

Routsias-JG; Sakarellos-Daitsiotis-M; Tsikaris-V; Sakarellos-C; Moutsopoulos-HM; Tzioufas-AG Δομικές, μοριακές και ανοσολογικές ιδιότητες των γραμμικών Β-επιτόπων του αυτοαντιγόνου Ro60kD. *Scan-J-Immunol.* 1998; 47:280-287

Σε ασθενείς με Συστηματικό Ερυθρηματώδη Λύκο (ΣΕΛ) και πρωτοπαθές Σύνδρομο Sjogren (πΣΣ) ανευρίσκονται σε υψηλή συχνότητα αντισώματα κατά της πρωτεΐνης Ro 60kD. Στη μελέτη αυτή μελετήθηκαν διεξοδικά οι αντιγονικές και δομικές ιδιότητες των δύο κύριων επίτοπων του αντιγόνου Ro60kD: TKYKQRNGWSHKDLLRSHLKP (169-190) και ELYKEKALSVE-TEKLLKYLEAV (211-232). Για το λόγο αυτό συνετέθησαν συνθετικά πεπτιδικά ανάλογα των δύο επιτόπων, ανάλογα της ομόλογης με τον 169-190 επίτοπο περιοχής των HLA DR1, DR3, DR5, DQ1 και DQ2 απλοτύπων και μια σειρά από εικοσιδύο 22-μερή πεπτίδια για κάθε επίτοπο όπου κάθε αμινοξύ υποκαταστάθηκε ανεξάρτητα από τα άλλα με αλανίνη. Με τη βοήθεια ενός μεγάλου αριθμού ορών από ασθενείς με ΣΕΛ και πΣΣ βρέθηκε ότι αντισώματα κατά των δύο επιτόπων ανευρίσκονται στο 45% των ασθενών, ενώ η αρχική τους ειδικότητα για νόσο διατηρείται. Εντοπίσθηκαν τα «σημαντικά» για την δέσμευση του αντισώματος αμινοξέα στα W¹⁷⁷, S¹⁷⁸, H¹⁷⁹, K¹⁸⁰, L¹⁸³, L¹⁸⁵ και S¹⁸⁶ για τον επίτοπο 169-190 και στα E²²³, L²²⁶, K²²⁷ και Y²²⁸ για τον επίτοπο 211-232. Η αντιγονικότητα του HLA DR3 πεπτιδίου RPDAEYWNSQKDLLEQKRGR βρέθηκε να είναι παρόμοια με αυτή του ομόλογου επιτόπου 169-190 του Ro60kD (κυρίως στους ΣΕΛ ορούς). Μελέτη των δομικών χαρακτηριστικών με κυκλικό διχρωισμό έδειξε ότι ο επίτοπος 211-232 του Ro 60kD παρουσιάζει χαρακτηριστικά α-έλικας ενώ ο επίτοπος 169-190 και το ομόλογο με αυτόν πεπτίδιο του HLA DR3, κατέχουν μικρότερο ποσοστό α-έλικας και μια δομή β-στροφής.

Τα αποτελέσματα αυτά υποδεικνύουν ότι η διαγνωστική αξία των επιτόπων του Ro60kD θα μπορούσε να αποδειχθεί χρήσιμη στη κλινική πράξη. Η εκτεθειμένη στα αυτοαντισώματα δομή του ομόλογου με τον επίτοπο 169-190 HLA DR3 πεπτιδίου προσφέρει έναυσμα για περαιτέρω μελέτη του ρόλου που παίζουν στη διέγερση της αυτοάνοσης αντίδρασης οι συσχετιζόμενοι με την αντί-Ro/SSA απόκριση HLA απλότυποι.

Structural, Molecular and Immunological Properties of Linear B-Cell Epitopes of Ro60KD Autoantigen

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Antibodies to Ro60KD protein are found with high frequency in sera from patients with systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS). Two major epitopes of the Ro60KD antigen, the TKYKQRNGWSHKDLLRSHLKP (169–190) and the ELYKEKALSVETEKLLKYLEAV (211–232), were synthesized and their antigenic and structural properties were studied. Using a large panel of SLE and pSS patients' sera, it was found that the anti-Ro60KD reactivity of both Ro60KD epitopes is rather limited ($\approx 45\%$), although they retain their original disease specificity. The epitope p.169–190 possessed sequence similarity with the peptide RPDAEYWNSQKDLLEQKRGR, shared in the β -chain of different HLA-DR molecules, among them the HLA-DR3 (which is associated with anti-Ro/Sjögren's syndrome A (SSA) response in patients with SLE). The antigenicity of the HLA-DR3 RPDAEYWNSQKDLLEQKRGR peptide was found to be similar to the 169–190 homologous Ro60KD epitope, recognized mainly by SLE sera. Structural studies showed that the 211–232 Ro60KD epitope exhibits pronounced helical characteristics, while the 169–190 epitope and the HLA-DR3 homologous peptide possess a somewhat lower percentage of α -helix. A β -folded structure was identified in the latter two peptides. Although the diagnostic value of the reported Ro60KD epitopes seems to be rather limited, correlations with other ribonucleoprotein epitopes (La/Sjögren's syndrome B, Ro52KD) may prove complementary to each other and valuable in clinical use. The ordered structure of the HLA-DR3 homologous peptide, exposed to the autoantibody binding, may offer an initiative in further investigation of the role of the HLA haplotypes, associated with the anti-Ro/SSA response, in the autoimmune stimulus.

