Tzioufas-AG; Yiannaki-E; Sakarellos-Daitsiotis-M; **Routsias-JG**; Sakarellos-C; Moutsopoulos-HM ȸəʤɔɑɡ Ùʤɟ ŰŮəɟʤəŰɜ ɨɡ űɥɝ Űűɨɣɥɝ űɥɝ ŭɞűɟ ŭɝűəŰɥ ɜɏűɥɝ **έναντι του La/SSB: χαρτογράφηση επιτόπων και χαρακτηρισμός τους.**

Clin-Exp-Immunol. 1997; 108; 191-198

Σε ασθενείς με Συστηματικό Ερυθηματώδη Λύκο (ΣΕΛ) και πρωτοπαθές Σύνδρομο Sjogren (πΣΣ) ανευρίσκονται σε υψηλή συχνότητα αντισώματα κατά της πρωτεΐνης La/SSB 48kD. Στη μελέτη αυτή παρουσιάζεται η λεπτομερής αντιγονική χαρτογράφηση της La/SSB πρωτεΐνης. Για το σκοπό αυτό συνετέθησαν 20μερή συνθετικά πεπτίδια που κάλυπταν, με αλληλοεπικάλυψη οκτώ αμινοξέων, την πλήρη αλληλουχία της πρωτεΐνης. Αντισώματα τάξεως IgG απομονώθηκαν από ορούς πέντε ασθενών με ΣΕΛ και τεσσάρων ασθενών με πΣΣ και ελέγχθηκαν ενάντια στα εφιππεύοντα συνθετικά πεπτίδια. Τα πεπτίδια που εμφάνισαν υψηλή αντιδραστικότητα με την κεκαθαρμένη IgG βρέθηκαν να κατέχουν τις αλληλουχίες 145-164, 289-308, 301-320 και 349-368 της πρωτεϊνης La/SSB. Ο προσδιορισμός του ελάχιστου μήκους των αντιγονικών καθοριστών που επέτρεπε την αναγνώριση από τα αντισώματα αποκάλυψε την ακριβή εντόπιση των αντίστοιχων επιτόπων: ^{145}HKAFKGSI^{154}, ^{291}NGNLQLRNKEVT^{302}, ^{301}VTWEVLEGEVEKEAL-KKI^{318}, και ^{349}GSGKGKVQFQGKKTKF^{364}. Εξετάσθηκε η ομολογία πρωτοταγούς δομής των επιτόπων με τη βοήθεια βάσεων δεδομένων πρωτεϊνών. Ο επίτοπος ^{145}HKAFKGSI^{154} παρουσιάζει 83.3% ομολογία με το ^{139}HKGFKGVD^{146} τμήμα της βασικής πρωτεΐνης της μυελίνης (MBP) και 72% ομολογία με την περιοχή YKNFKGTI της ανθρώπινης DNA πολυμεράσης II. Συνθετικά πεπτίδια με τις παραπάνω αλληλουχίες βρέθηκαν να αντιδρούν διασταυρωτά με τα αντισώματα αντι-La/SSB. Ελέγθηκαν 63 οροί με αντι-La/SSB αντισώματα από ασθενείς με πΣΣ ή ΣΕΛ, 35 οροί με χωρίς αντι-La/SSB αντισώματα από ασθενείς με πΣΣ ή ΣΕΛ και 41 οροί από υγιείς αιμοδότες όμοιας ηλικίας και φύλου, ενάντια σε βιοτινυλιωμένα συνθετικά πεπτιδικά ανάλογα. Τα αντι-La/SSB ανιχνεύθηκαν σε διαφορετικές συχνότητες για κάθε επίτοπο και κυμαίνονταν από 20% για τον επίτοπο ^{145}HKAFKGSI^{154} έως 100% για τον επίτοπο ^{349}GSGKGKVQFQGKKTKF^{364}. Η ολική ευαισθησία και ειδικότητα των τεχνικών ELISA βρέθηκε να είναι 93.6% και 85.6% αντίστοιχα.

Συμπερασματικά τα αντί-La/SSB αυτοαντισώματα αποτελούν ένα ετερόλογο πληθυσμό ο οποίος στοχεύει διαφορετικούς γραμμικούς B-επιτόπους στη La/SSB πρωτεΐνη. Ο επίτοπος ^{145}HKAFKGSI^{154} εμφανίζει μοριακή - αντιγονική ομοιότητα με τμήματα δυο άλλων αυτοαντιγόνων: της ανθρώπινης MBP και DNA τοποισομεράσης II. Τέλος τα συνθετικά πεπτιδικά ανάλογα μπορούν να χρησιμοποιηθούν με υψηλή ευαισθησία και ειδικότητα για την ανίχνευση των αντι-La/SSB αντισωμάτων.

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Fine specificity of autoantibodies to La/SSB: epitope mapping, and characterization

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SUMMARY

The B cell epitope mapping of La/SSB was performed using 20 mer synthetic peptides overlapping by eight amino acids covering the whole sequence of the protein. IgG, purified from sera of five patients with systemic lupus erythematosus (SLE) and four sera from patients with primary Sjögren's syndrome (pSS) were tested against the overlapping synthetic peptides. Peptides highly reactive with purified IgG were those spanning the regions 145–164, 289–308, 301–320 and 349–368 of the La protein. Determination of the minimum required length of the antigenic determinants disclosed the following epitopes: ^{147}HKAFKGSI^{154}, ^{291}NGNLQLRNKEVT^{302}, ^{301}VTWEVLEGEVEKEALKKI^{318} and ^{349}GSGKGKVQFQGKKTKF^{364}. Predicted features and molecular similarities of the defined epitopes were investigated using protein databases. The La epitope ^{147}HKAFKGSI^{154} presented 83·3% similarity with the ^{139}HKGFKGVD^{146} region of human myelin basic protein (MBP) and 72% similarity with the fragment YKNFKGTI of human DNA topoisomerase II. Peptides corresponding to these sequences cross-reacted with anti-La/SSB antibodies. Sixty-three sera with anti-La/SSB antibodies from patients with pSS or SLE, 35 sera without anti-La/SSB antibodies from patients with SS or SLE and 41 sera from age/sex-matched healthy blood donors were tested against biotinylated synthetic epitope analogues in order to determine their sensitivity and specificity for the detection of anti-La/SSB antibodies. Anti-La/SSB were detected with various frequencies ranging from 20% to epitope ^{147}HKAFKGSI^{154} to 100% to epitope ^{349}GSGKGKVQGKKTKF^{364}. The overall sensitivity and specificity using all assays with the synthetic peptides were found to be 93·6% and 85·6%, respectively. In conclusion, antibodies to La/SSB constitute a heterogeneous population, directed against different linear B cell epitopes of the molecule. The epitope ^{147}HKAFKGSI^{154} presents molecular similarity with fragments of two other autoantigens, i.e. human MBP and DNA topoisomerase II. Finally, synthetic epitope analogues exhibit high sensitivity and specificity for the detection of anti-La/SSB antibodies.

