Clin Exp Immunol 1994; 95:397–407

Epitope mapping with synthetic peptides of 52-kD SSA/Ro protein reveals heterogeneous antibody profiles in human autoimmune sera

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(Accepted for publication 26 November 1993)

SUMMARY

The reactivity of autoantibodies present in the sera of 489 patients with Sjögren's syndrome (SS), systemic lupus erythematosus (SLE) and other autoimmune diseases was investigated by ELISA using recombinant 52-kD SSA/Ro protein (rRo52) and 39 overlapping synthetic peptides representing the entire sequence of Ro52. We report that IgG antibodies reacting with rRo52 were present in the sera of a large number of patients with SS (67% of patients with primary SS and 46% of patients with SS associated with SLE), whereas they were less frequent (10–25%) in SLE, rheumatoid arthritis (RA), juvenile chronic arthritis (JCA) and mixed connective tissue disease (MCTD), and absent in scleroderma. Among the 39 peptides tested, five were recognized by sera from 30–65% of patients with SS, namely peptides representing residues 2–11, 107–122, 107–126, 277–292 and 365–382. Patients with JCA had raised levels of IgG antibodies reacting with peptides 2–11 and 365–382, and 51% of patients with MCTD had raised levels of IgG antibodies reacting with peptide 365–382. None of the five peptides was recognized by more than 20% of sera from patients with SLE and RA. Interestingly, and of importance in the field of diagnostic tests based on peptides, the reactivity of antibodies to the Ro52 synthetic peptides varied greatly according to the origin of sera. Inhibition experiments using either patients' sera or antibodies induced in rabbits against Ro52 peptides showed that the four domains 2–11, 107–122, 277–292 and 365–382 are accessible on the surface of the Ro52 protein. These regions may thus be involved in the induction of specific antibodies in autoimmune patients.

Keywords SSA/Ro antigen synthetic peptides B cell epitopes Sjögren's syndrome systemic lupus erythematosus

INTRODUCTION

In the past few years, many investigations have attempted to identify specific regions in protein antigens which are recognized by autoantibodies using patients' sera or MoAbs generated from autoimmune mice, and recombinant or synthetic protein fragments as antigens. The fine specificity of anti-SSA/Ro and anti-SSB/La antibodies has been particularly studied [1,2]. These antibodies are detected primarily in patients with systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS), and are of great interest because they occur in congenital heart block and neonatal lupus which are complications of these diseases [3]. Depending on the techniques used for their detection and on the ethnic origin of patients, anti-SSA/Ro antibodies have been described in 15–70% of patients with SLE, 35–96% of patients with SS, and 15–28% of patients with rheumatoid arthritis (RA) [1].

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The SSA/Ro antigen is present in low quantity in the cell as a ribonucleoprotein (RNP) complex which contains uridine-rich RNA designated Y RNA (for a recent review, see [4]). In HeLa cells and human lymphocytes, there are four RNAs (hY1, hY3, hY4 and hY5) which are transcribed by RNA polymerase III and whose sizes range from 83 to 112 nucleotides. A characteristic feature of Ro RNP particles is their heterogeneity. They differ between species, between cells within the same species (for example between human lymphocytes, erythrocytes and thrombocytes) and even within a cell [1,4]. The function of these particles remains unknown.

Results from several laboratories have demonstrated that the protein moiety which bears the SSA/Ro antigenic epitopes corresponds to two major proteins, namely SSA/Ro 60-kD protein (Ro60) and SSA/Ro 52-kD protein (Ro52). In human erythrocytes, Ro60 and Ro52 are replaced by two analogous but antigenically distinct proteins of 60 and 54 kD. A subset of SSB/La 50-kD (La) protein is the third protein known to be associated, at least transiently, with Ro RNP complex [5]. A

fourth protein, calreticulin, which migrates at 60 kD in SDS-PAGE but whose sequence corresponds to a mol. wt of 46 kD has also been described [6,7]. However, although other groups have confirmed that anti-calreticulin antibodies can be found in the serum of autoimmune rheumatic patients, the question of whether calreticulin is a constitutive component of the Ro particle remains controversial [8–10].

Several B cell epitopes on La and Ro60 have been identified at the level of short amino acid sequences [11–14], and evidence is also accumulating to show that a number of B cell epitopes on La and Ro are conformational [15,16]. Itoh *et al.* [16] have recently suggested that anti-Ro52 antibodies are mainly directed towards the denaturated protein, whereas anti-Ro60 antibodies are conformation-dependent.

Ro52 was identified by using conditions of PAGE permitting the separation of Ro52 and La (which normally migrate in a single band) and the subsequent detection by immunoblotting of specific antibodies binding these two proteins [9,17,18]. Two clones encoding Ro52 were isolated and characterized [19,20]. No homology between Ro60 and Ro52 was observed, but a striking homology was found between the three proteins Ro52, mouse T cell down-regulatory protein rpt-1 and human transforming protein ret-finger protein (rfp). In Ro52 there is no RNA recognition motif, as there is in La and Ro60, and in the amino terminal half of the molecule four putative zinc-finger motifs and a leucine zipper domain (residues 211–232) can be found.

Based on the structure of Ro52 described by Chan *et al.* [20], we have synthesized 39 overlapping peptides covering the entire sequence of Ro52 and analysed their reactivity by ELISA with the sera of 489 patients with systemic autoimmune diseases. Peptides were tested under different conditions, either immobilized on the plastic surface of microtitre plates or free in solution. Patients' sera were tested in parallel with immobilized or free recombinant Ro52 (rRo52). ELISA reactivity with rRo52 and Ro52 peptides was compared with data obtained in Western blot and counter-immunoelectrophoresis (CIE). Antibodies against Ro52 synthetic peptides were induced in rabbits, allowing us to map the location of particular domains in the free protein. Our data indicate that five linear peptides of Ro52 are recognized by IgG antibodies from SS patients, whereas none of the 39 Ro52 peptides was recognized by more than 20% of lupus sera. The frequency of occurrence of antibodies binding these five peptides in SS was found to vary according to the origin of the sera, thus showing a heterogeneity of anti-Ro52 antibody response.

MATERIALS AND METHODS

SSA/Ro proteins and synthetic peptides

The Ro52 cDNA clone described by Bozic *et al.* [21] was subcloned into vector pET-3b, and after transformation of *Escherichia coli* BL21 (DE3) pLysS with this construct, Ro52 was expressed by isopropyl-β-D-galactopyranoside (IPTG) induction. Ro52 was purified according to the method described by Adam *et al.* [22]. Affinity-purified Ro60 and La proteins were obtained from Immunovision (Springdale, AR; ref. SSA-3000 and SSB-3000 respectively). Their purity was checked by SDS-gel electrophoresis [14]. These proteins were used for both ELISA and immunoblotting. For CIE, bovine spleen extract (Sigma, St Louis, MO; ref. S-9005) and rabbit thymus extract (Northeast, Uxbridge, UK; ref. 41008-1) were used as a source of Ro and La antigens, respectively.