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INTRODUCTION

Antibodies to Ro/SSA are usually found in sera from patients with systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS). These antibodies are directed against a ribonucleoprotein particle consisting of cytoplasmic RNAs (hyRNAs) in conjunction with a protein of 60KD molecular weight (Ro60KD). Another protein of 52KD molecular weight (Ro52KD) is also considered to participate in the complex via protein–protein interaction with the Ro60KD protein [1, 2]. The autoimmune response to Ro/SSA is characterized by a remarkable heterogeneity and a precise definition of the anti-Ro/SSA

specificity may provide potential insights into understanding the origin of these autoantibodies [3]. Diverse approaches have been used to map B-cell epitopes in Ro/Sjögren's syndrome A (SSA), including testing of autoimmune sera either on overlapping synthetic peptides in ELISA or with deletion mutants of recombinant proteins by Western blot or by immunoprecipitation [4–7].

In a previous study, using overlapping 22-mer peptides and the epitope scanning technique according to Geysen's method, we have identified two discrete linear epitopes of the Ro60KD protein, the TKYKQRNGWSHKDLLRSHLKP (169–190) and the ELYKEKALSVETEKLLKYLEAV (211–232), recognized by sera from SLE and pSS patients respectively. The minimal required

peptide length for optimal antibody recognition was also defined (N^{175} GWSHKDLLR¹⁸⁴ and K^{216} ALSVETEKLLKYLEAV²³²) [8]. These data are in agreement with recent results from other laboratories [9], which showed that monoclonal antibodies (MoAbs) against Ro60KD recognize distinct epitopes in the region between the RNP motif and the putative zinc finger domain (182–236). Other studies have also shown that sera from pSS patients react preferentially with epitopes located between residues 139–326 and 181–320 of the Ro60KD protein [7, 10].

Although the immunogenic stimulus which contributes to the generation of autoantibodies remains in most cases unknown, it is possible that peptides corresponding to antigenic epitopes may mimic short regions on the surface of macromolecular structures, rendering them accessible to the immune system and thus contributing to the generation of autoantibodies. Hence, using protein database analysis, it was shown [8] that the epitope spanning the sequence 175–184 presents molecular similarity with a sequence YWNSQKDLLQ which is found in the β -chain of a diverse array of HLA-DR molecules. Among them, HLA-DR3 is highly related to the anti-Ro/SSA response [11, 12].

In this report the molecular and biochemical characteristics of the TKYKQRNGWSHKDLLRSHLKP (169–190) and the ELYKEKALSVEKLLKYLEAV (211–232) Ro60KD synthetic peptide epitopes, as well as their diagnostic value for the detection of anti-Ro/SSA antibodies, are presented, using a large panel of sera from SLE and pSS patients.

MATERIALS AND METHODS

Patient sera and IgG purification. We tested 61 sera from patients with SLE and pSS for their antibody specificity, with counterimmunoelectrophoresis, immunoblot and a commercially available anti-Ro/SSA ELISA (Diastat anti-Ro/SSA: Shield Diagnostics, London, UK). All patients fulfilled the revised criteria for the classification of SLE [13] and pSS [14]. In all, 31 SLE and 30 pSS sera gave anti-Ro60KD positive reactions by all methods. For the alanine (Ala) substitution experiments, immunoglobulin (Ig)G from sera purified by protein-A sepharose column was concentrated at 200 μ g/ml, and dialyzed against phosphate-buffered saline (PBS), pH 7.3. Concentrations of IgG were evaluated by radial immunodiffusion (Boehringer-Mannheim, Mannheim, Germany).

Purified human IgG with anti-p.216–232 and anti-p.175–184 activity was obtained after passage of the whole IgG fraction through CNBr-activated sepharose 4B columns covalently attached to the KALSVEKLLKYLEAV and NGWSHKDLLR peptides, respectively [8]. The columns were washed with PBS, pH 7.2, and after elution with HCl-Gly, pH 2.7, the specific IgG was neutralized, concentrated at 20 μ g/ml and dialyzed against PBS. The IgG concentration was measured by radial immunodiffusion (Boehringer-Mannheim).

Peptide synthesis: pin-bound Ala-substituted peptide epitopes. All 22-mer peptide analogues were prepared in duplicate according to the method of Geysen *et al.* [15] for each of the Ro60KD linear epitopes previously described [8]. Each residue of the Ro60KD epitopes was individually replaced by alanine. The synthesis was performed on prederivatized polyethylene pins [16, 17] and the protocols used were based on the principles of the solid-phase peptide synthesis of Merrifield [18], using the N^α -fluorenylmethoxycarbonyl (Fmoc) protecting group

strategy [19, 20]. For the accurate dispensing of different solutions at the coupling stages, self-made computer software was used. In each synthesis cycle two control peptides, VRLRWNPADYGGIKKIRL and VRLRWAPAAAYGGIKKIRL, were synthesized. The first one was specifically recognized by anti-VRLRWNPADYGGIKKIRL MoAbs, while the second one did not react.

