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Calreticulin synthetic peptide analogues: anti-peptide antibodies in autoimmune rheumatic diseases

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SUMMARY

Autoantibodies in sera from patients with systemic lupus erythematosus (SLE) and onchocerciasis recognize calreticulin (CaR), a calcium-binding protein, as antigen. In this study we present the immunological properties of two synthetic peptides prepared to correspond to the 1–24 and 7–24 amino acid sequence of CaR. In contrast to information previously reported for the recombinant protein, the CaR-peptide analogues appeared immunoreactive to anti-Ro/SSA autoimmune sera. Human sera from patients with SLE, Sjögren's syndrome (SS), rheumatoid arthritis (RA), as well as mixed connective tissue disease (MCTD), demonstrated a positive autoimmune response (binding of antibodies), to the CaR-peptide analogues. These findings suggest that anti-calreticulin autoantibodies are not restricted to any disease specificity.

Keywords calreticulin Ro/SSA autoimmune rheumatic diseases synthetic peptides

INTRODUCTION

Autoimmune rheumatic diseases are often associated with a variety of autoantibodies directed against cytoplasmic or nuclear components. Among them, the Ro/SSA antigen which was initially reported by Clark *et al.* [1] is a common target of an autoimmune response in systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) [2,3]. The Ro/SSA antigen is composed of at least two immunologically distinct proteins, 60 and 52 kD, non-covalently bound to a family of small RNAs [4,5].

Lieu *et al.* described a 60-kD protein [6] which was thought to be the 60-kD Ro/SSA. Synthetic peptides corresponding to the amino terminal sequence of this protein, as well as the isolated protein, were selectively recognized by anti-Ro/SSA monospecific sera. In addition, rabbit anti-peptide sera demonstrated a behaviour identical to the anti-Ro/SSA sera in Western blot, immunofluorescence and RNA precipitation. Thus, it was proposed that a major autoepitope of the 60-kD Ro/SSA resided within the amino terminal region [6–11]. However, isolation of a c-DNA encoding the 60-kD protein [11] revealed that this was human calreticulin (CaR) [12–14], a calcium-binding protein, which was also cloned [15,16].

The groups of Deutscher [17] and Ben-Chetrit [18], working independently, isolated the 60-kD Ro/SSA clone, which was found to be completely different from that of CaR. The *in vitro* translated and bacterially expressed protein of the reported clone not only interacted with anti-Ro/SSA autoantibodies in immunoblot and immunoprecipitation [17–20], but also bound the hY1 RNA *in vitro* [17,20]. On the other hand, Rokeach *et al.* [21,22] using c-DNA, encoded the human homologue of CaR. It did not interact with the anti-Ro/SSA monospecific sera, although it was reactive to specificities such as anti-Ro/SSA (and anti-Sm and/or anti-nRNP). It was also found that this protein did not interact either via protein–RNA or protein–protein interaction with the Ro/SSA RNPs. The discordance of the results with those previously published [6–11] may be related to the copurification of both the 60-kD Ro/SSA and CaR [21]. Nevertheless, this assumption does not explain the anti-Ro/SSA reactivity of the synthetic peptides corresponding to the amino terminal part of CaR [7,9].

The present study was designed to investigate if sera from patients with rheumatic diseases recognized CaR, as well as to gain further insight into autoimmunity. We report on both the synthesis and immunological examination of two peptides homologous to the amino terminal of CaR and corresponding to the 1–24 and 7–24 amino acid sequence of the protein.

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PATIENTS AND METHODS

Patient sera

Thirty-three sera from patients with SLE, 27 with SS, 28 with rheumatoid arthritis (RA), and 18 with mixed connective tissue disease (MCTD) (diagnosis fulfilled the criteria previously described [23–26]) were submitted to counter immunoelectro-

phoresis. The following specificities of autoantibodies were found: 49 anti-Ro/SSA, 16 anti-Ro/SSA and anti-La/SSB, 13 anti-nRNP. Sixty-nine normal sera were collected from healthy bank donors.

Peptide synthesis and purification of the CaR analogues

Two peptides composed of residue 1–24 (SP24) and 7–24 (SP18) of the amino terminal sequence of CaR Glu[1]-Pro-Ala-Val-Tyr-Phe-Lys[7]-Glu-Gln-Phe-Leu-Asp-Gly-Asp-Gly-Trp-Thr-Ser-Arg-Trp-Ile-Glu-Ser Lys[24] were synthesized.