For the synthesis of Ro52 peptides, assembly of the protected chains was carried out on a small scale (25 μmol) with a home-made multichannel peptide synthesizer [23] according to a classical Fmoc methodology. Homogeneity of peptides was assessed by analytical high performance liquid chromatography (HPLC) on a nucleosil C8 column, 5 μm (3·9 × 150 mm), using a triethylammonium phosphate buffer system. Electro spray mass spectra were obtained as described previously [24].

An additional gly-lys arm was added at the C-terminus tryptophan residue 11 of peptide 2–11 to allow the peptide to be conjugated to bovine serum albumin (BSA). Conjugation was performed using N-succinimidyl 3-[2-pyridyl dithio] propionate (SPDP) [25]. The yield of coupling was determined by measuring spectrophotometrically the release of 2-thiopyridone.

Antisera to Ro52 peptides and human sera

In order to induce anti-peptide antibodies, rabbits were given s.c. inoculations with BSA-conjugated peptides 2–11 and 463–475, and unconjugated peptide 277–292 in the presence of Freund's complete adjuvant (FCA) for the first and subsequent injections. In the case of peptides 107–122 and 365–382, rabbits were immunized by multiple i.d. injections [26]. Peptides (250 μg) were mixed with 3 mg methylated BSA (Calbiochem, La Jolla, CA; ref. 455451) and emulsified in a mixture containing Freund's incomplete adjuvant (FIA) and heat-killed tuberculosis bacillus (15 mg in 4 ml) in a final proportion v/v/v of 1/0·5/0·5. The animals were bled on a fortnightly basis from the third injection in the case of rabbits injected subcutaneously, and from the third week after the single series of injections for rabbits immunized intradermously. A prebleeding of each animal was performed and tested as control in each assay.

Human sera were from 141 unselected patients with active and quiescent SLE, 89 patients with primary SS (pSS), 57 patients with secondary SS (sSS), 70 patients with RA, 79 patients with juvenile chronic arthritis (JCA), 43 with mixed connective tissue disease (MCTD), 10 with scleroderma and from 36 healthy volunteers. These sera were obtained from the hospital centres supervised by two of us (D.A.I. and O.M.), from Drs N. Abuaf (Hôpital Rothschild, Paris, France), J. L. Pasquali (CHU Strasbourg, France), C. André (Hôpital Henri-Mondor, Créteil, France) for scleroderma and A. M. Prieur (Hôpital Necker, Paris) for JCA sera. Sera from Polish patients were obtained from Professor H. Maldyk (Institute of Rheumatology, Warsaw, Poland) and sera from US patients were a gift from Drs M. Reichlin (Oklahoma Health Sciences Centre, Oklahoma City, OK) and R. S. Schwartz (Tufts University, Boston, MA). All patients with SLE fulfilled four or more of the American Rheumatism Association classification criteria for the disease [27]. The patients with SS each met criteria described elsewhere [14,28]. The diagnosis for the Sjögren's patients was confirmed by labial minor salivary gland biopsy in most cases, and clinicians were extremely careful to distinguish between primary and secondary SS. Among the 57 patients classified as sSS, 26 collected in the centre of D.A.I. also had SLE, and 31 obtained from Poland also had RA. Among the 43 sera of patients classified as MCTD, 15 were from the collection of Dr W. J. Van Venrooij (University of Nijmegen, The Netherlands), 10 were from Dr N. Abuaf and have been described previously [29], and 18 were from Dr M. Reichlin.

Table 1. Reactivity in ELISA of autoimmune sera with rRo52 and Ro52 synthetic fragments

Sera source	Patients (*n*)	rRo52 (%)	Ro52 peptides 2–11 (%)	107–122 (%)	277–292 (%)	365–382 (%)
pSS	89	**66·7**	14·5	11·6	**31·9**	18·8
SS +SLE	26	**46·1**	**65·4**	**30·8**	**53·8**	**61·6**
SS +RA	31	29·0	6·4	3·2	6·4	12·9
SLE	141	18·4	14·9	5·7	16·3	19·1
RA	70	14·3	7·1	5·7	2·8	11·4
JCA	79	24·0	**30·4**	10·1	21·5	**39·2**
MCTD	43	18·6	20·9	16·3	20·9	**51·2**
Scleroderma	10	0	0	10·0	0	10·0
NHS	36	0	0	0	0	0

Values are expressed as percentage of positive sera among total tested sera (*n*). The sera were considered positive when the OD values were $\geq 0{\cdot}30$. Only the IgG antibody response was tested. Patient sera were diluted 1:1000 with PBS-T-bovine serum albumin (BSA).

pSS, Primary Sjögren's syndrome; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; RA, rheumatoid arthritis; JCA, juvenile chronic arthritis; MCTD, mixed connective tissue disease; NHS, normal human serum.

ELISA

For the test of human sera, ELISA microtitre plates (Falcon, ref. 3912) were coated at 37°C with the following antigens diluted in 0·05 M carbonate buffer pH 9·6: purified Ro60 and La (1 μg/ml), rRo52 (20 ng/ml), and Ro52 synthetic peptides (2 μM). In each assay, patient sera were also tested in a non-coated well incubated with coating buffer as control. The subsequent steps of the test were as described previously [14] using patient sera diluted 1:1000 and goat anti-human IgG conjugated to horseradish peroxidase (HRP) diluted 1:30 000 in PBS-T. The final reaction was visualized by addition of 3,3′,5,5′ tetramethyl benzidine in the presence of H_2O_2.

The cut-off points of the assays were determined with a series of 36 sera from healthy individuals. The mean OD values were 0·032 (s.d. 0·028), 0·037 (s.d. 0·037) and 0·036 (s.d. 0·045) for Ro60, Ro52 and La respectively. In the case of Ro52 peptides, the highest mean OD value was obtained with peptide 422–438 (0·172, s.d. 0·036). For convenience, when sera were screened with the 39 peptides of Ro52 and with rRo52, they were considered positive when the OD values were higher than 0·30. When this threshold value was used, none of the normal sera was found positive (Table 1). All samples were systematically tested in at least two independent assays. The average intra- and interplate coefficients of variation were $\leq 5\%$. For calculations, all OD values >3 were considered as 3·0. 'Mean' OD values correspond to the arithmetic mean of all OD values, including values under the cut-off line for positivity.