Keywords B cell epitopes La/SSB Sjögren's syndrome molecular mimicry systemic lupus erythematosus

INTRODUCTION

Autoantibodies to La/SSB are often found in sera of patients with primary Sjögren's syndrome (pSS) and systemic lupus erythematosus (SLE). La/SSB is a ribonucleoprotein particle consisting of a 408 amino acid protein component, with a calculated molecular weight of 47 kD in association with virtually any RNA polymerase III transcripts. Therefore, La/SSB particles can include human cytoplasmic RNAs (hyRNAs-RoRNAs), U1RNA, 5SRNA, tRNA, and several viral RNAs, including VA-RNAs and EBER-RNAs encoded by adenovirus and Epstein–Barr virus (EBV), respectively [1].

Despite progress in the complete characterization and molecular cloning of La/SSB protein, the fine specificity of B cell epitopes is not well defined. In previous studies, the antigenic regions of La/SSB were detected by molecular biological techniques. In fact, Chan *et al.* [2,3], using controlled proteolytic degradation with *Staphylococcus aureus* V8 protease, identified two antigenically independent sets of protease-resistant peptides termed X and Y. Sturgess *et al.*, using cDNA encoding 87% of La/SSB protein, identified a major antigenic epitope within the 103 aa of the C-terminal portion of the protein [4]. Rauh & Lührmann [5] suggested three independent immunodominant regions, spanning the 284–292, 293–345 and 346–383 sequences of La/SSB. St Clair *et al.* [6], using fusion proteins encoded by La/SSB cDNA fragments, defined three antigenic regions comprising the sequences 1–107, 111–242 and 242–408 (called LaA, LaC and LaD), respectively. More recently, Bini *et al.* [7] defined two immunodominant epitopes, one each in the N- and C-terminal halves of the protein.

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The purpose of the present study was to investigate the fine specificity of antibodies to La/SSB derived from sera of both pSS and SLE patients, using 20 mer overlapping synthetic peptides covering the entire sequence of the protein. Molecular similarities of the defined epitopes to other unrelated molecules were also investigated. Finally, a potential clinical use of the antigenic epitope synthetic analogues to detect antibodies to La/SSB is also reported.

PATIENTS AND METHODS

Patients

IgG purified by protein A Sepharose chromatography (Pharmacia, Uppsala, Sweden), from five patients with SLE [8] and four patients with pSS [9], was used for the epitope mapping of La/SSB. In all sera anti-La/SSB positivity was evaluated by counterimmunoelectrophoresis, immunoblot and ELISA (Shield Diagnostics, London, UK). Four sera were found to be also positive for both anti-Ro52kD and anti-Ro60kD antibodies, three sera were positive for anti-Ro52kD antibodies and two positive for anti-Ro60kD antibodies. IgG, purified from serum of a patient with SLE and a patient with pSS, positive for anti-Ro60kD and anti-Ro52kD antibodies, respectively, but negative for anti-La/SSB antibodies, as well as IgG from three healthy individuals, negative for autoantibody reactivity, were used as controls for the epitope mapping. IgG was used instead of whole serum, since preliminary experiments showed that the background was substantially reduced.

Sensitivity and specificity of the defined antigenic epitopes, for the detection of anti-La/SSB antibodies, were tested using 39 anti-La/SSB sera of pSS patients (all females, mean age 56 ± 7 years) and 24 anti-La/SSB$^+$ sera of SLE patients (21 females, three males, mean age 42 ± 8 years). Twenty sera contained also anti-Ro60kD antibodies, 34 sera had antibodies to Ro52kD and nine sera had, in addition to anti-La/SSB antibodies, antibodies to both Ro60kD and Ro52 proteins. Thirty-five sera of pSS and SLE patients negative for anti-La/SSB antibodies were used as disease controls. These sera contained different autoantibodies, as follows: 35 had antibodies to Ro60kD, nine had antibodies to Ro52, five had antibodies to U1RNP, three antiSm antibodies, eight anti-dsDNA antibodies, and 12 sera has IgM rheumatoid factor. Finally, 41 sera from age- and sex-matched healthy blood donors were used as negative controls.

Peptide synthesis

Pin-bound peptides. Sixty-eight sequential 20 mer peptides overlapping by eight amino acids each one to the other and covering the whole sequence of La/SSB protein were prepared in duplicate according to the method of Geysen *et al.* [10]. The peptides were prepared in duplicate in order for the same serum to be tested twice, at the same time in the ELISA experiments. Synthesis was performed on prederivatized polyethylene pins (Cambridge Research Biochemical Inc., London, UK) and the protocols are based on the principles of solid-phase peptide synthesis, as described by Merrifield [11], using the Na-fluorenylmethoxycarbonyl (FMOC) protecting group strategy. In each cycle of synthesis, control peptides PLAG(G)n and GLAG(G)n of various lengths (2 to 16 amino acids) were synthesized. All PLAG(G)n control peptides were specifically recognized by anti-PLAG MoAbs (Cambridge Research Biochemical Inc.), while GLAG(G)n control peptides did not bind to MoAbs. In addition, the accuracy of the peptide synthesis was directly determined by amino acid analysis of two different peptides, after cleavage from the polyethylene pins, according to Cohen & Stydous [12].

Biotinylated soluble peptides. Biotinylated soluble peptides corresponding to the defined epitopes HKAFKGSI (147–154), NGNLQLRNKEVT (291–302), VTWEVLEGEVEKEALKKI (301–318) and GSGKGKVQGKKTKF (349–364) were synthesized by standard solid-phase methods using benzydrylamine anchor-bond and resin and Na-Boc/benzyl side chain protection [13]. Amino acid couplings were performed by the dicyclohexyl carbodiimide (DCC)/hydroxybenzotriazole (HOBt) procedure using a ratio amino acid /DDC/HOBt/resin 3/3/3/1. Deprotection of the a-amino groups from the Nat-Boc protecting groups was performed using trifluoroacetic acid, followed by diisopropylethylamine for neutralization. Biotinylation of the peptides was carried out by coupling d-biotine to the N-terminus and, after Boc-deprotection, following the standard solid-phase procedure. The biotinylated 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 peptides were extracted from the resin using 2 M acetic acid, and after lyophilization they were subjected to partition chromatographic purification, using a Sephadex G-25 column, equilibrated with 2 M aqueous acetic acid. Elution of the peptides was performed using a homogeneous mixture of *n*-butanol/pyridine/acetic acid/H_2O (BPyAW) in a ratio 4:1:1:2 v/v. The homogeneity of the peptides was determined by thin layer chromatography in BPyAW (4:1:1:2 v/v), while 1 and 2D ^{1}H-NMR spectra as well as amino acid analysis confirmed their purity and identity.