Solid-phase synthesis of the SP24 and SP18 using N^{α}-t-Boc-N^{ε}-(2-chloro-CBz)-L-lysine-PAM resin (1% cross-linked divinylbenzene-styrene, 1·5 g, 0·39 mep/g) anchor bond and N^{α}-t-Boc/benzyl-side chain protection was carried out by standard methods [27]. Amino acid couplings were performed by the dicyclohexylcarbodiimide (DCC)/hydroxybenzotriazole: (HOBT) procedure using a ratio of amino acid/DCC/HOBT/resin (3/3/3/1). Deprotection of the N^{α}-t-Boc protecting group was carried out using trifluoroacetic acid (TFA) followed by diisopropylethylamine for neutralization. After synthesis, the peptides were cleaved from the resin with anhydrous HF in the presence of anisole and phenol (10% v/v) as scavengers at 0°C for 1 h. The peptides were extracted from the resin with 2 M acetic acid and lyophilized to give 0·85 g of the 24 SP (52% yield) and 0·57 g of the 18 SP (45% yield).

Purification of the crude peptides was achieved by anion exchange chromatography on diethyl amino ethyl (DEAE) resin, using as eluent gradient of NH_3 0·1 M/H_2O. The peptides were homogeneous as determined by thin-layer chromatography in MeOH/$CHCl_3$/27% NH_3 (2/2/1) ($R_{f_{SP24}}$=0·73, $R_{f_{SP18}}$=0·67) and Pyr/But/ACOH/H_2O (5/5/1/4) ($R_{f_{SP24}}$=0·56, $R_{f_{SP18}}$=0·49). The overall yield after purification was 480 mg (30% yield) for the 24 SP and 320 mg (25% yield) for the 18 SP. Appropriate 1 D and 2 D ^{1}H-NMR spectra confirmed the identity of the peptides.

Coupling of peptide to carrier protein

Peptide SP24 was coupled with bovine serum albumin (BSA) via a tyrosine residue with bisdiazobenzidine, as described by Scheidtman [28]. The uncoupled peptide and the coupling reagents were removed by dialysis against PBS at 4°C for 2 days.

ELISA

The modified solid phase assay (ELISA) of Engvall & Perlman [29] was applied. The SP18 and SP24 peptide (1 mg/ml) in ethanol (or PBS at pH *ca* 7·2) was added to the wells (50 μl/well) of the Nunc microplates and incubated overnight at 4°C. The microplates were washed with PBS-Tween 20 and the remaining binding sites were coated with 0·5% BSA, 1·5% ovalbumin and 0·1% Tween 20 in PBS (blocking buffer) for 2 h. After washing with PBS-Tween 20, diluted sera (1:50) with blocking buffer were added to the wells and incubated for 3 h. The plates were washed again (PBS-Tween 20), and goat anti-human IgG (0·5 mg/1 ml) conjugated to alkaline phosphatase (1:1000 in blocking buffer) was added to the wells. Subsequently, the plates were incubated for 2 h followed by washing and addition of the *p*-nitrophenol phosphate substrate at 37°C. The enzymatic reaction was terminated with the addition of NaOH 4 N (50 μl/well), and the optical density was measured at 405 nm.

Binding units are reported (Figs 1 and 2) without any subtraction of background binding, since normal and patient sera showed very low and almost identical binding levels to BSA and control peptides.

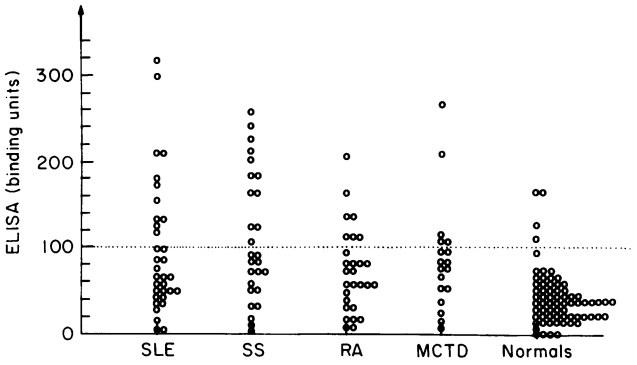

Fig. 1. Binding of sera from patients with systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), rheumatoid arthritis (RA), and mixed connective tissue disease (MCTD) to the 24 SP calreticulin (CaR) peptide analogue. Dotted line represents s.d. above the mean binding levels of normal control sera.