Peptide synthesis: pin-bound HLA-DR sequences homologous to the Ro60KD (169–190) epitope. All five 22-mer peptides representing the Ro/SSA homologous regions of the HLA-DR1, -DR3, -DR5, -DQ1 and -DQ2 were synthesized in duplicate on prederivatized polyethylene pins following the preceding protocols [15–20].

Peptide synthesis: soluble peptides. Peptides corresponding to the full length of the Ro60KD epitopes TKYKQRNGWSHKDLLRSHLKP (169–190) and ELYKEKALSVEKLLKYLEAV (211–232), as well as to the HLA-DR3 RPDAEYWNSQKDLLQKRGR homologous to the Ro60KD (169–190) epitope, were synthesized by stepwise solid-phase synthesis (SPPS) on a phenylacetamidomethyl (PAM) resin [21, 22]. We carried out N^α -Boc/Bzl side-chain protection (Boc, *t*-butyloxycarbonyl; Bzl, benzyl) by standard methods. Histidine was introduced as N^α -Boc-L-His Tos (Tos, *p*-toluenesulphonyl) and lysine as N^α -Boc-L-Lys Fmoc. All protected amino acids were coupled using a molar ratio of 3:3:3:1 for amino acid:1-hydroxybenzotriazole: N,N' -dicyclohexylcarbodiimide:resin. Completion of the coupling reactions was ensured by the use of the ninhydrin test [21, 23]. Deprotection of the N^α -t-Boc protecting groups was performed using trifluoroacetic acid (TFA) followed by diisopropylethylamine (DIPEA) for neutralization. After synthesis, the peptides were cleaved from the resin with anhydrous hydrogen fluoride (HF) in the presence of anisole and phenol (10% v/v) as scavengers at 0°C for 1 h. The crude peptides, extracted from the resin with 2 M acetic acid, were lyophilized and purified using preparative high performance liquid chromatography (HPLC) on a C-18 column [24]. A programmed gradient elution with the following solvents was used: (A) H₂O/0.1% TFA; (B) CH₃CN/0.1% TFA (CH₃CN: acetonitrile) (Fig. 1). The purity of the peptides was confirmed by analytical HPLC and one- and two-dimensional (1D and 2D) proton nuclear magnetic resonance spectroscopy (¹H-NMR).

Peptide synthesis: biotinylated soluble peptide. The peptide corresponding to the restricted length of the Ro60KD epitope KALSVEKLLKYLEAV (216–232), elongated by an additional tetrapeptide spacer SGSG from the N-terminus end, was also prepared by the SPPS

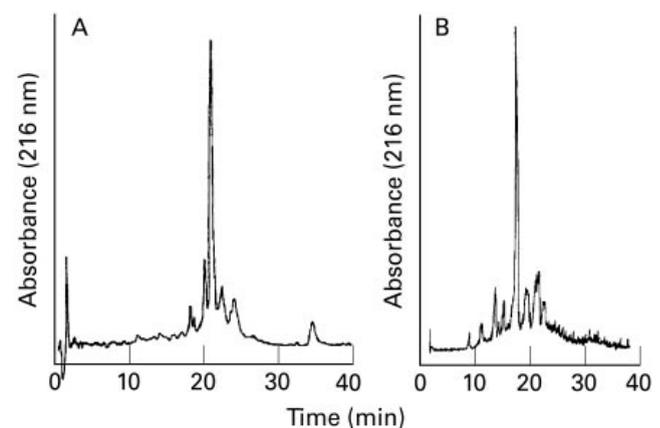


Fig. 1. Analytical HPLC of the TKYKQRNGWSHKDLLRSHLKP (169–190) epitope (A) and the ELYKEKALSVEKLLKYLEAV (211–232) epitope (B) on a reverse-phase C-18 column.

previously described [21]. Biotinylation of the peptide was carried out on the resin by coupling d-biotine to the N-terminus amino group, after deprotection by Boc, following the standard solid-phase coupling procedure [25]. The biotinylated peptide was cleaved from the resin using the HF protocol and purified by preparative HPLC, (A) H₂O/0.1% TFA; (B) CH₃CN/0.1% TFA. The homogeneity of the peptide was confirmed by analytical HPLC and 1D and 2D ¹H-NMR spectroscopy.

Soluble peptides anchored to a sequential oligopeptide carrier. The peptide TKYKQRNGWSHKDLLRSHLKP corresponding to the 169–190 epitope sequence of the Ro60KD protein was coupled to the (Lys-Aib-Gly)₂ sequential oligopeptide carrier (SOC₂) from the Lys-N^εH₂ groups (for details see Refs 26–28). The obtained dimeric compound was dialyzed against water using dialysis tubes with a molecular mass cut-off of ≈1500 (Sigma Chemicals, St Louis, MO, USA). The purity of the peptide was confirmed by analytical HPLC and amino acid analysis.