Cell extract and immunoblotting

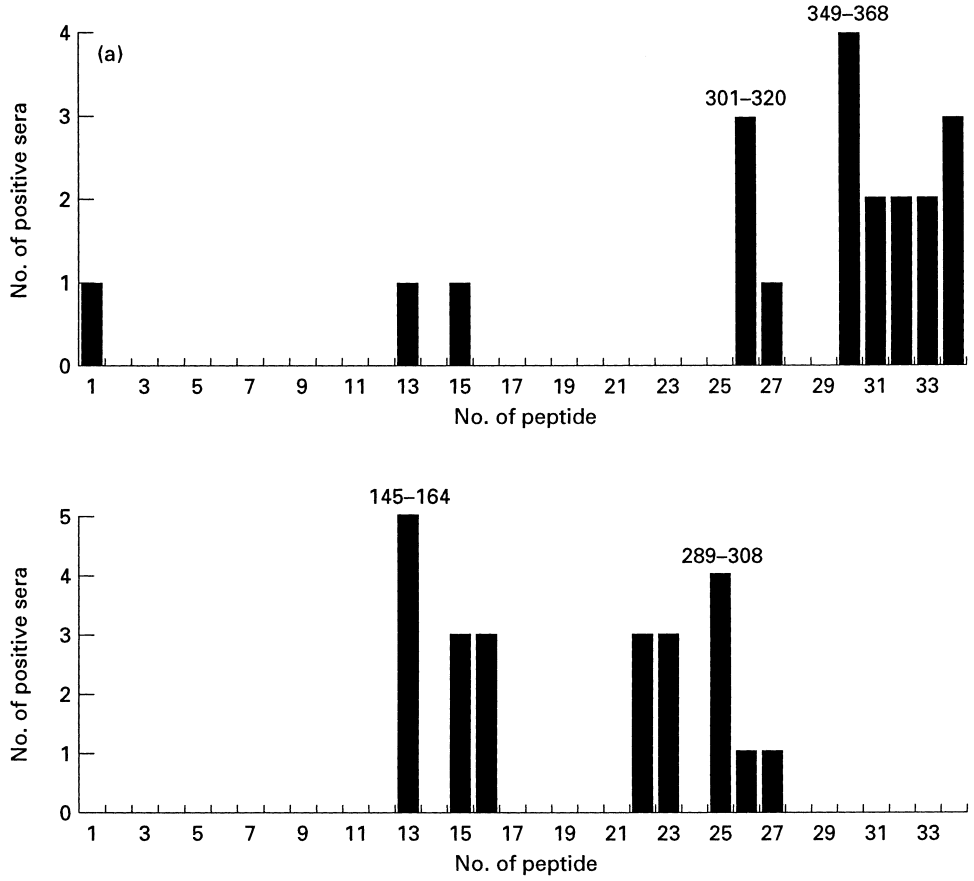
Cytoplasmic extract was prepared from cultured HeLa cells as described by Habets *et al.* [14]. Samples of extracts were applied to SDS–PAGE, followed by electrotransfer to nitrocellulose. The nitrocellulose blots were cut into strips which were subsequently blocked with non-fat milk 5% at 4°C overnight. Afterwards, anti-human IgG conjugated to horseradish peroxidase (HRP) was added (1:1000 in blocking buffer) and allowed to react for 1 h. The colour was developed by adding a substrate solution of 4-chloro-L-naphthol to the strips.

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, 1% albumin and 1% ovalbumin to block non-specific binding. IgG concentration of 200 μg/ml (which corresponds to an estimated IgG concentration in serum dilution 1:50) was added to the wells and incubated overnight at 4°C. This dilution was selected because it exhibited the highest specific binding. After washing with PBS containing 0·5% Tween 20, anti-human IgG, conjugated to HRP (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 sulfonic acid (ABTS) and absorbance of the colour was measured at 405 nm. Subsequently, bound antibodies were removed from the rods by sonication for 30 min in water bath with 0·1 M sodium dihydrogen phosphate, 1% 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 HRP followed by the addition of substrate solution (ABTS). In all cases the final optical density (OD) was equal to the background.

Biotinylated soluble peptides. ELISA, using biotinylated peptides, was as follows. Ninety-six-well polystyrene plates (Nunc, Roskilde, Denmark) were pretreated with 5 μg/ml streptavidin (100 μl/well) and kept overnight at 37°C. Afterwards, 100 μl bovine serum albumin (BSA) 2% in PBS pH 7·3 were added per well and the plates were incubated at 37°C for 1 h. Biotinylated peptides (5 μg/ml) were added in the wells and incubated for 1 h at room temperature. After three washings with PBS–0·1% Tween 20, sera were added in dilution 1:50, in BSA 2% in PBS (100 μl/well). After a 3-h incubation and three washings with PBS–0·1% Tween 20, alkaline phosphatase-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark), diluted 1:6000 in BSA 2% in PBS, was added per well. Following 2 h incubation at room temperature and three washings, 50 μl substrate (*p*-nitrophenyl-phosphate in diethanolamine buffer) was added and the reaction was read at 405 nm after 30 min. The background of each individual serum was



(b)

Antigenic epitopes	Mean OD ± s.d. SLE anti-La/SSB $n = 5$	SS anti-La/SSB $n = 4$	Normals $n = 3$	OD, disease control $n = 2$
145–164	0·822 ± 0·201	0·707 ± 0·062	0·167 ± 0·020	0·202
289–308	0·750 ± 0·141	0·549 ± 0·076	0·151 ± 0·043	0·241
301–320	0·527 ± 0·121	1·239 ± 0·433	0·134 ± 0·028	0·232
349–368	0·153 ± 0·046	1·167 ± 0·103	0·087 ± 0·034	0·061

