Terzoglou AG, **Routsias JG**, Sakarellos C, Sakarellos-Daitsiotis M, Moutsopoulos HM, Tzioufas AG. ***ũɟ ŬɛɛɜɷɛɑŮ ɑŰɛ´ɛɜüɨɛ üɜŬűɛɟ ŮŰɷɩ ɜ ŬgŰɛŬɜŰŵɧɜɣ ɜ (La/SSB και βασική πρωτεϊνη της μυελίνης) με υψηλού βαθμού μοριακή ομοιότητα προκαλούν διαφορετικές χυμικές ανοσολογικές απαντήσεις*** . J Autoimmun. 2003 Aug;21(1):47-57.

Η περιοχή 147-154αα της πρωτεΐνης La/SSB παρουσιάζει 83% ομοιότητα αμινοξικής αλληλουχίας με την περιοχή 139-146αα της ανθρώπινης βασικής πρωτεΐνης της μυελίνης (ΒΠΜ). Ο σκοπός της μελέτης αυτής ήταν να διερευνήσουμε τον επιπολασμό και την σημασία των αντισωμάτων, που υπάρχουν στον ορό ασθενών με αυτοάνοσα συστηματικά νοσήματα, έναντι και των δύο αυτών επιτόπων και να συγκρίνουμε τις χυμικές ανοσολογικές αποκρίσεις, που παρήχθησαν από κουνέλια, τα οποία εμβολιάστηκαν με αυτούς τους επιτόπους. Τα δύο ομόλογα πεπτίδια 147-154αα της La/SSB και 139-146αα της ΒΠΜ συντέθηκαν προσδεδεμένα σε τετραμερείς ολιγοπεπτιδικούς φορείς και χρησιμοποιήθηκαν για τον εμβολιασμό λευκών κουνελιών Νέας Ζηλανδίας. Αντισώματα κατά των πεπτιδίων, με τα οποία έγιναν οι εμβολιασμοί, καθώς και κατά των πεπτιδίων που αντιπροσωπεύουν άλλους προσδιορισμένους Β-κυτταρικούς επιτόπους της La/SSB (289-308αα, 349-364αα), κατά της ολόκληρης ανθρώπινης πρωτεΐνης ΒΠΜ (αΒΠΜ) και της ανασυνδυασμένης ανθρώπινης πρωτεΐνης La/SSB (rechLa), προσδιορίσθηκαν με την χρήση ειδικών πειραμάτων ELISA. Οροί από 45 ασθενείς με πρωτοπαθές σύνδρομο Sjögren (πσΣ), 49 με Συστηματικό Ερυθηματώδη Λύκο (ΣΕΛ), και 18 με Ρευματοειδή Αρθρίτιδα (ΡΑ) ελέγχθηκαν έναντι των δύο συνθετικών πεπτιδίων και έναντι της αΒΠΜ. 22% των ορών από ασθενείς με πσΣ, 27% των ασθενών με ΣΕΛ και κανένας από τους ασθενείς με ΡΑ αντέδρασαν κατά του La/SSB επιτόπου. 27% από τους πσΣ ορούς, 22% από τους ΣΕΛ και 17% από του ΡΑ ορούς έδειξαν θετική αντίδραση κατά του πεπτιδίου της ΒΠΜ. Τέλος 19% από τους πσΣ, 30% από τους ΣΕΛ και 38% από τους ΡΑ ορούς αντέδρασαν κατά της αΒΠΜ. 35 μέρες μετά της ανοσοποίηση των κουνελιών με τον επίτοπο της La/SSB, είχαν παραχθεί αντισώματα κατά και των τριών επιτόπων της La/SSB, του πεπτιδίου της ΒΠΜ και των πρωτεϊνών αΒΠΜ και rechLa. Τα κουνέλια που είχαν ανοσοποιηθεί με το πεπτίδιο της ΒΠΜ παρήγαγαν αντισώματα κατά του πεπτιδίου, με το οποίο ανοσοποιήθηκαν και του ομολόγου La/SSB πεπτίδιου, σε μικρό χρονικό διάστημα μετά από την ανοσοποίηση, ενώ τα αντισώματα κατά των άλλων La/SSB επιτόπων, καθώς και κατά των δύο ολόκληρων πρωτεϊνών, παρήχθησαν αργότερα. Πειράματα αναστολής σε ορούς κουνελιών με υψηλή δραστικότητα κατά της αΒΠΜ, χρησιμοποιώντας το πεπτίδιο της ΒΠΜ ως αναστολέα, αποκάλυψαν ότι το 80% της ανοσολογικής δραστικότητας των ορών απολέσθει. Συμπερασματικά ένα σημαντικό ποσοστό ανθρώπινων αυτοανόσων ορών αντέδρασε έναντι και των δύο πεπτιδίων της La/SSB και της ΒΠΜ, καθώς και έναντι της αΒΠΜ. Όταν το πεπτίδιο 147-154αα της La/SSB χρησιμοποιήθηκε για ανοσοποιήσεις ζώων, προκάλεσε μια άμεση εξάπλωση επιτόπων, η οποία συμπεριελάμβανε και τις δύο πρωτεΐνες ΒΠΜ και La/SSB. Αντίθετα ο επίτοπος της ΒΠΜ προκάλεσε μια καθυστερημένη ανοσολογική απόκριση έναντι των άλλων La/SSB επιτόπων. Έτσι, παρά το γεγονός ότι αυτά τα δύο πεπτίδια παρουσιάζουν μοριακή ομοιότητα, προκαλούν διαφορετικές ανοσολογικές αποκρίσεις.