The ELISA used to measure the binding of rabbit antibodies to Ro52 synthetic peptides was performed with plates coated as described above and with an anti-rabbit immunoglobulin HRP conjugate (Jackson Labs, Bar Harbor, ME; ref. 111-035-003) diluted 1:40 000.

For double antibody sandwich assay where the antigen rRo52 is attached to two different layers of antibodies, either rabbit antiserum directed against Ro52 peptide 463–475 or patient sera diluted 1:5000 in PBS-T were first incubated overnight at 4°C in wells of ELISA microtitre plates. After repeated washings with PBS-T and blocking of remaining sites on the plastic by incubation with 10 mg/ml BSA in PBS-T for 1 h at 37°C, plates were again washed three times with PBS-T and allowed to incubate during 1 h at 37°C with rRo52 (20 ng/ml). After repeated washings with PBS-T, patient sera or rabbit anti-Ro52 peptide antisera diluted 1:500–1:1000 with PBS-T-BSA were added for 1 h at 37°C. The detection of human or rabbit antibodies binding to Ro52 was performed as above.

The antigenic activity of peptides was also measured by ELISA using fluid-phase inhibition. After incubating the peptides for 1 h at 37°C and then overnight at 4°C with diluted human or rabbit sera, the mixture was added to wells precoated with rRo52 or with peptides (see legend for details) and incubated for 1 h at 37°C. Further steps of the assay were as described above.

Immunoblotting and CIE

Antibodies to Ro52 were detected by Western blotting using rRo52 as substrate. The latter protein was run in a 12·5% SDS-polyacrylamide gel followed by electrotransfer to nitrocellulose in 20% methanol Tris-glycine buffer pH 8·3. The blotted strips were incubated with test sera diluted 1:500–1:1000 in PBS-T containing 0·5% casein. The final reaction was revealed by means of anti-human (1:2000) or anti-rabbit (1:5000) IgG antibodies conjugated to alkaline phosphatase (Sigma; ref. A 9544 and A 9525, respectively) and BCIP-NBT colour development substrate. Precipitating antibodies to Ro and La were screened undiluted by CIE with bovine spleen extract and rabbit thymus extract as antigen source for Ro and La, respectively.

RESULTS

Ro52 synthetic peptides

Thirty-nine overlapping peptides covering the entire sequence of Ro52 as determined by Chan *et al.* [20] were selected for synthesis (Fig. 1). The degree of purity of the peptides assessed

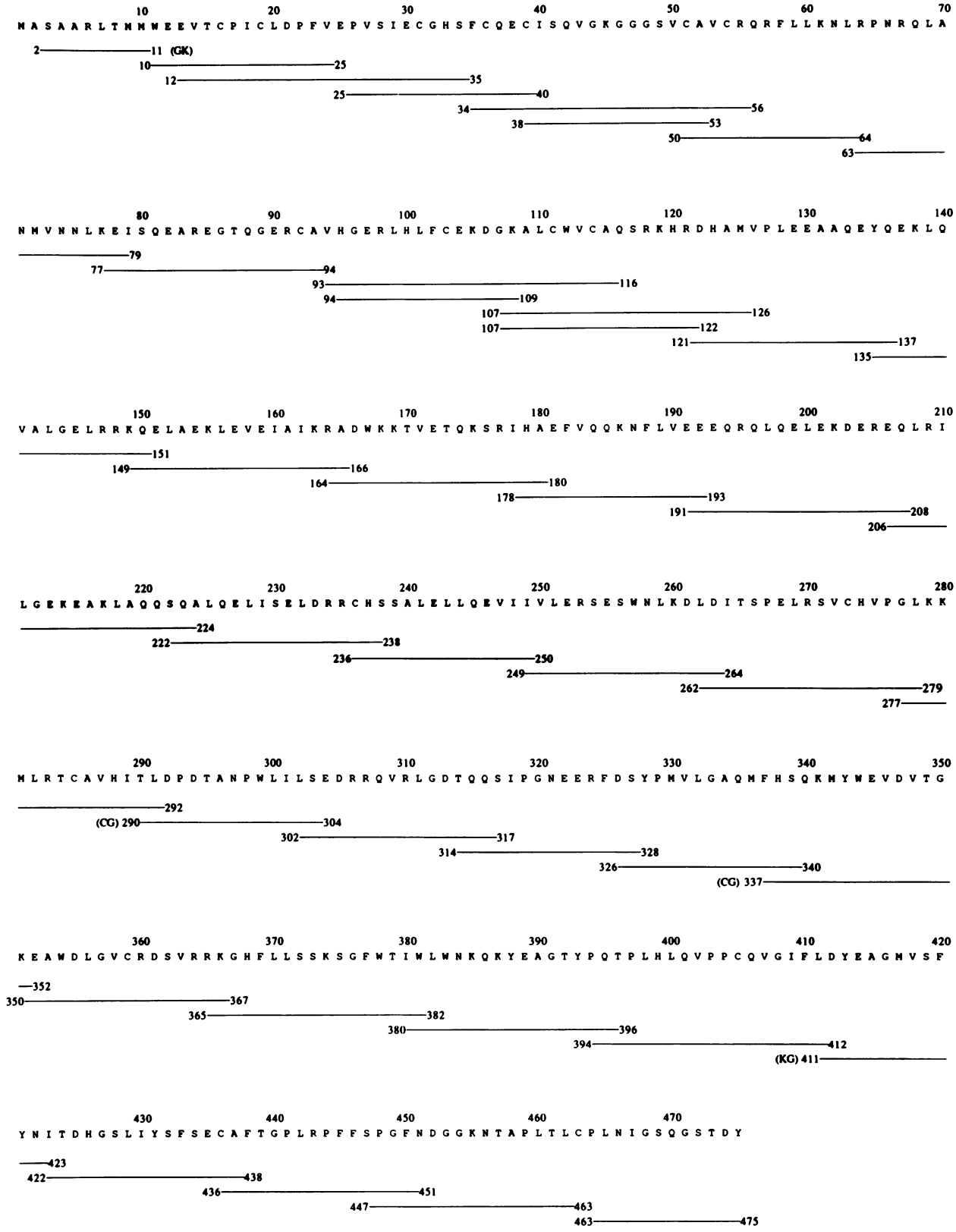


Fig. 1. Sequence of Ro52 derived from Chan *et al.* [20] and location of synthetic peptides used for epitope mapping. Additional amino acid residues introduced for further coupling purpose are indicated in parentheses. Four potential zinc fingers exist in Ro52. They are located in peptides 12–35, 34–56, 93–116 and 107–126.

by HPLC was at least 85%. The net peptide content was used as a basis of molarity calculation. Amino acid analysis of each peptide and FAB-mass spectra performed with the most important peptides showed that the purified products had the expected composition.