ELISA: pin-bound peptides. Peptides covalently attached to polyethylene rods were tested for antibody binding by ELISA in 96-well microtitre plates. Rods were immersed in sodium phosphate buffer (PBS), pH 7.2, containing 0.1% Tween 20, 2% albumin to block non-specific binding. In all, nine affinity-purified IgG (five from pSS and four from SLE sera) were diluted with the preceding buffer at a concentration of 200 µg/ml, added to the wells and incubated overnight at 4°C. After washing with PBS containing 0.1% Tween 20, anti-human IgG conjugated to horseradish peroxidase (1:1000 dilution in blocking buffer) was added and incubated for 1 h at 20°C. The rods were again washed and the presence of antibodies was detected using a substrate solution of 2,2' azino-cis3-ethylbenzothiazoline sulphonic acid (ABTS) and the absorbance of the colour was measured at 405 nm. Subsequently, the bound antibodies were removed from the rods by sonication for 30 min in a water bath with 0.1 M sodium dihydrogen phosphate, 1% sodium dodecyl sulphate (SDS) and 0.1% 2-mercaptoethanol at 60°C, and the rods were used again or dried for storage. In order to determine whether the antibodies from the previous experiment were completely dissociated from the peptides, IgG-free rods were incubated with anti-human IgG conjugated to horseradish peroxidase followed by the addition of substrate solution (ABTS). In all cases the final optical density (OD) was equal to the background.

ELISA: soluble peptides. Microtitre polystyrene plates (Nunc, DK4000 Roskilde, Denmark) were coated with 100 µl of peptide solution (5 µg/ml) and kept at 37°C for 4 h, allowing the solution to evaporate to dryness. In the case of biotinylated peptides, the wells were pretreated with 100 µl of 5 µg/ml streptavidine. After blocking the remaining binding sites with 2% bovine serum albumin (BSA) and 0.1% Tween 20 in PBS at room temperature for 1 h, the plates were incubated with diluted sera (1:50 in blocking buffer) overnight at 4°C. The plates containing non-biotinylated peptides were washed and treated as previously described for the pin-bound peptides.

Biotinylated peptide solutions (5 µg/ml) were added in the wells and incubated for 1 h at room temperature. After three washes with PBS/0.1% Tween 20, sera were added in dilution 1:125 in 2% BSA/PBS (100 µl per well). This dilution was selected after preliminary experiments, because it was considered as the best discriminatory point between positive and negative controls (average OD value of positive, 1.20 nm; average OD value of normal, 0.10 nm; OD value using only streptavidine, background 0.10 nm). After incubation for 1 h and three washes with PBS/0.1% Tween 20, we added horseradish peroxidase conjugated to anti-human IgG, diluted 1:1000 in 2% BSA/PBS, per well. Following incubation for 1 h at room temperature and three washes, 50 µl ABTS substrate was added and the reaction was read at 405 nm after 20 min. The background of each individual serum was determined

in parallel experiments in streptavidine-pretreated peptide-free ELISA plates.

In all ELISA experiments using soluble-free, soluble biotinylated or pin-bound peptides, positive reactions were considered to be those that gave OD greater than the mean OD of the negative control plus three standard deviations (SD).

Circular Dichroism (CD) experiments and modelling. All CD spectra were measured on a spectropolarimeter computer assisted under constant nitrogen flash. Data were expressed as mean residue ellipticity [8]. The concentrations of the peptide solutions were taken from weighed amounts and 8.0–8.5 × 10⁻⁴ solutions were used for all experiments.

The coordinates of the HLA-DR1 heterodimer [29] were obtained from the Protein Database Brookhaven (PDB). Modelling was performed on a Silicon Graphics Indy workstation using the program Insight II (Biosym Technologies, San Diego, CA, USA). Conformations of the substituted amino acids in the HLA-DR1 homologous sequence were selected from a library of rotamers using the appropriate rotamer for each case and the energy minimization was also obtained for each substituted residue. The energy minimization of the Ro/SSA peptide model was received using the Discover program (Biosym Technologies).

Animal immunizations. New Zealand white rabbits, two for each peptide used, were immunized by subcutaneous injection with peptides NGWSHKDLLR (175–184) and KALSVEKLLKYLEAV (216–232). The rabbits received 500 µg of peptide in complete Freund's adjuvant (1:1) on day 0 and in incomplete Freund's adjuvant (1:1) on days 14 and 28, and they were bled on day 35. The antisera were used without purification. We used ELISA assays as previously described to test the antisera for their ability to recognize the Ro60KD peptides.

RESULTS

Alanine substitutions

In order to define the antigenic role of each amino acid in the Ro60KD epitopes, all residues were individually replaced by alanine and the obtained pin-bound peptides were tested (Fig. 2) against affinity-purified IgG from five pSS and four SLE patients' sera with anti-Ro60KD reactivity. All IgG fractions were purified by affinity chromatography using the synthetic epitopes p.175–184 and p.216–232. Alanine was selected because of its low antigenicity and small stereochemical volume. Substitution of E²²³, L²²⁶, K²²⁷ and Y²²⁸ by alanine resulted in significant loss of antigenicity of the epitope ELY-KEKALSVEKLLKYLEAV (211–232). The antibody-binding capacity of the TKYKQRNGWSHKDLLRSHLKP (169–190) was slightly affected when W¹⁷⁷, S¹⁷⁸, H¹⁷⁹, K¹⁸⁰, L¹⁸³, L¹⁸⁵ and S¹⁸⁶ were substituted by alanine.