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Linear epitopes of two different autoantigens-La/SSB and myelin basic protein—with a high degree of molecular similarity, cause different humoral immune responses

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Abstract

The region 147–154aa of La/SSB presents 83% sequence similarity with the 139–146aa region of the human myelin basic protein (MBP). The aim of this study was to investigate the prevalence and significance of antibodies against both epitopes in sera from patients with systemic autoimmune diseases, and to compare the humoral responses produced after rabbit immunization. Peptides 147–154aa of La/SSB and 139–146aa of the MBP were attached on tetrameric sequential oligopeptide carriers and used for immunizations of New Zealand White rabbits. Antibodies to immunizing peptides, as well as to the peptides corresponding to other previously defined La/SSB epitopes (289–308aa, 349–364aa), to the intact human MBP (hMBP) and to the recombinant human La/SSB (rechLa) proteins, were identified using specific ELISA assays. Sera from 45 patients with Sjogren's syndrome (pSS), 49 with Systemic Lupus Erythematosus (SLE), and 18 with Rheumatoid Arthritis (RA) were tested against the two peptides and the hMBP. Twenty-two per cent of sera from pSS patients, 27% of SLE patients, and none from RA sera reacted with the La epitope; 27% from pSS sera, 22% of SLE sera, and 17% of RA sera gave a positive reaction against the MBP peptide. Finally, 19% of pSS, 30% of SLE, and 38% of RA sera reacted with the hMBP. Thirty-five days after immunization of rabbits with the La epitope, antibodies were produced against all three La/SSB peptides, the MBP peptide, and the hMBP and rechLa proteins. Rabbits immunized with the MBP peptide produced antibodies against the immunizing peptide and the mimicking peptide of La shortly after immunization, whilst antibodies against the other La epitopes and the two intact proteins were produced later. Inhibition experiments in rabbit sera with high reactivity against hMBP, using the MBP peptide as inhibitor, revealed that 80% of serum reactivity was abolished. In conclusion, a significant proportion of human autoimmune sera reacted with both La and MBP derived peptides, as well as with hMBP. La 147–154aa peptide, when used for animal immunizations, induces a fast epitope spreading involving both La and MBP. In contrast, the mimicking MBP epitope induces a delayed response against the other La epitopes. Thus, despite the fact that these peptides present molecular similarity, they induce different immune responses.