Study of sera from patients with SS, SLE and other rheumatic diseases with recombinant Ro52 protein and Ro52 synthetic peptides

First, sera from 489 autoimmune patients and 36 sera from normal individuals were tested systematically for the presence of IgG antibodies reacting with rRo52 and the 39 Ro52 synthetic peptides (Table 1). Using a cut-off value corresponding to 0·3 OD unit, it was found that 67% of pSS sera reacted with rRo52. The OD range was 0–⩾3·0 and the mean OD was 1·31 (s.d. 1·16). Antibodies reacting with rRo52 were also detected in a large number of sera from patients with sSS who also had SLE (46·1%; OD range 0–⩾3·0; mean OD 1·04, s.d. 1·29) and in 29% of patients with sSS associated with RA (OD range 0–2·15; mean OD 0·46, s.d. 0·71). Ro52 reacting antibodies were found in 24% of patients with JCA, 19% of patients with MCTD, 18% of unselected patients with active and quiescent SLE, and 14% of patients with RA. No activity with rRo52 was found in sera from patients with scleroderma.

All these sera (positive or negative with rRo52) were tested with the 39 Ro52 synthetic peptides described in Fig. 1. For testing, all peptides were directly adsorbed on ELISA plates except for peptide 2–11 which was first conjugated to BSA via SPDP (coupling molar ratio BSA:peptide 1:21 as determined from the amino acid composition of the final conjugate) before incubation in wells of microtitre plates. Peptides were found to be satisfactorily attached to the plastic surface of ELISA plates by using antibodies induced in rabbits against these peptides (a detailed study of the reactivity of these antisera with peptides and the Ro RNP particle will be described elsewhere). Peptides could thus be used as antigens in this ELISA format for testing patient sera. Among the 39 Ro52 synthetic peptides tested, five were recognized by IgG antibodies from autoimmune patients, namely peptides 2–11, 107–122, 107–126, 277–292 and 365–382 (Table 1). The peptide 107–126 was slightly less well recognized by patients' sera than peptide 107–122. Therefore, in the following results, only the reactivity of peptide 107–122 is described. The other peptides were recognized by less than 5% of patients' sera. Thirty-two per cent of sera from patients with pSS ($n=89$) reacted with peptide 277–292 (OD range 0–2·42; mean OD 0·35, s.d. 0·53). Patients with sSS who also had SLE possessed antibodies reacting with the four peptides 2–11, 107–122, 277–292 and 365–382. The respective OD range for the four peptides was 0·04–⩾3·0, 0·02–0·59, 0·06–1·20 and 0·09–1·33, and the mean ODs were 0·59 (s.d. 0·57), 0·24 (s.d. 0·14), 0·41 (s.d. 0·31) and 0·47 (s.d. 0·35), respectively. In patients with SLE, RA, scleroderma or in patients with sSS associated with RA, reaction with the four peptides was found in less than 20% of sera. Patients with JCA possessed IgG antibodies reacting with peptide 2–11 (OD range 0·02–0·80; mean OD 0·30, s.d. 0·18) and 365–382 (OD range 0–1·07; mean OD 0·33, s.d. 0·17) and patients with MCTD possessed IgG antibodies reacting with peptide 365–382 (OD range 0–1·70; mean OD 0·47, s.d. 0·36). The binding of IgG antibodies present in pSS sera to rRo52 and Ro52 peptides is shown in Fig. 2.

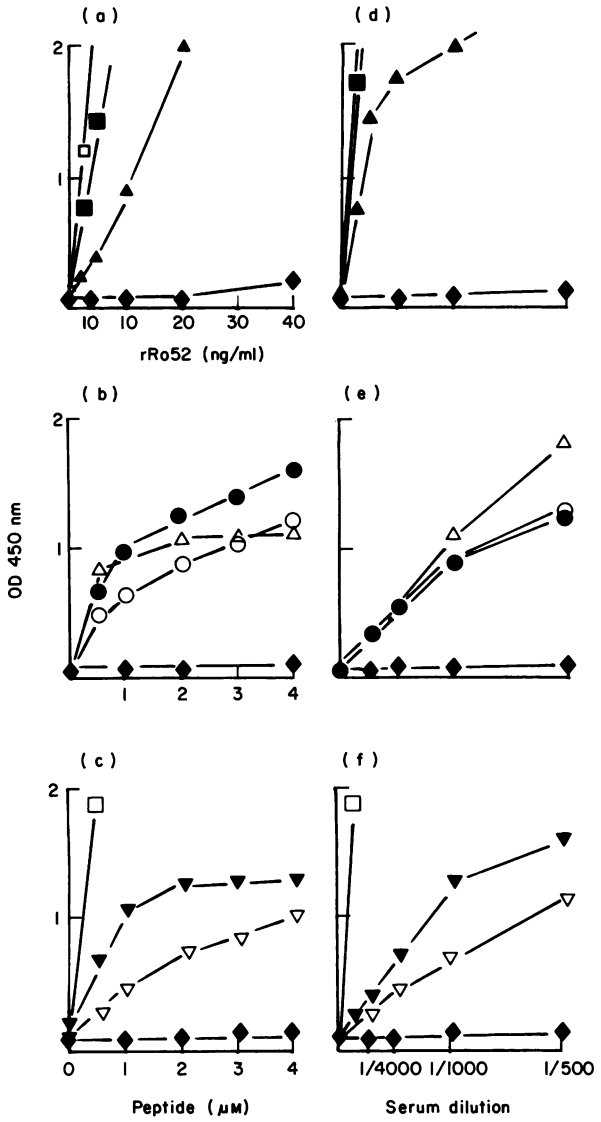


Fig. 2. Binding in ELISA of eight different primary Sjögren's syndrome (pSS) sera (○, △, □, ▽, ●, ▲, ■, ▼) and one normal human serum (◆) to rRo52 (a, d), peptide 277–292 (b, e) and peptide 365–382 (c, f) of Ro52. In a, b, c, patient sera were diluted 1:1000 and allowed to react with various concentrations of rRo52 or Ro52 peptides; in d, e, f, various dilutions of patient sera were used with 20 ng/ml of rRo52 and 2 μM of peptide. Only IgG antibodies were tested.