Fig. 1. (a) Epitope mapping of La/SSB autoantigen, using overlapping synthetic peptides. Purified IgG from primary Sjögren's syndrome (pSS) sera (upper panel) reacted with peptides corresponding to 301–320 and 349–368 fragments of La/SSB. In contrast, IgG derived from systemic lupus erythematosus (SLE) sera (lower panel), with anti-La/SSB reactivity, reacted with peptides 145–164 and 289–308, and parts of La/SSB. Three normal sera and two sera from patients with anti-Ro/SSA antibodies, but without anti-La/SSB antibodies, were used as negative controls. (b) The mean optical density produced (OD) from the reaction of four major antigenic epitopes reacting with IgG from five patients with SLE and anti-La/SSB antibodies, four pSS patients and anti-La/SSB antibodies, three normal human sera and two sera from patients with SLE without anti-La/SSB antibodies.

determined with parallel experiments in streptavidin-pretreated, peptide-free ELISA plates. The real binding was calculated by subtracting the mean OD of the sample in the non-peptide-coated wells from the mean OD of the peptide-coated wells. Preliminary experiments, using different concentrations of all reagents, were used to define the optimal conditions of all ELISAs developed. The cut-off point of positivity was calculated as the mean OD of the 41 normal sera + 3 s.d.

Inhibition assays

In order to investigate whether the synthetic epitope analogues can inhibit antibody binding to native La protein, a serum with high anti-La/SSB activity, reacting with all four synthetic epitope analogues, was pretreated in dilution 1:500 for 3 h at 37°C and overnight at room temperature with each peptide individuaIly, and a mixture of all four synthetic peptides (concentrations ranging from 0·01 to 5 μg/ml). Subsequently, all sera were tested in ELISA (Shield Diagnostics) for anti-La/SSB activity.

Alanine substitutions, computer predictions and homology search

In order to define which amino acids play an essential role for the antigenicity of the identified epitopes, residues were individually replaced by alanine and the newly produced peptides were tested against IgG with anti-La/SSB reactivity. Alanine was selected because of its low antigenicity and small stereochemical volume.

Prediction of the hydrophilicity, flexibility and T and B cell antigenic profiles, using the La/SSB primary structure, was achieved by the EPIPLOT program [15]. Prediction of the secondary structure of La/SSB was made using the PHDsec profile neural network method [16].

The antigenic peptide sequences were compared against the Swiss-PROT, NBRF/PIR, PRF and GENPEPT databases using Fasta [17] and Smith & Waterman [18] algorithms at GENOMENET-FASTA and EMBL-FASTA-BLITZ servers. The non-identical amino acids were scored with PAM 250 and PAM 100 matrices [19] and the gap inclusions were allowed in Smith–Waterman searching.

Statistical analysis

The OD of each sample including normal sera was expressed in units according to the formula:

$$\text{Sample (units)} = \frac{\text{OD sample} - \text{OD background}}{\text{mean OD} + 3\,\text{s.d. of normal sera}} \times 100$$

According to this formula, 100 U was the cut-off point for each assay. Statistical differences between patients and normals were calculated using the non-parametric *t*-test. The sensitivity of the ELISAs with the synthetic peptides was calculated as follows:

$$\text{Sensitivity (\%)} = \frac{\text{number of positive samples}}{\text{total samples}} \times 100$$

The specificity was calculated according to the formula

$$\text{Specificity (\%)} = \left(1 - \frac{\text{number of positive disease controls}}{\text{number of total disease controls}}\right) \times 100$$

RESULTS

Identification and restriction pattern of antigenic determinants

Thirty-four 20 mer peptides, overlapping by eight residues covering the sequence of La/SSB, as determined by Chambers *et al.* [20] and Chan *et al.* [21] were prepared in duplicate. Six additional pairs of 20 mer peptides representing differences in the sequence, as reported by other investigators [4,5,22], were also synthesized. All pin-bound peptides were tested against purified IgG from anti-La/SSB$^+$ sera of pSS and SLE patients. Five out of five SLE IgG recognized peptides spanning the 145–164 and 289–308 sequences of La/SSB. Furthermore, all IgG from pSS patients recognized the region 349–368, while three out of four IgG fractions reacted also with the region 301–320 of the La/SSB protein (Fig. 1a,b).

Determination of the minimum required length of the antigenic determinants was performed by the synthesis of two sets of multiple length peptides for each 20 mer antigenic epitope. Each set of peptides was obtained by serially subtracting two amino acids from either the amino or carboxyterminal end of the initially reactive peptides. The points beyond which anti-La/SSB IgG failed to bind were considered the ends of the antigenic epitope (Fig. 2). Thus, refinement of the peptide TLHKAFKGSIFVVFDSIESA (145–164) resulted finally in the peptide HKAFKGSI (147–155). Restriction of the peptide ANNGNLQRNKEVTWEVLEG (289–308) revealed the antigenic epitope NGNLQLRNKEVT (291–302). Similarly, the initially reactive peptides VTWEVLEGEVEKEALKKIIE (301–320) and GSGKGKVQFQGKKTKFASDD (349-368) were finally restricted to VTWEVLEGEVEKEALKKI (301-318) and GSGKGKVQFQGKKTKF (349–364), respectively.

The specificity of the defined epitopes was investigated by inhibition experiments. It was shown that in the presence of synthetic peptides the binding of anti-La/SSB antibodies in native La/SSB was reduced in a dose-dependent manner by 12% to 43% (Fig. 3).

Alanine substitutions, predicted features and molecular similarities of the epitopes

The alanine-substituted peptide epitopes were tested against IgG with anti-La/SSB activity, and the antibody-binding capacity in three out of four antigenic epitopes was not affected, except in the case of W303 substitution. Substitution of W303 by alanine resulted in significant loss of antigenicity of the peptide ANNGNLQLRNKEVTWEVLEQ (289–308).

Prediction of the secondary structure of the epitopes suggested that the sequences 147–154 and 301–308 attain helical conformations containing β-loop structures. In addition, the epitope 147–154 belongs to part of the RNA binding site of La/SSB protein. All four epitopes presented features of high antigenicity [23], had high ratios of hydrophilic amino acids, commonly found in B cell epitopes [24], and moderate to high flexibilities [25]. The epitopes HKAFKGSI (147–154) and VTWEVLEGEVEKEALKKI (301–318) possessed features of putative T cell epitopes by three of four methods used for T cell epitope predictability [26–28]. The biochemical characteristics of the epitopes strongly suggested that they are located on the surface of the La/SSB protein.