Anti-Ro60KD reactivity of the HLA-DR sequences, homologous to the Ro60KD (169–190) epitope

The cross-reactivity of the autoantibodies directed against the 169–190 epitope of Ro60KD with the HLA-DR haplotypes [11, 12] which contained the Ro60KD-mimicking molecules was investigated. Hence the HLA-DR1, -DR3, -DR5, -DQ2 and -DQ1 sequences were prepared and tested (Fig. 3). The DR1 and DR3 homologous sequences showed anti-Ro60KD reactivity

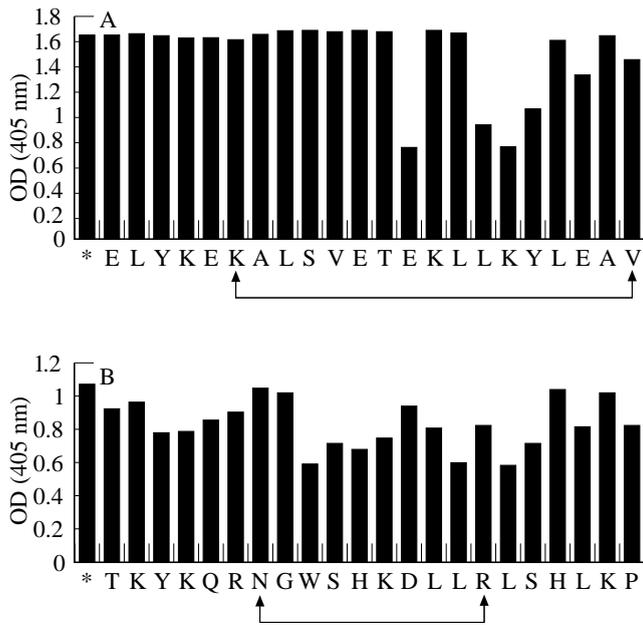


Fig. 2. ELISA of the pin-bound Ala-substituted peptide epitopes. (A) Affinity-purified IgG from anti-KALSVE... and anti-Ro60KD positive pSS sera; (B) Affinity-purified IgG from anti-NGWSHKDLLR (175–184) and anti-Ro60KD positive SLE sera. Each column represents the average OD values. On the horizontal axis is given the amino acid substituted each time by alanine. Arrows show the restricted epitopes. The first column (*) shows the average of each epitope, (A) 211–232 and (B) 169–190.

equal to that of the 169–190 epitope. These DR molecules share a local identity with the 169–190 epitope comprising residues W¹⁷⁷, S¹⁷⁸ and the sequence KDLL (180–183). In contrast the DR5, DQ2 and DQ1 sequences did not react with the anti-Ro60KD IgG. The major difference between reacting and non-reacting sequences was that in the former, the KDLL alignment was present (see lower panel of Fig. 3).

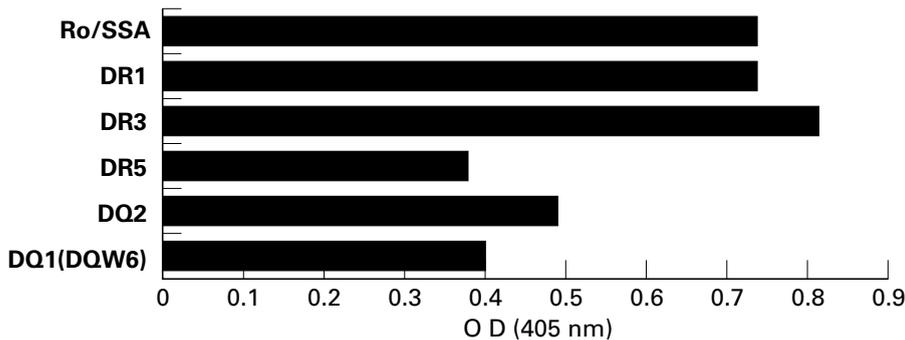


Fig. 3. Anti-Ro60KD reactivity of the HLA-DR sequences. Top, anti-Ro60KD reactivity of the pin-bound 169–190 Ro60KD epitope, the HLA-DR1, -DR3, -DR5, -DQ2 and -DQ1 sequences. This experiment has been performed using affinity-purified anti-NGWSHKDLLR IgG. Bottom, shared local sequence similarities of the 169–190 Ro60KD epitope, the HLA-DR1, -DR3, -DR5, -DQ2 and -DQ1.

Structural characteristics of the Ro60KD epitopes and the HLA-DR3 homologous peptide

The CD spectra of the TKYKQRNGWSHKDLLRSHLKP (169–190), ELYKEKALSVEKLLKYLEAV (211–232) and HLA-DR3 RPDAEYWNSQKDLLEQKRGR homologous to the 169–190 peptide are given in 4. All three peptides showed two negative bands around 200 and 220 nm typical of a random coil in water. In a mixture of 50:50 trifluoroethanol (TFE):H₂O the peptides adopt helical conformation, as can be evaluated from the negative bands at ≈220 (nπ* transition) and ≈208 nm, as well as from the positive one (ππ* transition) at ≈190 nm [30, 31]. According to Greenfield & Fasman’s analysis [32] the helix content deduced from the molar ellipticity at 208 nm was estimated at 33% for the 211–232 peptide and around 23% for the other two. Comparable helical conformations exhibited the Ro60KD epitopes and the HLA-DR3 homologous peptide in an amphiphilic environment (10 mM SDS), which can be roughly considered as a micellar medium [33]. The higher intensity of the 208 nm band compared to that of the 220 nm observed for the peptide 169–190 and the homologous HLA-DR3 peptide can be attributed to the contribution of a type III β-turn [34].