Reactivity of sera with Ro52 synthetic peptides varies with the origin of sera

In Table 2 patients are subdivided according to the origin of sera. The results show that in a comparable number of sera from pSS patients collected in different centres in France, England, Poland and the USA, equivalent incidences of sera reacting with rRo52 (52–80%) were found. In contrast, raised levels of IgG antibodies reacting with peptide 277–292 were found in pSS sera collected in France, whereas raised levels of antibodies reacting with peptide 365–382 were found in pSS sera collected in England and Poland. Although pSS sera collected in the USA reacted strongly with rRo52 (OD range 0–⩽3·0; mean OD 1·79, s.d. 1·19), very few sera reacted with Ro52 peptides. It is possible that these sera react preferentially with conformational epitopes

Table 2. Reactivity of autoimmune sera in ELISA with rRo52 and Ro52 synthetic peptides according to origin of sera

Sera source	Patients (*n*)	rRo52 (%)	Ro52 peptides 2–11 (%)	107–122 (%)	277–292 (%)	365–382 (%)
pSS						
France	23	**52·2**	4·5	27·3	**56·5**	9·0
England	25	**72·0**	24·0	4·0	28·0	**32·0**
Poland	20	**80·0**	5·0	5·0	15·0	**65·0**
USA	21	**76·2**	14·3	4·8	9·5	13·6
SLE						
France	36	22·2	8·3	5·5	5·5	8·3
England	39	10·2	17·9	5·1	28·2	9·5
Poland	49	14·3	18·4	6·1	14·3	10·2
USA	28	20·7	7·1	0	10·7	7·1
MCTD						
France	10	20·0	0	3·0	10·0	3·0
The Netherlands	15	13·3	0	0	6·7	**53·3**
USA	18	27·8	**55·6**	16·7	**38·9**	**61·1**

pSS, Primary Sjögren's syndrome; SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease.

on Ro52 protein or with linear epitopes missed in the 39 peptides tested in this work.

A similar comparison was performed with MCTD sera collected in France ($n = 10$), in The Netherlands ($n = 15$) and in the USA ($n = 18$) (Table 2). While the reaction of these sera with rRo52 was fairly low, MCTD sera reacted with three Ro52 peptides. A large number of MCTD sera collected in The Netherlands and in the USA reacted with peptide 365–382 (OD range 0–0·67 and 0·11–1·29, respectively; mean ODs 0·28 (s.d. 0·19) and 0·47 (s.d. 0·30), respectively). MCTD sera collected in the USA also reacted with peptides 2–11 and 277–292 (OD range 0·08–0·84 and 0–0·83, respectively; mean ODs 0·31 (s.d. 0·21) and 0·29 (s.d. 0·21), respectively). The results are illustrated in Fig. 3.

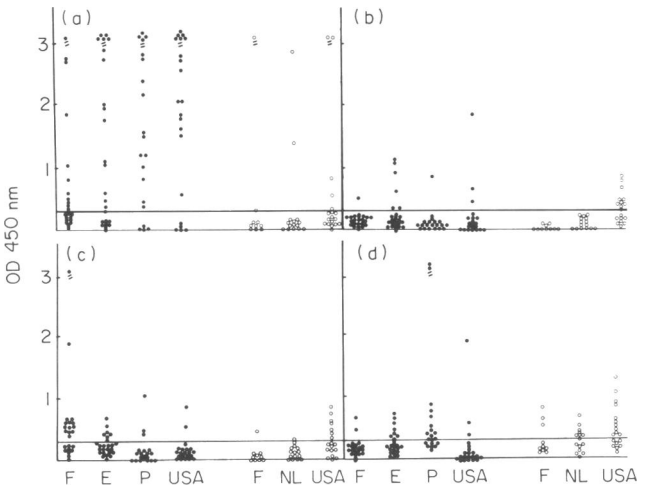


Fig. 3. Binding in ELISA of rRo52 and Ro52 peptides to primary Sjögren's syndrome (pSS) (●) and mixed connective tissue disease (MCTD) (○) sera according to the origin of the patient sera. Only IgG antibodies were tested. The cut-off line corresponds to 0·3 OD unit. F, France; E, England; P, Poland; NL,The Netherlands. (a) rRo52. (b) Peptide 2–11. (c) Peptide 277–292. (d) Peptide 365–382.

Reactivity of sera with Ro52 synthetic peptides in different ELISA formats

The reactivity of a series of patients' sera selected randomly with the four peptides 2–11, 107–122, 277–292 and 365–382 was also studied by ELISA inhibition experiments (Table 3), where the free peptides were used to inhibit the binding of antibodies to rRo52. Depending on the pSS serum tested, peptides were found to possess up to 49% inhibitory activity. When tested in inhibition assay under similar conditions, neither other Ro52 peptides nor peptide 149–158 of tobacco mosaic virus protein (TMVP) used as control showed any activity with pSS sera. As shown in Table 3 with four pSS sera, rRo52 was recognized in direct ELISA as well as in inhibition assay with rRo52 in the liquid phase. In contrast, as far as Ro52 peptides are concerned, it seems that peptides were generally better recognized when they were adsorbed on to microtitre plates. In a number of cases, however, peptides were recognized in their free form and not when they were directly adsorbed to the plastic (e.g. peptide 2–11/serum 24; peptides 2–11, 107–122 and 277–292/serum K215; Table 3).

rRo52 inhibited the reactivity in ELISA of human sera to the homologous rRo52 protein immobilized and possibly at least partially denatured on the plastic surface. In the same ELISA test, neither Ro60 nor La protein inhibited in their free form the binding of antibodies to immobilized rRo52, indicating that there is no cross-reaction between denatured Ro52 and native Ro60 or La proteins.

