evidence of molecular mimicry involvement in the generation of autoantibodies during Chagas disease. This was substantiated by the following facts: (i) chronic Chagas heart disease anti-JL5 antibodies reacted with the parasite and host ribosomal P proteins; (ii) the autoreactive specificity of the anti-JL5 antibodies was directed to the highly conserved C terminal region defined by R-13; (iii) the significant correlation between anti-R-13 antibody levels and anti-*T. cruzi* antibody titres; (iv) the prevalence of the IgG1 isotype in the IgG subclass distribution of the anti-R-13 and anti-*T. cruzi* antibody response; and (v) the specificity of the anti-R-13 response.

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