Modelling studies based on the X-ray structure of the HLA-DR1 showed the presence of a folded structure (Fig. 5) around the KDLL sequence of the Ro60KD epitope (169–190) and the HLA-DR1 homologous region. The calculated values of the φ and ψ angles [35] for the peptide backbone of the Ro60KD KDLL sequence are in good approximation with those obtained for the homologous sequence of the HLA-DR3. In fact, the φ and ψ values of the D and L residues are very similar to those obtained for a type III β-turn (φ, –60°, –30°; ψ, –60°, –30°) [36].

Reactivity of antisera to the NGWSHKDLLR (175–184) and KALSVEKLLKYLEAV (216–232) peptide epitopes

The reactivity of the rabbit antisera to the peptides 175–184 and 216–232 was evaluated by ELISA using as antigens the 175–184

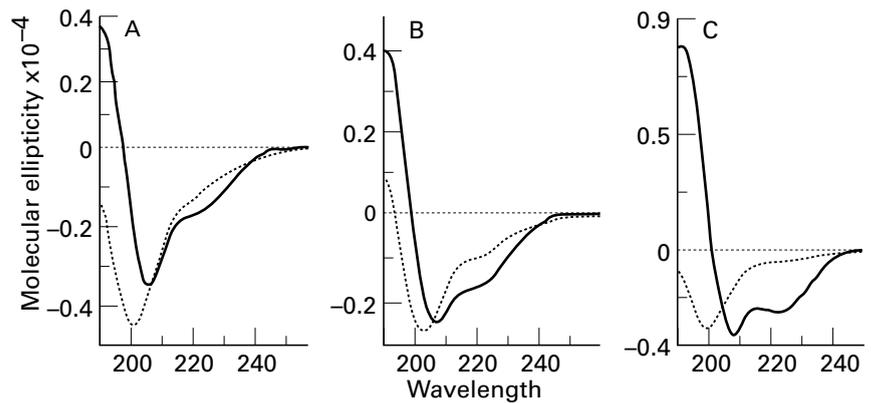


Fig. 4. Circular dichroism spectra of (A) TKYKQRNGWSHKDLLRSHLKP (169–190); (B) ELYKEKALSVEKTEKLLKYLEAV (211–232); and (C) HLA-DR3 RPDAEYWNSQKDLLEQKRGR in H₂O (---) and 50:50 TFE/H₂O (—). Concentration 8.0–8.5 × 10⁻⁴ M.

and 216–232 soluble peptide epitopes. Antisera obtained from immunized rabbits with the 175–184 peptide and tested in dilution 1:100 recognized only the homologous immunizing peptide 175–184 (OD values: Rabbit 1, 0.70 nm; Rabbit 2, 0.60 nm; control rabbit, 0.05 nm). These antisera did not cross-react with the 216–232 peptide (OD values: Rabbit 1, 0.03 nm; Rabbit 2, 0.05 nm). Antisera from the animals immunized with the 216–232 peptide recognized the 216–232 sequence (OD values: Rabbit 1, 0.80 nm; Rabbit 2, 0.75 nm; control rabbit 0.03 nm), while they did not react with the 175–184 sequence (OD values: Rabbit 1, 0.02 nm; Rabbit 2, 0.03 nm). None of the antisera obtained showed non-specific binding.

Prevalence of anti-peptide antibodies in autoimmune sera

Using the soluble synthetic epitopes of the Ro60KD protein in ELISA, the prevalence of anti-peptide antibodies in SLE and pSS sera was investigated (Fig. 6). Antibodies to TKYKQRNGWSHKDLLRSHLKP (169–190) were found in 17 out of 31 SLE sera (55%) and 10 out of 30 pSS (33%) sera with anti-Ro60KD reactivity. This epitope (169–190) was

recognized by 27 out of 61 anti-Ro60KD positive sera (44%). Antibodies to ELYKEKALSVEKTEKLLKYLEAV (211–232) were detected in 11 out of 31 SLE sera (35%) and 16 out of 30 pSS (53%) sera. The 211–232 epitope was recognized by 27 out of 61 anti-Ro60KD positive sera (44%).

Antibodies to the biotinylated KALSVEKTEKLLKYLEAV (216–232) epitope were detected in two out of 31 SLE sera (6%) and nine out of 30 pSS sera (30%). This epitope was recognized by 11 out of 61 anti-Ro60KD positive sera (18%). The TKYKQRNGWSHKDLLRSHLKP (169–190) epitope conjugated to the synthetic carrier SOC₂ was also tested in ELISA experiments. All individual steps for this ELISA were the same as for the ELISA for soluble-free peptides. The same sera were tested against the SOC₂ backbone carrier alone and the background OD was subtracted from the sample OD. It was found that 11 out of 29 SLE sera (38%) and 17 out of 27 pSS sera (63%) are recognized by the dimeric form of the 169–190 epitope, which also gave a positive anti-Ro60KD reaction in 28 out of 56 sera (50%). No correlation was found between anti-peptide antibodies and any particular clinical or serologic feature in the patient population studied.