Identification of the primary structure of La/SSB epitopes made possible the use of protein databases to search for sequences shared with other proteins of unrelated origin. The epitope HKAFKGSI (147–154) was found to be highly conserved in mammals. This epitope had an 83·3% similarity to the human myelin basic protein (MBP) in the region 139–146 (HKGFKGVD) and 72% similarity to the fragment 914–921 (KNFKGTIQ) of the autoantigen topoisomerase II. These observations prompted us to construct new peptides containing the above mentioned sequences

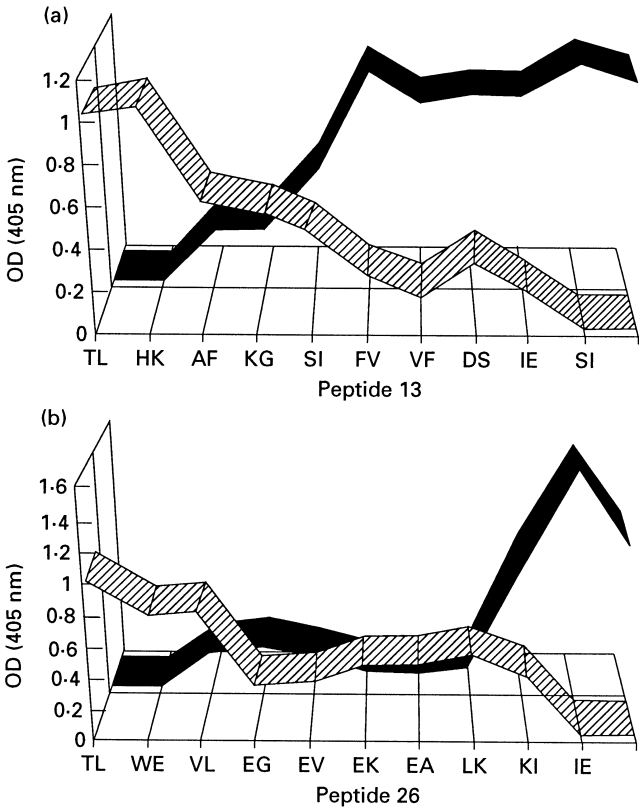


Fig. 2. The refinement of epitopes HKAFKGSI (147–155) (a) and VTWEVLEGEVEKEALKKI (301–318) (b) are shown. The hatched line represents the ELISA optical density (OD) after substraction of amino acids from the –NH2 terminus and the black line denotes the ELISA OD after substraction of amino acids from the –COOH terminus, from the initially reactive 20 mer peptides. The amino acids beyond the points where anti-La/SSB IgG abolished its binding capacity have been considered as the borders of the epitopes, since these amino acids were essential in preserving the antigenicity of the peptides.

and test them against IgG with anti-La/SSB specificity. As shown in Fig. 4a, IgG with anti-La/SSB activity reacted not only with the La/SSB epitope, but also with the peptides derived from human MBP and topoisomerase II sequences. Furthermore, pretreatment of anti-La/SSB IgG reacting with the p147–154, in a concentration 250 μg/ml, resulted in a significant reduction of antibody binding to both topoisomerase II and human MBP-related peptides (Fig. 4b). In contrast, p289–308, which was used as control peptide, of La/SSB did not inhibit anti-La/SSB binding.

Prevalence of antipeptide antibodies in autoimmune sera

Using the biotinylated antigenic epitope analogues in ELISAs, the prevalence of antipeptide antibodies in sera of SLE and pSS was investigated. Given that the peptides partly inhibited the binding of antibodies to native La/SSB protein, it is obvious that these epitopes are targets for a significant population of anti-La/SSB antibodies. Antibodies to HKAFKGSI (147–154) were found in 12/24 (50%) of SLE sera with anti-La/SSB reactivity, and 7/39 (18%) of pSS sera with anti-La/SSB antibodies. None of the 35 disease control sera, negative for anti La/SSB antibodies, was found to be positive to this epitope. Antibodies to NGNLQLRNKEVT (291–302) antigenic epitope were detected in 9/24 (37·5%) of SLE

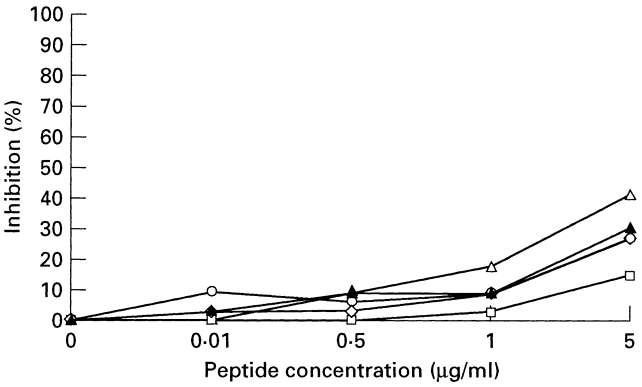


Fig. 3. Inhibition of anti-La/SSB antibody binding in anti-La/SSB ELISA, using as inhibitors different concentrations of all four synthetic epitope analogues. Mixed antigen denotes mixture of all synthetic peptides (each one contributing to the solution by 25%). Anti-La/SSB$^+$ serum was used in dilution 1:500. △, Amino acids (aa) 349–364; ○, aa 301–318; □, aa 147–154; ◊, aa 291–302; ▲, mixed peptides.

sera and 8/39 (20%) of pSS sera with anti-La/SSB reactivity, and none of the disease control sera. Antibodies to VTWEVLEGEVE-KEALKKI (301–318) were found in 19/24 (79%) and 27/39 (69%) of SLE and pSS sera positive for anti-La/SSB antibodies,