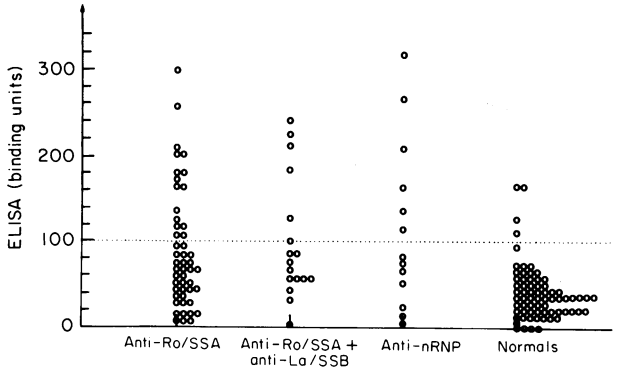

Fig. 2. Binding of sera from patients with anti-Ro/SSA, anti-Ro/SSA-anti-La/SSB, anti-nuclear ribonucleoprotein particle (nRNP) autoantibody specificities to the 24 SP calreticulin (CaR) peptide analogue. Dotted line represents 3 s.d. above the mean binding levels of normal control sera.

Dot blot

The Towbin technique [30] modified as follows was used. One microlitre of the water peptide solutions (2 mg/ml) was spotted on Immobilon polyvinyledene difluoride (PVDF) membranes, incubated with blocking buffer for 2 h and then with diluted sera (1:50) in blocking buffer at 4°C overnight. After washing with PBS-Tween 20, a solution of goat anti-human IgG conjugated to peroxidase was added and allowed to react for 1 h. The membranes were washed and the colour was developed by adding a substrate solution of 4-chloro-1-naphthol.

Counter immunoelectrophoresis

The method was performed as described by Bunn *et al.* [31], using calf thymus extract as antigen source.

RESULTS

Qualitative analysis of autoantibodies, against the CaR peptide analogues (SP24, SP18)

The qualitative analysis of the autoantibodies directed against the prepared peptides SP18 and SP24 was performed using Dot

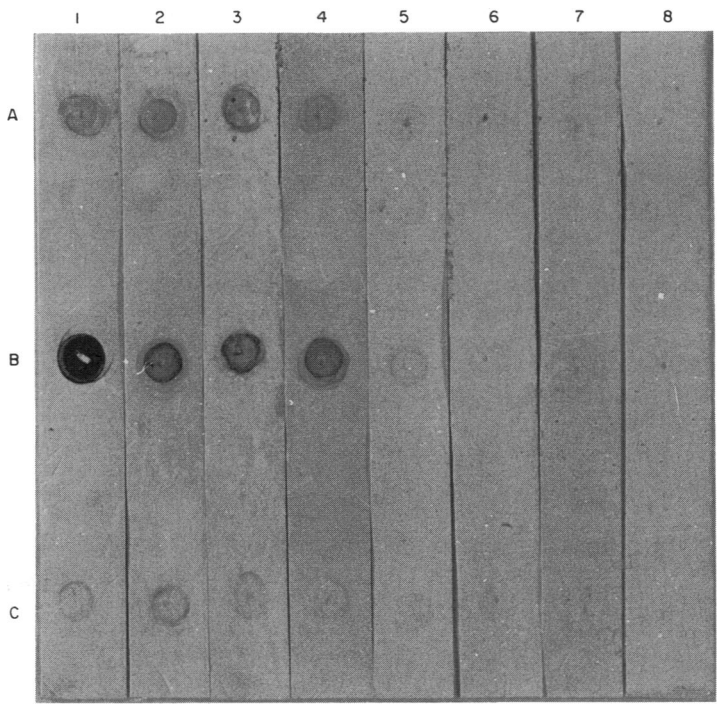

Fig. 3. Dot blots on the calreticulin (CaR) peptide analogues 24 SP (row A), 18 SP (row B), control peptides (row C, also see text). Human autoimmune patient sera (lanes 1–4), normal human sera (lanes 5–8).

blot (Fig. 3). Normal sera did not recognize any of the synthetic peptides, while sera from human autoimmune patients were found to react selectively with them. In contrast, the control peptides did not demonstrate any antigenic reactivity to either normal or patient sera (Fig. 3).

Antigenicity of the CaR peptide analogues

Human autoimmune patient sera. The solid-phase assay method (ELISA) was applied for the quantification of anti-SP antibodies in autoimmune patient sera. Thirty-three percent of patients with SLE, 40·7% of patients with SS, 25% of patients with RA, 27·7% of patients with MCTD and 5·8% of normals were found to be positive for the anti-SP24 antibodies (Fig. 1).