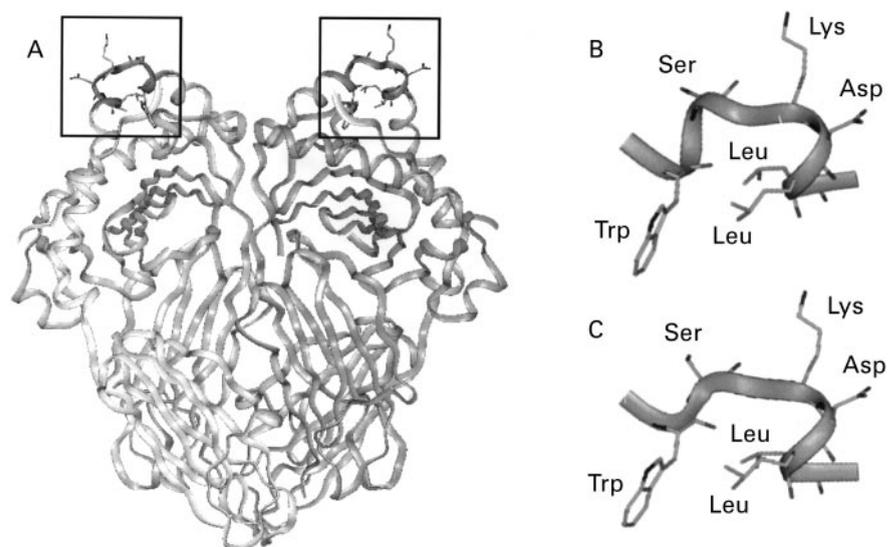


Fig. 5. Stereoviews of (A) the HLA-DR1 dimer; (B) the HLA-DR1 homologous to the 169–190 Ro60KD epitope; and (C) the Ro60KD common sequence.

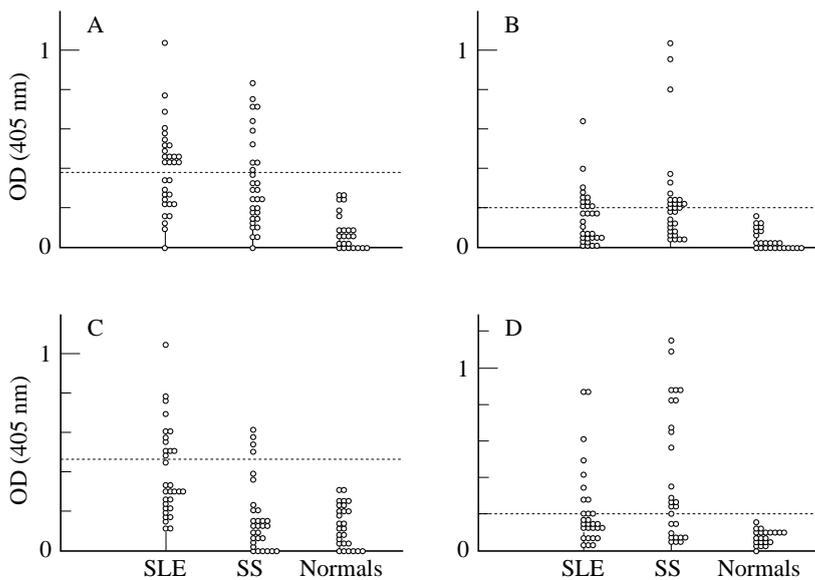


Fig. 6. Prevalence of anti-peptide antibodies to (A) TKYKQRNGWSHKDLLRSHLKP (169–190), (B) ELYKEKALSVEKLLKYLEAV (211–232), (C) HLA-DR3 RPDAEYWNSQKDLLEQKRGR, and (D) TKYKQRNGWSHKDLLRSHLKP coupled to SOC₂ synthetic peptides in sera from patients with SLE and pSS with anti-Ro60KD antibodies. The dotted line represents the cut-off point, set by the mean OD of the normals plus three standard deviations.

The HLA-DR3 RPDAEYWNSQKDLLEQKRGR peptide homologous to the 169–190 epitope of the Ro60KD autoantigen was recognized by 12 out of 31 SLE sera (39%) and four out of 30 pSS sera (13%). Statistical analysis of the binding levels of the SLE and pSS sera to the HLA-DR3 peptide showed a significant preference of the SLE sera ($t = 4.28$, $P = 3.5 \times 10^{-5}$).

In all the reported ELISA assays 25 normal sera negative for anti-Ro60KD antibodies were used as negative controls.

DISCUSSION

The identification of B-cell epitopes of autoantigens can provide information both on the mechanisms involved in the stimulation of autoantibody production and the nature of structures that could be targeted by autoantibodies. The Ro60KD autoantigen has attracted considerable research interest over several years since a significant proportion of autoimmune sera contain antibodies directed against it. In an effort to understand the molecular, biochemical and immunological properties of the protein we have synthesized and studied the major epitopes [8] of this autoantigen as well as an HLA-DR3 fragment homologous to the Ro60KD epitope (169–190).

Using a large panel of SLE and pSS anti-Ro60KD positive sera, the prevalence of anti-peptide antibodies was investigated. It was found that the TKYKQRNGWSHKDLLRSHLKP (169–190) epitope is mainly recognized by SLE sera (55%), while the ELYKEKALSVEKLLKYLEAV (211–232) epitope is preferentially recognized by pSS sera (53%). These findings indicate a rather limited disease preference of each synthetic epitope and are in agreement with our previous results of the Ro60KD epitope mapping [8]. However, the sensitivity of both epitopes for the detection of anti-Ro60KD antibodies remains limited (44%). When the 169–190 epitope was coupled to a synthetic soluble carrier (SOC₂) the anti-Ro60KD reactivity was slightly enhanced (50%), while its disease specificity decreased

(38%). On the other hand, antisera obtained from animals immunized with the NGWSHKDLLR (175–184) and KALSVE-TEKLLKYLEAV (216–232) peptides (restricted epitopes) showed almost 100% reactivity against the full-length epitopes (p.169–190 and p.212–232, respectively).