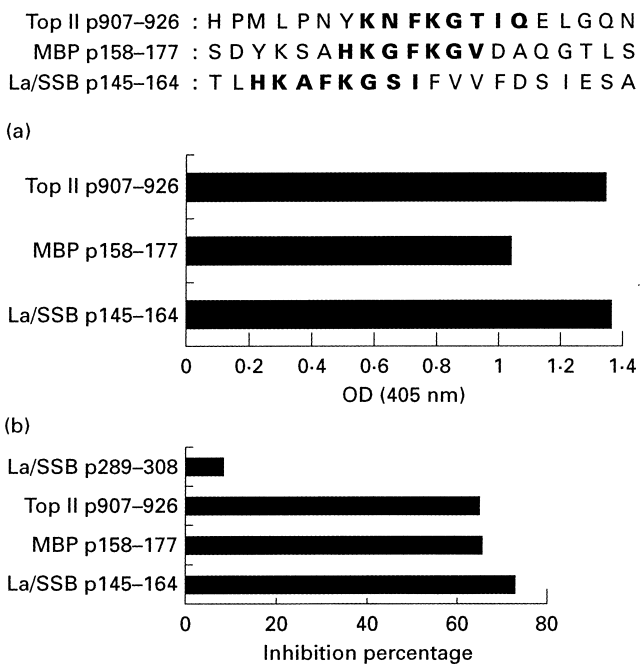


Fig. 4. (a) Reaction of anti-La/SSB antibodies with peptides derived from human myelin basic protein (MBP) and topoisomerase II. Bold letters denote sequence similarities between La/SSB, topoisomerase II and human MBP. Two normal human sera tested in dilution 1:50 did not exceed optical density (OD) of 0·150 in all three peptides. (b) Inhibition of anti-p145–164 antibodies binding in polyethylene pins coated with p145–164 of La/SSB, p158 –177 of human MBP, p907–926 of DNA topoisomerase II and p289–308 of La/SSB. IgG concentration 200 μg/ml. As inhibitor p147–154 (HKAFKGSI) of La/SSB was used at a concentration of 250 μg/ml. The inhibition values were: p145–164 of La/SSB, 73%; p158–174 of human MBP, 65%; p907–926 of DNA topoisomerase II, 64%; p289–308 of La/SSB was used as control peptide and did not inhibit the antibody binding substantially.

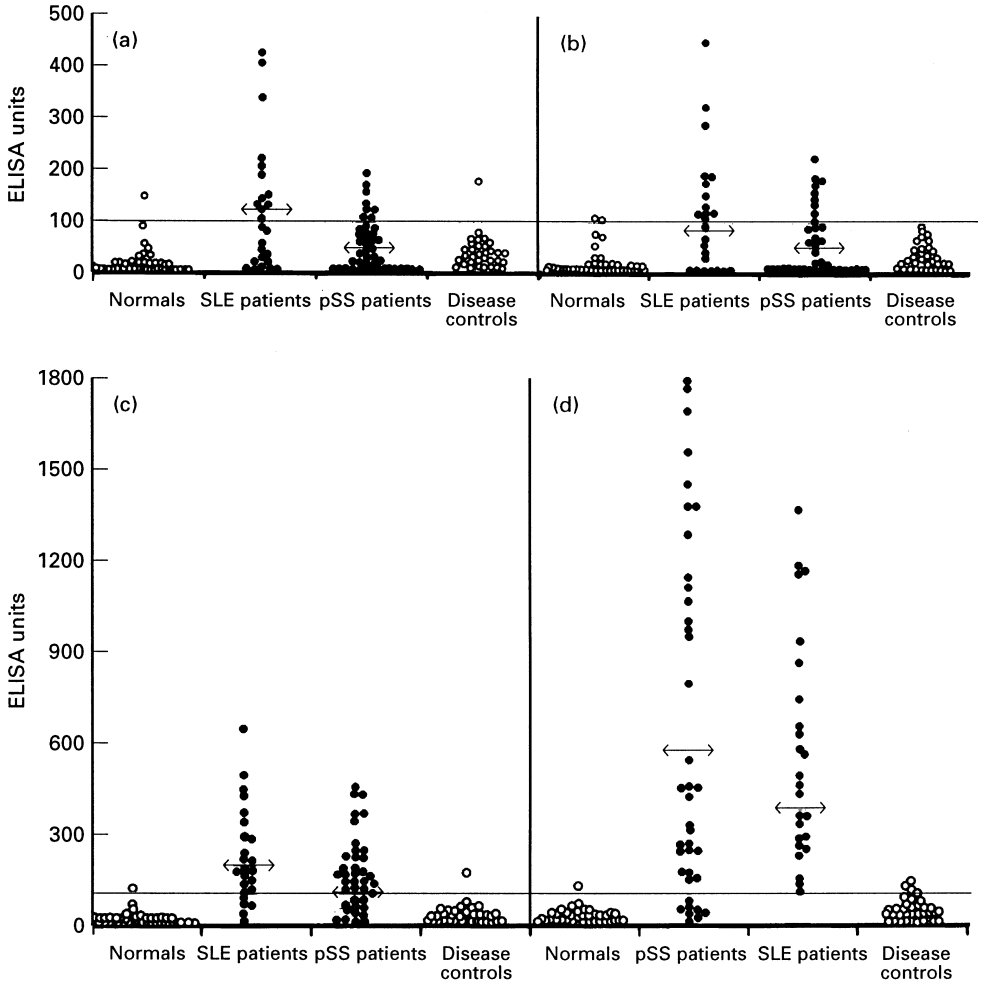


Fig. 5. Prevalence of antibodies to: HKAFKGSI (147–154) (a), NGNLQLRNKEVT (291–302) (b), VTWEVLEGEVEKEALKKI (301–318) (c) and GSGKGKVQFQGKKTKF (349–364) (d) synthetic epitope analogues in sera from patients with primary Sjögren's syndrome (pSS) and systemic lupus erythematosus (SLE) with anti-La/SSB antibodies, patients with pSS and SLE without anti-La/SSB antibody (disease controls) and normal controls. The cut-off point (100) was calculated using the mean binding units (see Patients and Methods) of 41 normal human sera + 3 s.d. ○, αLa-negative; ●, αLa-positive.

respectively, as well as 1/35 (2·9%) of disease-negative controls. Finally, antibodies to GSGKGKVQFQGKKTKF (349–364) peptide were detected in all SLE sera (100%) with anti-La/SSB reactivity and 32/39 (82%) pSS sera and 4/35 (11·4%) disease controls (Fig. 5). Four of 41 normal sera (9·7%) reacted also with the peptides.

Seven of 63 anti-La/SSB$^+$ sera reacted with all peptides, 16/63 sera reacted with three peptides, 31/63 reacted with two peptides and 5/63 with one peptide. Four of 63 sera did not react with any of the peptides. ELISA with the peptide 349–364 exhibited the highest sensitivity (88%) and high specificity (88·6%). Detection of anti-La/SSB antibodies using the peptide 301–318 revealed a sensitivity of 73% and specificity of 69%. The concordance between these two assays was 70%. ELISA with the other two peptides, 147–154 and 291–302, showed remarkably lower sensitivities (25% and 27%, respectively); but none of these peptides reacted with either disease control or normal control sera. The overall sensitivity and specificity, using all assays with the synthetic peptides, were found to be 93·6% and 85·6%, respectively. None of the assays could discriminate between pSS and SLE anti-La/SSB$^+$ patients.