Location of Ro52 domains in native Ro52 protein

When rRo52 was used as inhibitor and peptides 2–11, 107–122, 277–292 and 365–382 as antigen, rRo52 was found to possess inhibitory activity for the binding of patients' antibodies to each of four peptides (Fig. 4). This result demonstrates that these four regions are at least partly exposed when rRo52 is tested in the

Table 3. Reactivity of sera with rRo52 and Ro52 synthetic peptides in different ELISA formats

	rRo52		2–11		107–122		277–292		365–382	
Serum	Direct	Inh	Direct	Inh	Direct	Inh	Direct	Inh	Direct	Inh
24	2·73	76%	0·16	47%	0·31	5%	0·60	45%	0·86	49%
11	2·68	49	0·20	19	0·34	12	0·53	1	0·49	38
23	1·04	46	0·18	14	0·40	3	0·49	4	0·66	3
K215	⩾3·0	89	0·15	28	0·13	29	2·79	29	0·53	37

In direct ELISA, microtitre plates were coated with rRo52 (20 ng/ml) or Ro52 peptides (2 μM) and allowed to react with patient sera diluted 1:1000. In inhibition (Inh) of ELISA reaction, rRo52 (10 ng/ml) was adsorbed to the plastic plate and allowed to react with patient sera diluted 1:10000 and preincubated with various concentrations of rRo52 (up to 500 ng/ml; homologous inhibition) or free peptides 2–11, 107–122, 277–292 and 365–382 (1–25 μM); per cent of inhibition ⩽20% was considered not significant.

liquid phase. By using a double antibody sandwich ELISA where the antigen rRo52 was attached to a first layer of antibody (serum from patient K215 diluted 1:5000) and antibodies raised against peptide 2–11 as a second layer of antibody, we confirmed that the N-terminal region of Ro52 is readily accessible in native protein. Furthermore, by using this strategy with antibodies raised against peptide 463–475, we also found that the latter region is exposed in Ro52. In contrast, rabbit antibodies directed against peptides 107–122, 277–292 and 365–382 did not react with rRo52 in this ELISA format. This lack of reactivity may result from the type of presentation of rRo52 in the fluid phase (rRo52 is presented via antibodies of the serum

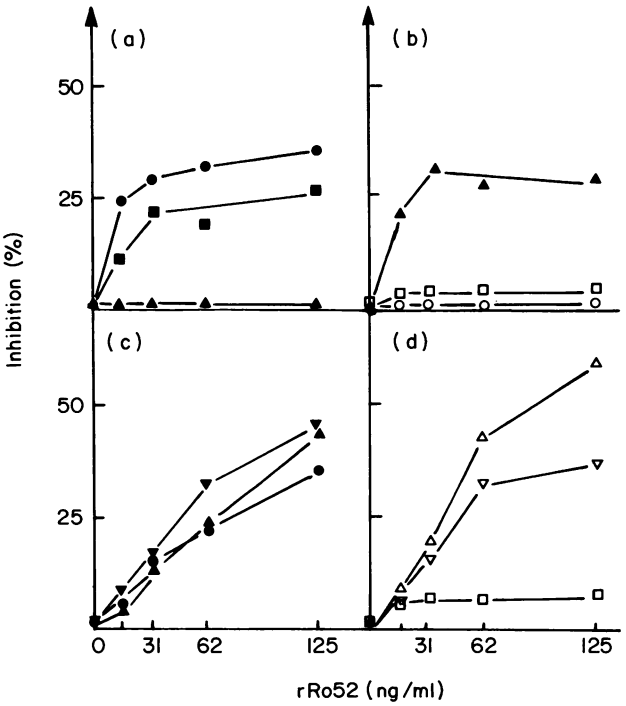


Fig. 4. Inhibition of the ELISA reaction between eight different primary Sjögren's syndrome (pSS) sera (●, ■, ▲, ▼, ○, □, △, ▽) and peptides 2–11 (a), 107–122 (b), 277–292 (c) and 365–382 (d) by increasing concentrations of rRo52. pSS sera were diluted 1:10000. Peptides 107–122 and 365–382 (1 μM) and 0·5 μM peptides 2–11 and 277–292 were used for coating plates.Only IgG antibodies were tested. Binding of normal human sera was insignificant.

K215 in the double sandwich assay while the protein is completely free when tested by ELISA inhibition experiment), or from individual affinities of rabbit anti-peptide antibodies used as probes.

Comparison of results obtained in CIE, immunoblotting and ELISA

For this comparison, sera from 20 SS patients were selected and tested in parallel in CIE with Ro and La antigens, Western blot with rRo52, direct ELISA with Ro60, rRo52, Ro52 synthetic peptides and La, and double-antibody sandwich ELISA with rRo52. In Table 4, sera were arranged according to their pattern of reactivity with rRo52 and Ro52 synthetic peptides in direct ELISA; the subsequent reactivities in the four groups of sera (five sera/group) are +/+, +/−, −/+ and −/− respectively. In this series of selected sera we found a complete agreement between the reactivity of sera with rRo52 in both ELISA and immunoblotting assays. Furthermore, among the 20 sera, seven reacted with rRo52 presented in double-antibody sandwich ELISA by rabbit antibodies against the C-terminal Ro52 peptide (residues 463–475). Interestingly, all sera positive in double-antibody sandwich ELISA were also positive in direct ELISA and Western blot with rRo52. Ten sera negative with rRo52 in double-antibody sandwich ELISA were also negative in direct ELISA and Western blot. Thus, in this series of sera, only three sera (Nos 5, 7 and 8) were found to react with immobilized rRo52 and not with native rRo52.

In the comparison involving Ro in CIE, rRo52 in Western blot, and Ro60 and rRo52 in ELISA, the data presented in Table 4 show that a better (but not complete) agreement is found when Ro60 rather than rRo52 in direct ELISA is compared with Ro in CIE. The reactivity of sera in immunoblotting is illustrated in Fig. 5. The reactivity of antisera induced in rabbits against the four peptides 2–11, 107–122, 277–292 and 365–382 is shown.

DISCUSSION

A number of studies have been devoted to the identification of cellular structures which may induce the specific production of antibodies in patients with systemic autoimmune diseases. Several previous reports have shown that certain autoantibody subsets recognize molecular complexes or proteins in their native form, whereas others react preferentially with the denatured forms of the protein. Although it has been shown that

Table 4. Data obtained in counter immunoelectrophoresis (CIE), immunoblotting assay and with Ro52 protein and Ro52 peptides in ELISA

Patient no*	CIE†		Immunoblot with rRo52‡	Direct ELISA§ with							Double antibody sandwich ELISA¶ with rRo52
						Ro52 synthetic peptides					
	Ro	La		Ro60	rRo52	2–11	107–122	277–292	365–382	La	
1	−	−	+++	+++	+++	+	−	+	+	+	++
2	+	+	++	+	+++	+	−	+	+	+	++
3	+	+	++++	++	+++	−	−	+	−	+	++
4	+	+	+	++	+++	++	−	−	−	−	++
5**	+	+	+++	++	++	+	−	+	+	−	−
6	−	−	+++	++	+	−	−	−	−	−	+
7	−	−	++	−	+++	−	−	−	−	−	−
8	−	−	+	−	++	−	−	−	−	−	−
9	−	−	++++	−	++	−	−	+	−	−	+
10	+	+	+++	++	++	−	−	−	−	+	+
11**	−	−	−	+	−	++	+	++	++	+	−
12**	−	−	−	−	−	+	−	+	++	−	−
13**	+	−	−	−	−	++	−	−	−	−	−
14**	+	+	−	+	−	+	−	−	−	−	−
15	−	−	−	−	−	+	−	+	+	−	−
16**	+	−	−	−	−	−	−	−	−	−	−
17**	−	−	−	−	−	−	−	−	−	−	−
18**	−	−	−	−	−	−	−	−	−	−	−
19	−	−	−	−	−	−	−	−	+	−	−
20	−	−	−	+	−	−	−	−	−	−	−

* Patients had a primary Sjögren's syndrome (pSS) except those marked (**) who had a Sjögren's syndrome (SS) associated with systemic lupus erythematosus (SLE).