The alanine substitutions showed that the residues crucial for the antibody binding of the 211–232 Ro60KD epitope are within the 216–232 region, which was identified as the minimal required peptide length for optimal antibody recognition. In the case of the 169–190 Ro60KD epitope, residues significant for the antibody recognition (W¹⁷⁷, S¹⁷⁸, H¹⁷⁹, K¹⁸⁰ and L¹⁸³) are within the restricted epitope and are also homologous (except H¹⁷⁹) to the HLA-DR sequences. Residues L¹⁸⁵ and S¹⁸⁶, which also seem to play a role in the binding, are not involved in the restricted length (175–184), and this is possibly one of the reasons for the limited antibody recognition of the restricted epitope. Nevertheless, none of the reported substitutions resulted in a complete loss of the epitope antigenicity.

The most prominent conclusion which can be extracted from the presented data is that sera from SLE and pSS patients contain antibodies against conformational, rather than linear, epitopes. In fact, the majority of anti-Ro60KD positive sera do not react with the synthetic epitope analogues. Given that anti-Ro60KD antibodies are more frequently directed against conformational epitopes [37], it is possible that the sequences 169–190 and 211–232 belong to a conformational Ro60KD epitope, retaining a part of its reactivity when tested separately. On the other hand, the possibility that the peptides may have a different conformation, when bound to the antibody, cannot be excluded. It should be noted, however, that different lengths of the same peptides tested against the same IgG with anti-Ro60 activity present almost the same reactivity [8].

The similarities of the anti-Ro60KD reactivity of the pin-bound DR1 and DR3 to the 169–190 Ro60KD epitope, compared to the pin-bound DR5, DQ2 and DQ1, could be attributed

to the common shared amino acid sequence W¹⁷⁷, S¹⁷⁸ and KDLL (180–183). The same holds true for the soluble DR3 peptide, which is mainly recognized by SLE positive sera. Nevertheless, direct evidence of HLA-DR3/Ro60 cross-reaction can be demonstrated by binding of affinity-purified anti-p.169–190 antibodies in HLA-DR3 positive cells.

Conformational studies of both Ro60KD epitopes and the DR3 homologous peptide, using CD spectroscopy, showed a pronounced helical structure for the 211–232 soluble epitope (33%) and a lower helical content for the other two peptides (23%). These results are in agreement with the computer-predicted secondary structure for the Ro60KD protein [8, 38]. They also indicate a rather common structural character for the 169–190 Ro60KD epitope and its homologous HLA-DR3 sequence, which was further substantiated by the modelling studies. The KDLL homologous sequences together with the residues W and S are probably localized at a highly exposed part of the corresponding proteins, accessible to the antibody binding. In this segment (60–69), hydrophilic residues S, K and D are directed towards the outer surface of the protein, while hydrophobic residues W, L and L proceed to the interior, thus stabilizing the folded structure by hydrophobic interactions. In this regard, it is more likely that the KDLL sequence, shared in all these reactive peptides, plays a significant role for antibody binding via peptide conformation rather than the primary sequence.

Molecular mimicry between HLA molecules and autoantigens is not known. It should be noted, however, that the NZB.H-2 mice with a mutation in MHC class II β -chain (I-A β bm12 mutation), which involves the residues 68, 71 and 72 of I-A β -chain, presented high titres of anti-DNA antibodies, in contrast to the wild-type NZB-H-2b and NZB-H-2d mice, which do not possess anti-DNA reactivity in their sera. The authors suggest that mutations of mice MHC β -chain in the region 66–72, when superimposed on an appropriate genetic background, may significantly influence the immune response towards autoantibody production [39]. The molecular similarity between human MHC β -chain and Ro60 presented in our study, involves the region 63–72 of the human MHC β -chain. Although the shared residues are not identical, as for the bm12 mutation, it still remains to be elucidated whether patients with antipeptide antibodies and HLA-DR3 present alterations in this region of HLA-DR3 β -chain. Nonetheless, a number of studies have shown that antibodies against exposed regions of the MHC can activate B cells or macrophages through dimerization and cross-linking of these molecules [40, 41]. In fact, recent reports have demonstrated that cross-linking of HLA-DR antigens on small, resting B cells induces a rapid expression of the B 7–1 costimulatory molecule with subsequent activation of allogenic CD4 T cells via the CD28 molecule [42].

In conclusion, the TKYKQRNGWSHKDLLRSHLKP (169–190) and the ELYKEKALSVETEKLLKYLEAV (216–232) Ro60KD synthetic epitopes exhibit a rather limited anti-Ro60KD reactivity, either due to a particular heterogenous and complex immune response, or due to their participation as parts

of a discontinuous epitope [37]. The antigenic similarity of the HLA-DR3 RPDAEYWNSQKDLLEQKRGR peptide with the homologous 169–190 Ro60KD epitope deserves emphasis since systemic autoimmune diseases are highly associated with this HLA class II (HLA-DR3) alloantigen.

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