DISCUSSION

In the present study, an attempt was made to define the linear antigenic epitopes of La/SSB autoantigen. The fine B cell epitope mapping of autoantibodies to La/SSB was performed using overlapping synthetic peptides. The length of the peptides used was rather long (20 amino acids), since in previous studies of Ro60kD epitopes it was shown that long peptides exhibited higher specificity [29] compared with overlapping octapeptides [30]. In fact, the mimotope scanning of Ro60kD molecule, using 22 mer overlapping synthetic peptides, revealed two discrete, disease-specific epitopes (one associated with SS and the other with SLE), compared with 14 epitopes defined by the octapeptides. Thus, antigenic epitope formation is probably influenced by both the primary structure of a given synthetic epitope, along with the peptide secondary structure. In this regard, NMR studies have revealed that even small peptides, such as the PPGMRPP epitope of the Sm autoantigen [30], can possess more than one conformation [31].

During the past few years, several investigators have performed epitope mapping of La/SSB autoantigen, using either fusion proteins of various lengths or c-DNA deletion mutants.

Their results did not lead to a clear definition of B cell epitopes of La/SSB protein, since they were only assigned to long stretches of polypeptides. It was shown, however, that antibodies to La/SSB, binding to a given B cell epitope, did not react with antibodies directed to other epitopes, suggesting that an anti-La/SSB response comprises independent anti-La/SSB antibody populations [32]. These observations strongly suggest that the production of anti-La/SSB antibodies is attributable to an antigen-driven process. The stimulating antigen could be a part of the native or denatured La/SSB protein. In this regard, the definition and the exact determination of B cell epitopes is of particular importance in order to delineate the mechanisms accounting for anti-La/SSB antibody production.

The epitopes reported in this study are linear epitopes varying in length from 8 to 18 amino acids. It has been previously estimated that a small but significant proportion of antibodies directed to discontinuous epitopes of a given antigen are also able to react with linear peptide fragments of the protein [33]. The relevance of the linear epitopes described in this study has been confirmed in two ways. First, they reacted with a large panel of autoimmune sera with anti-La/SSB antibodies, but not with normal or disease control sera. Second, using inhibition assays it was shown that the antibody binding to native La/SSB was reduced in the presence of synthetic epitope analogues. Our results agree with previous reports regarding the locations of the epitopes. In fact, the epitope HKAFKGSI (147–155) belongs to the protein region containing the RNA recognition motif (RRM) of La/SSB; antibodies to this fragment of the protein have been described previously [34]. The RRM, spanning amino acids 111–187 of La/SSB protein, is included in the LaC epitope (amino acids 111–242), as described previously [20]. Experiments with non-overlapping and overlapping peptides corresponding to this region have shown that antibodies directed to this fragment of the protein bind in conformational determinants [35]. Our study adds further to our knowledge of the antigenicity of this region, defining also a linear epitope (147–155) consisting of eight amino acids.

The region 291–318 of La/SSB consists of two different continuous linear epitopes (291–302 and 301–318). Previous studies have shown that this fragment is an immunodominant epitope of the protein, since both Rauh & Lührmann [5] and Sturgess *et al.* [4] have shown that this region exhibits the highest binding of antibodies to La/SSB. In contrast, St Clair *et al.* [6], based on a quantitative analysis of the binding of anti-La/SSB antibodies in three different fragments of the molecule, showed that the LaD region (amino acids 242–408) presented the lowest binding compared with the two other fragments. However, it is not possible to compare directly the levels of anti-La/SSB in these studies, since all of them have used only limited numbers of sera. The epitope 349–364 also belongs to the LaD region. It is noteworthy that the vast majority of sera from both SLE and SS patients reacted with the synthetic peptide corresponding to this epitope. This region belongs also to one of the previously described immunodominant epitopes of the protein [20].

The epitope HKAFKGSI (147–154) presented sequence similarity with fragments of two other, unrelated autoantigens, MBP and topoisomerase II. Interestingly, antibodies to La/SSB which bind to 147–154 epitope cross-react with the peptides corresponding to the similar epitopes of MBP and topoisomerase II. MBP can induce experimental allergic encephalomyelitis after immunization [36], while antibodies to topoisomerase II have been described in sera of patients with idiopathic interstitial lung disease [37]. Despite the fact that none of the patients with SLE or SS had evidence of central nervous disease or interstitial lung disease, this cross-reaction between fragments of different autoantigens should be further investigated. Our data add further to this concept, showing that an immunodominant epitope of La/SSB possesses molecular similarity to a fragment of laminin A.

One of the most interesting findings in this study is that all but four of the 63 sera of patients with either pSS or SLE and anti-La/SSB antibodies react at least with one synthetic peptide analogue of the La/SSB linear epitopes. The four non-reacting sera probably contain antibodies directed towards either conformational or long linear epitopes of the protein. Most positive sera bind to two or more synthetic epitope analogues, reinforcing the concept that antibodies to La/SSB consist of heterogeneous groups. The second conclusion is that the use of synthetic peptides as substrates for the identification of anti-La/SSB antibodies is a promising method. In fact, the positivity rate in healthy blood donors is low, comparable to that observed in assays based on recombinant La/SSB [38]. Furthermore, disease control sera (i.e. sera from SS and SLE patients, without anti-La/SSB antibodies) also possessed low reactivity, resulting in the high specificity of the assays.

In conclusion, epitope mapping of La/SSB disclosed that antibodies to La/SSB are directed towards different linear epitopes of the molecule. The binding of antibodies derived from pSS sera presented a different pattern compared with antibodies derived from SLE sera. The linear epitopes with the highest reactivity belong to fragments of the protein which previously have been assigned as immunodominant epitopes of La/SSB. Peptide-based assays may prove useful in the detection of anti-La/SSB antibodies. In this respect, a large number of sera in a prospective study and a higher structural order of peptides used as substrates (i.e. peptides connected in sequential oligopeptide carriers (SOC) [39] are currently under investigation.

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REFERENCES

1 Pruijn CJM, Slobbe RL, van Venrooij WJ. Structure and function of La and Ro RNPs. Mol Biol Rep 1990; **14**:43–48.
2 Chan EKL, Francoeur AM, Tan EM. Epitopes, structural domains and asymmetry of amino acid residues in SSB/La nuclear protein. J Immunol 1986; **136**:3744–9.
3 Chan EKL, Tan EM. The small nuclear ribonucleoprotein SSB/La binds RNA with a conserved protease-resistant domain of 28 kilodaltons. Mol Cell Biol 1987; **7**:2588–94.
4 Sturgess AD, Peterson MG, McNeilage LJ, Whittingham S,Coppel RL. Characteristics and epitope mapping of a cloned human autoantigen La. J Immunol 1988; **140**:3212–8.
5 Rauh AJG,Lührmann R. At least 3 distinct B cell epitopes reside in the C-terminal half of La protein, as determined by a recombinant DNA approach. Eur J Immunol 1988; **18**:2049–57.
6 St Clair EW, Pisetsky DS, Reich CF,Keene JD. Analysis of autoantibody binding to different regions of the human La antigen expressed in recombinant fusion proteins. J Immunol 1988; **141**:4173–80.
7 Bini P, Chu J-L, Okolo C, Elkon K. Analysis of autoantibodies to recombinant La(SSB) peptides in systemic lupus erythematosus and primary Sjögren's syndrome. J Clin Invest 1990; **85**:325–33.