† Patient sera were screened undiluted with bovine spleen extract and rabbit thymus extract as antigen source for Ro and La respectively.

‡ Patient sera were diluted 1:1000 and IgG antibodies only were tested. Intensity of reaction was evaluated by comparison with internal standards as − to ++++.

§ IgG autoantibodies measured in ELISA with antigens adsorbed on the plastic surface of microtitre plates and sera diluted 1:1000; −, OD 450 nm <0·30; +, 0·3–0·75; ++, OD 0·75–2.0; +++, OD ⩾2·0.

¶ Rabbit antiserum directed against peptide 463–475 of Ro52 was used at a 1:50 000 dilution as the first layer of antibody and patient sera diluted 1:1000 were used as the second layer of antibody.

some antibodies binding native complexes are able to inhibit function [30], it is not known whether these antibodies play a direct role in the pathogenesis of systemic diseases, and if so, whether they play a greater role than antibodies recognizing rather other states, somewhat denatured, of the polypeptides as they may be presented, for example, at the cell surface. Investigations aimed at identifying, with short protein fragments, epitopes recognized by autoantibodies can thus provide information dealing with both the mechanisms involved in the stimulation of antibody production and the type of structure that could be targeted by autoantibodies.

Ro52 is a constitutive protein of the Ro RNP particle which has been claimed to be a dominant target for antibodies in mothers with infants with complete heart block [3]. Furthermore, the presence of anti-Ro52 antibodies alone would be indicative of SS whereas the presence of anti-Ro60 alone without anti-Ro52 antibodies would be indicative of SLE [17,31]. Using a direct ELISA, we report that IgG antibodies reacting with rRo52 are present in the serum of a large number of patients with SS, while they are less frequent (10–25%) in SLE, RA, JCA and MCTD. Elevated levels of anti-Ro52 antibodies in SS were also found in sandwich and inhibition ELISA, i.e. assays which reveal antibodies to the native form of the antigen [16]. Among the 39 overlapping Ro52 synthetic peptides tested in this study, four were recognized by sera of patients with SS, namely peptides 2–11, 107–122, 277–292 and 365–382 (as well as peptide 107–126 to a lower extent). The latter peptides were recognized by patient antibodies in direct ELISA assay, in double-antibody sandwich ELISA or in ELISA inhibition assay where peptides were immobilized on the solid-phase or free in the liquid-phase. Peptide 365–382 is included in the Ro52 rfp domain also present in several nuclear proteins (Fig. 6). Peptide 277–292 is present in a larger region (residues 216–292) shown by Bozic *et al.* [21] to be essential for reactivity with autoantibodies. In the case of the study by Bozic *et al.*, however, these antibodies were predominantly from SLE patients. We found no antigenicity with patients' sera to peptides covering the leucine zipper domain (residues 211–232). Likewise, the peptides encompassing completely three of the four putative zinc-finger domains (peptides 12–35, 34–56 and 93–116) were not, or only rarely, recognized by antibodies of autoimmune patients. The observation that peptide 107–122

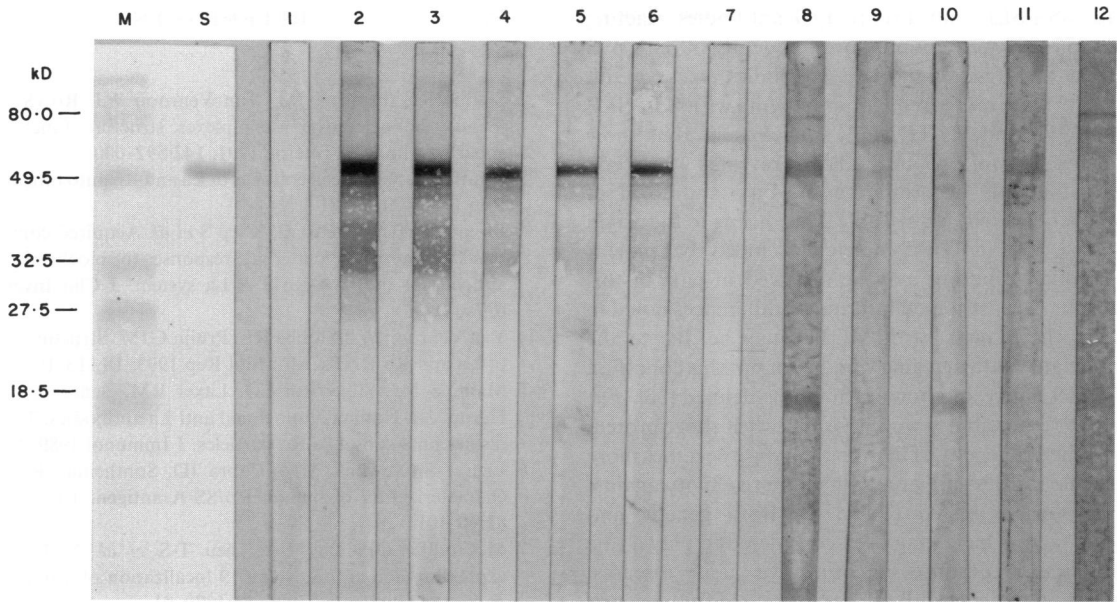
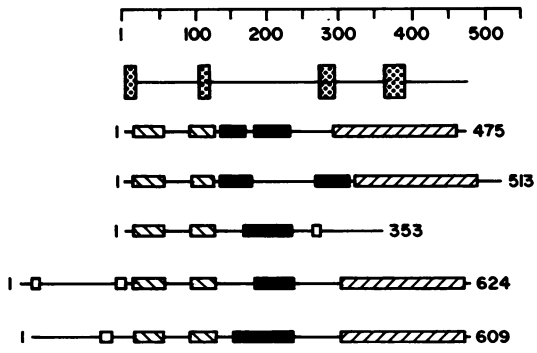