Keywords: Cross reaction; Epitope spreading; La/SSB; Myelin basic protein; Sjogren's syndrome

1. Introduction

Systemic autoimmune diseases are characterized by the presence of a vast array of autoantibodies directed against organ and non-organ specific autoantigens. Amongst them, autoantibodies to the cytoplasmic RoRNP complexes are frequently found in patients with primary Sjogren's syndrome (pSS) and Systemic Lupus Erythematosus (SLE). Most of these autoantibodies are directed against the protein components of the complex, Ro52, Ro60, and La48 kD [1–3]. During the past several years, an effort has been undertaken by others and ours

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laboratories, in order to identify the fine specificity of autoantibodies against RoRNPs. The identification of the major epitopes would allow us to define better the disease subgroups, to study in depth the structure and the biological properties of a given epitope, and to develop assays with higher sensitivity and specificity for the antibody detection [4].

Previous studies in our laboratory, using sera from patients with SLE and pSS and the mimotope peptide scanning technique revealed that the majority of sera bind to four distinct B-cell epitopes of La/SSB [5]. These epitopes lie within the regions 145–164aa, 289–308aa, 301–320aa, and 349–364aa [6]. Testing of a large number of human sera against peptide analogues corresponding to these regions, demonstrated that they have a high sensitivity and specificity for the detection of anti-La/SSB antibodies [6]. T-cell epitopes of La/SSB protein have also been described in the literature. They have been mapped within the sequences 61–84aa and 289–299aa of the molecule [7,8].

Examination of the aminoacid sequences of the four B-cell epitopes using protein databases has led to the discovery that the region 145–164aa of La presents 83% sequence similarity with the region 139–146aa of the human myelin basic protein (MBP). MBP is considered to be one of the major autoantigens in patients with multiple sclerosis (MS) [9,10]. MBP and La/SSB proteins share in common some immunological features. They are both disease specific autoantigens, they contain both B- and T-cell epitopes, and the immune response against them appears to be antigen driven. Finally, when fragments of these two proteins are used for animal immunizations, they can induce spreading of the immune response.

The aim of the current study was to investigate the humoral immune responses against both regions of La/SSB and MBP in sera from patients with autoimmune diseases and sera from immunized rabbits. Thereafter is shown that a significant proportion of SLE and pSS patients with anti-Ro/La immune responses possess also antibodies directed against the homologous with La/SSB region 139–146aa of MBP. In addition, La epitope was able to produce intramolecular spreading of the immune response against all La/SSB epitopes and the generation of specific antibodies targeting the MBP mimicking peptide, after immunization of the experimental animals. On the other hand the MBP peptide with the sequence similarity induced a delayed epitope spreading in comparison with the homologous La peptide. Thus, this study suggests that despite the high sequence similarity between two epitopes from two different autoantigens, they can induce different immune responses. Though, after a long postimmunization period of time it was observed that both peptides caused the development of the same antibodies.

Table 1
The physicochemical properties of the two homologous peptides of La/SSB (147–154aa) and MBP (139–146aa) proteins.

	La/SSB ^{147}HKAFKGSI^{154}	MBP ^{139}HKGFKGVD^{146}
Molecular weight	887.0	887.0
Theoretical pI	10.0	8.6
Hydrophobic residues	A, F, I	F, V
Positively charged residues	K, K	K, K
Negatively charged residues	None	D
Aliphatic index	61.25	36.25