8 Tan EM,Cohen AS, Fries JB *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982; **25**:1271–7.
9 Vitali C, Bombardieri S, Moutsopoulos HM *et al.* Preliminary criteria for the classification of Sjögren's syndrome. Arthritis Rheum 1993; **36**:340–7.
10 Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoofs PG. Strategies for epitope analysis using peptide synthesis. J Immunol Methods 1987; **202**:259.
11 Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. JACS 1963; **85**:2149–54.
12 Cohen SA, Stydous DJ. Aminoacid analysis utilising phenylsothyiocynate derivatives. Anal Biochem 1988; **174**:1–16.
13 Stewart JM, Young JD, eds. Solid phase peptide synthesis. Rockford: Pierce Chemical Co., 1984.
14 Habets WJ, De Rooij DJ, Hoet MH, Van de Putte LB, van Venrooij WJ. Quantitation of anti-RNP and anti-Sm antibodies in MCTD and SLE patients by immunobloting. Clin Exp Immunol 1985, **59**:457–66.
15 Mendez-Arias L,Rodriguez RA. BASIC microcomputer program for prediction of B and T cell epitopes in proteins. CABIOS 1990; **6**:101–5.
16 Burkhard R, Sander C. Prediction of protein secondary structure at better that 70% accuracy. *J Mol Biol* 1993; **232**:584–99.
17 Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 1988; **85**:2444–8.
18 Smith TF, Waterman MS. Identification of common molecular subsequences. J Mol Biol 1988; **147**:195–7.
19 Altschul SE. Amino acid substitution matrices from an information theoretic perspective. J Mol Biol 1991; **219**:555–65.
20 Chambers JC,Kenan D, Martin BJ, Keene JD. Genomic structure and amino acid sequence domains of the human La autoantigen. J Biol Chem 1988; **263**:18043–51.
21 Chan EKL,Sullivan KF, Tan EM. Ribonucleoprotein SSB/La belongs to a protein family with consensus sequences for RNA binding. Nucleic Acid Res 1988; **17**:2233–44.
22 Kohsaka H, Yamamoto K, Fujii H *et al.* Fine specificity of the human SSB/La protein : identification of a distinct autoepitope homologous to a viral gag polyprotein. J Clin Invest 1990; **85**:1566–74.
23 Welling GM, Weijer WJ, Van der Zee R, Welling-Wester. Prediction of sequential antigenic regions in proteins. FEBS Letters 1985; **188**:215–8.
24 Hopp TP, Woods KR. Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci USA 1981; **78**:3824–8.
25 Karplus PA, Schultz GE. Prediction of chain flexibility in proteins. Naturwissenschaften, 1985; **72**:212–3.
26 Margalit H, Spouge JL, Cornette JL, Cease KB, Delisi C, Berzofsky JA. Prediction of immunodominant helper T cell antigenic sites from the primary sequences. J Immunol 1987; **138**:2213–29.
27 Rothbard JB, Taylor WR. A sequence pattern comment to T cell epitopes. EMBO J 1988; **7**:93–100.
28 Sette A, Buus S, Appela E *et al.* Prediction of major histocompatibility complex binding regions of protein antigens by sequence pattern analysis. Proc Natl Acad Sci USA 1989; **86**:3296–300.
29 Routsias JG, Tzioufas AG, Sakarellos-Daitsiotis M, Sakarellos C, Moutsopoulos HM. Epitope mapping of the Ro/SSA 60KD autoantigen reveals disease specific antibody binding profiles. Eur J Clin Invest 1996 **26**:514–21.
30 Scofield RH, Harley JB. Autoantigenicity of Ro/SSA antigen is related to a nucleocapsid protein of vesicular stomatitis virus. Proc Natl Acad Sci USA 1991; **88**:3343–7.
31 Tsikaris V,Vlachoyiannopoulos PG, Panou E *et al.* Immunoreactivity and conformation of the P-P-G-M-R-P-P repetitive epitope of the Sm autoantigen. Int J Pep Prot Res 1996; **48**:319–27.
32 St Clair EN, Burch JA, Ward MM, Keene JD, Pisetsky DS. Temporal correlation of antibody responses to different epitopes of the human La autoantigen. J Clin Invest 1990; **85**:515–21.
33 Van Regenmortel MHV. Protein antigenicity. Mol Biol Reports 1992; **16**:133–8.
34 Rischmueller M, Mc Neilage LJ, McCluskey J, Gordon T. Human autoantibodies directed against the RNA recognition motif of La(SSB) bind to conformational epitope present on the intact La(SSB)/Ro(SSA) ribonucleoprotein particle. Clin Exp Immunol 1995;**101**:39–44.
35 Topfer F, Gordon T, McCluskey J. Intra- and inter-molecular spreading of autoimmunity involving the nuclear self-antigens La(SSB) and Ro(SSA). Proc Natl Acad Sci USA 1995; **92**:875–9.
36 Miller A, Al-Sabbagh A, Santos LMB, Pradhu-Das M, Weiner HL. Epitopes of myelin basic protein (MBP) that trigger TGF- β release following oral tolerization to MBP are different from encephalitogenic epitopes and mediate epitope-driven bystander suppression. J Immunol 1993; **151**:7307–15.
37 Meliconi R, Bestagno M, Sturani C *et al.* Autoantibodies to DNA topoisomerase II in cryptogenic fibrosing alveolitis and connective tissue disease. Clin Exp Immunol 1989; **76**:184–9.
38 Owe-Young RA, Horn S, Edmonds JP, Sturgess AD. Use of molecularly cloned human SSB antigen to detect anti SSB antibodies. J Autoimmun 1992; **5**:351–61.
39 Tsikaris V, Sakarellos C, Cung-Thong M, Marraud M, Sakarellos-Daitsiotis M. Concept and design of a new class of sequential oligopeptide carriers (SOC) for covalent attachment of multiple antigenic peptides. Biopolymer, 1996; **38**:291–3.