Fig. 5. Immunoblot analysis of primary Sjögren's syndrome (pSS) sera and rabbit antisera induced against the four peptides 2–11, 107–122, 277–292 and 365–382. rRo52 was used as antigen (S, rRo52 stained with Ponceau red) and only IgG antibodies were tested. M, prestained mol. wt markers (Biorad, ref 161-0305). The following strips were incubated with patients' sera diluted 1 : 1000 described in Table 4 : lane 1, NHS; lanes 2–4, sera from patients 1, 3 and 5 respectively; lane 5, serum from patient K215; lanes 6 and 7, sera from patients 10 and 19; lanes 8–11, antisera raised in rabbits against peptides 2–11, 107–122, 277–292 and 365–382 (dilution 1 : 500); lane 12, normal rabbit serum. The final reaction was revealed with GIBCO BRL NBT/BCIP-stable mix product as chromogenic substrate for the enzyme alkaline phosphatase.

could be recognized by patients' sera whereas the slightly longer peptide 107–126 was less well recognized, may be due to the fact that the longer peptide adopts a conformation that is different from that of the shorter peptide.

A number of sera contain antibodies reacting with Ro52 peptides and not with the parent protein. This observation has been described for other self proteins such as histones and protein D of Sm antigen [33,34]. Thus, according to the antigen used for their detection or according to the test used (solid- or liquid-phase assays), different antibody populations can be detected (Tables 3 and 4). In this study, however, we show that similar results are obtained when rRo52 is tested as an immobilized protein in ELISA or on Western blot. The lack of correlation of the results obtained in the latter assays with those obtained by CIE may be due to the absence of the Ro52 component in the test antigen, and to the fact that the routine test used Ro antigen extracted from bovine spleen, which is known to be less effectively recognized than human Ro [31,35].

Elevated levels of IgG antibodies reacting with peptide 2–11 were found in 65% of patients with SS associated with SLE (SS + SLE) and in 30% of patients with JCA, whereas the level of antibodies reacting with peptide 107–122 was increased in



Amino acid residues	Autoepitopes of Ro52	Zn fingers simil. (%)	Zn fingers ident. (%)	rfp domains simil. (%)	rfp domains ident. (%)
	Ro52				
	rfp	71·0	42·0	74·0	52·0
	rpt-1	71·0	49·0	–	–
	PwA33	64·5	35·0	72·5	48·0
	xnf7	61·4	37·0	71·0	45·0

Fig. 6. Schematic representation of the relationship between Ro52 and several other proteins. The putative structural and functional domains are shown as distinctive boxes: ▩, Zinc-finger motifs; ■, coiled coil domain; ■, ret-finger protein (rfp) domain; □, nuclear localization signal (NLS). In Ro52, a leucine zipper motif has been characterized in residues 211–232. Proteins compared with Ro52 are the human rfp, the mouse rpt 1 regulatory protein, Pw3, a nuclear protein of the loops of amphibian lampbrush chromosomes, and xnf7, a nuclear protein from Xenopus [32]. Alignment of proteins were made by M. Bellini (Pierre et Marie Curie University, Paris, France) with the program Clustal (Bysance-citi 2). To the right are given the overall identity and similarity of amino acids for each protein relative to Ro52 (zinc finger domain, residues 12–123 of Ro52; rfp domain, residues 290–465 of Ro52). Location of the four peptides 2–11, 107–122, 277–292, and 365–382 recognized by IgG antibodies from autoimmune patients is shown (▩).

patients with SS+SLE only (31%). IgG antibodies reacting with peptide 277–292 were found more frequently in both pSS (32%) and SS+SLE (54%), and IgG antibodies reacting with peptide 365–382 were more frequent in patients with SS+SLE (62%), JCA (39%) and MCTD (51%). The results thus show that the fine specificity of IgG antibodies as revealed with Ro52 peptides varied greatly according to the type and form of diseases and overall, as shown in Table 2, according to the origin of patients' sera. It cannot be completely excluded that patient selection, although random, may be involved in part in this intriguing result. At least some of the major differences noted in the results for the patients with MCTD may be due to the problems associated with agreeing whether or not this condition is a truly distinct entity and how it should be defined [36].

An alternative possibility would be that with short protein fragments, the heterogeneity of immune response becomes more detectable, while it is less apparent when the reactivity against the whole protein is tested (Table 2). Both genetic and environmental factors have long been considered to play a role in the autoimmune response in connective tissue diseases. Among Caucasian patients with pSS, an increased frequency of the human MHC antigens HLA-B8, Dw3, DR3 and sometimes DR2 has been found, and the presence of these haplotypes was reported to be strongly associated with the presence of autoantibodies against Ro antigen [37]. In contrast, Japanese anti-Ro-positive patients with pSS show higher frequencies of HLA-DRw8 and HLA-DRw52 [38], and Greek patients with pSS exhibit a high prevalence of HLA-DR5 [39]. Detailed studies of the patients' sera reactivity with Ro52 peptides in relation to HLA alleles expressed by these patients would help to clarify the present results. It will also be interesting to follow the possible association which may exist between clinical features, HLA alleles and the presence of particular peptide-reacting antibodies.

Because most evidence suggests that the anti-Ro response (as the immune response to several self molecular complexes) is antigen-driven, we have investigated whether the Ro52 peptides which are recognized by patients' sera encompassed regions of Ro52 that are accessible at the surface of Ro52 in solution. Our finding that the four domains 2–11, 107–122, 277–292 and 365–382 are indeed exposed in native Ro52 argues in favour of a mechanism in which Ro52 may be involved in the induction of antibodies. However, it is not known whether these regions are also accessible in the Ro RNP particle. Rabbit anti-peptide antibodies and affinity-purified human antibodies reacting with Ro52 peptides will represent valuable probes to target the Ro RNP particle in solution and map the location of particular domains of native Ro52.

ACKNOWLEDGMENTS

We thank Drs N. Abuaf, C. André, H. Maldyk, J. L. Pasquali, A. M. Prieur, M. Reichlin, R. S. Schwartz and W. J. Van Venrooij for providing patient sera, Ms J. Booker (St Thomas's Hospital, London, UK) for help with CIE experiments, and Dr M. Bellini (Pierre et Marie Curie University, Paris, France) for alignment of zinc finger proteins. We are grateful to Dr M. H. V. Van Regenmortel for his constant support and to Dr W. J. Van Venrooij for helpful comments on the manuscript. This work was supported in part by a grant from NATO (No 900515), from EEC (Human Capital and Mobility No ERB-CHRX-CT93-0176) and by Sanofi Diagnostics Pasteur.

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