The same sera, when allowed to interact with the BSA-SP24, exhibited greater binding levels compared with the free SP24 (data not shown), while no binding activity was detected for BSA. Control peptides were inactive to the normal and patient sera.

Human autoantibody specificities. Human autoimmune sera specific for various antigens (Ro/SSA, La/SSB, nRNPs) were tested (ELISA) for quantitative binding reactivity to the SP24 and SP18 CaR analogues. Overall, 28·5% of anti-Ro/SSA sera, 29·4% of anti-Ro/SSA and anti-La/SSB sera and 46·1% of anti-nRNP sera were positive to the anti-SP antibodies (Fig. 2). SLE and SS patients monospecific for anti-Ro/SSA were positive for the SP24 antibodies in 35% and 37·5%, respectively (data not shown). Control peptides did not show any antigenicity to normal and patient sera. The following control peptides were used in all experiments (Figs 1–3): (i) Trp-Asn-Pro-Asn-Asp-Tyr-Gly-Gly-Ile-Lys; (ii) Trp-Asn-Pro-d-Ala-Asp-Tyr-Gly-Gly-Ile-Lys; (iii) Arg-Leu-Gly-Arg-Leu-Gly; and (iv) Poly (Arg-Nva-Gly).

The immunological activity of both synthetic peptides was approximately the same in all cases, suggesting an identical antigenic determinant in the 1–24 and 7–24 amino acid sequence in CaR.

DISCUSSION

Two peptides composed of residues 1–24 (SP24) and 7–24 (SP18) from the amino terminal sequence of CaR were synthesized using solid-phase methodology. The CaR peptide analogues reacted in ELISA with sera from patients with SLE, SS, RA as well as MCTD (Fig. 1), which in agreement with previous observations suggested that anti-CaR autoantibodies are not restricted to any subset of the disease [7–9].

Both the SP18 and SP24 peptides were positive (ELISA) to specific human sera antibodies, such as anti-Ro/SSA, anti-Ro/SSA and anti-La/SSB and in some cases with anti-nRNP (Fig. 2) and anti-Sm (not shown in Fig. 2). However, the coexistence of anti-Ro/SSA autoantibodies with anti-nRNP and anti-Sm can not be excluded, since it is known that the 52-kD-Ro/SSA antibodies and subpopulations of the 60-kD-Ro/SSA antibodies specifically recognize human antigens, but fail to bind non-primate antigen which was used in the counter immunoelectrophoresis [32,33].

It is also interesting to note that the prepared CaR analogues appeared immunoreactive to anti-Ro/SSA autoimmune sera (Fig. 2), although the CaR encoded from c-DNA did not exert similar reactivity [21,22] (the encoded CaR interacted to anti-Ro/SSA (and anti-Sm and/or anti-nRNP sera)). These conflicting findings may be attributed to the additional amino terminal residues of the recombinant CaR, which are absent in the authentic protein [21]. In addition, it is probable that this amino acid sequence, rich in hydrophobic residues (signal peptide),

may influence the conformational mobility of the N-terminal portion of the molecule, which consequently may affect antibody recognition. On the other hand, we cannot exclude that the synthetic CaR peptide analogues may be more easily recognized in the anti-Ro/SSA autoimmune sera, compared with the whole protein, as a consequence of the significant degree of conformational freedom of the relatively short synthetic peptide moieties [34].

Even though CaR is not recognized by monospecific anti-Ro/SSA sera, there is little doubt that this protein is a human autoantigen [6,21,35]. Our results argue in favour of this observation, since antibodies against the synthetic CaR peptide analogues were detected in all the autoimmune rheumatic diseases (Fig. 1). It was also proposed that CaR is a heat shock/stress [15] protein and, therefore, may be related to the autoimmune mechanism [36]. From this point of view, it is not surprising that anti-peptide antibodies were detected in autoimmune sera (Fig. 1).

Taking into consideration the heterogeneous nature as well as the high molecular mass, as determined by gel filtration, of the Ro/SSA-RNP particle [37–39], one could pose the question whether CaR is a component of the ribonucleoprotein complex. Identification and synthesis of CaR epitope(s), as well as determination of their immunological properties, could answer the above question. Moreover, synthetic CaR peptide analogues could help elucidate the controversial observations presented by Rokeach [21] and McCauliffe *et al.* [8,9,11] and provide more insight into the cellular function(s) of CaR.

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