2. Materials and methods

2.1. Peptide properties and peptide synthesis

Regions 139–146aa of MBP and 147–154aa of La/SSB protein have been found to present a high degree of homology. Between these two homologous sequences, there are three aminoacid differences. More specifically an alanine (Ala), a serine (Ser), and an isoleukine (Ile) residue in the region of La/SSB is substituted by a glycine (Gly), a valine (Val), and an aspartic acid (Asp) residue respectively in the sequence of MBP. Table 1 demonstrates the physicochemical properties of these two regions as defined from their aminoacid sequences. One can see that these two peptides are quite similar, since they have the same molecular weight, identical pI, and they contain approximately the same aminoacid residues. The aliphatic index value demonstrates also that these two oligopeptides possess approximately similar hydropathic properties. From conformational studies of the hMBP molecule it has been found that the region of the protein that we have examined lies in a loop located to the outer surface of the protein, as illustrated in Fig. 1 [11,12]. Such surface exposed protein regions usually posses high mobility and they are more easily accessed by antibodies [13,14]. Thus, these protein regions are more likely to contain B-cell epitopes.

Peptide analogues of the three B-cell epitopes of human La/SSB protein ^{147}HKAFKGSI^{154} (pep147–154), ^{289}ANNGNLQLRNKEVTWEVLEG^{308} (pep289–308), and the ^{349}GSGKGKVQFQGKKTKF^{364} (pep349–364), as well as the ^{139}HKGFKGVD^{146} region of the MBP (MBP peptide), were constructed using the Fmoc strategy based on the solid-phase peptide synthesis [15]. Each of the synthesized peptides was attached to tetrameric sequential oligopeptide carriers (SOC_4) [16]. An irrelevant peptide IASRYDQL (corresponding to the sequence 250–257aa of Leismania glycoprotein gp63), attached to SOC_4 carrier [$(IASRYDQL)_4$-SOC_4] was also constructed, in order to be used as a control peptide for the study.

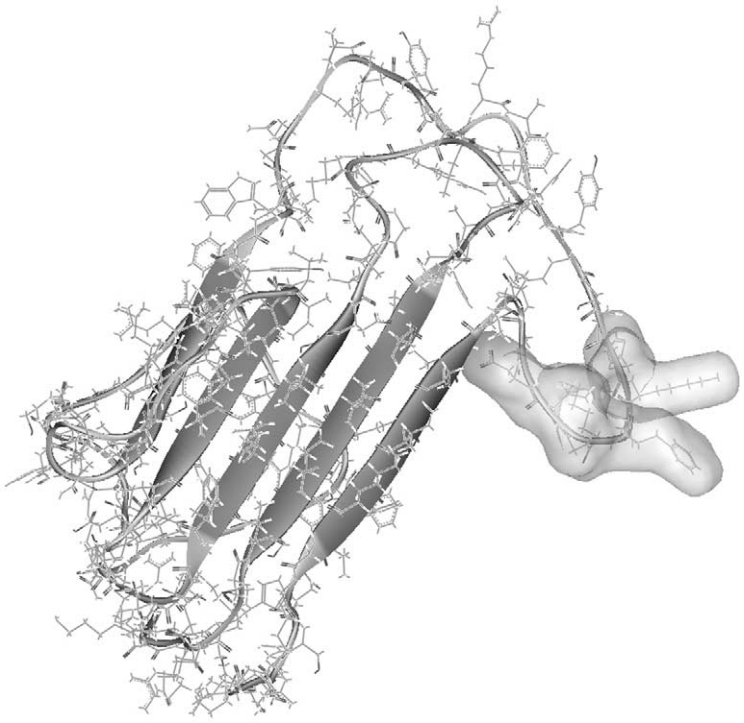

Fig. 1. Three dimension configuration of the human MBP molecule, as it is believed to be from experimental data. The protein region marked in gray is the region 139–146aa that is homologous with the pep147–154aa of La/SSB.

2.2. Preparation of La/SSB and hMBP intact proteins

Human MBP (hMBP), purified from human brain according to previously described methods [17], was purchased from Advanced ImmunoChemical Inc. Recombinant human La/SSB whole protein (rechLa) was constructed using the methodology that has been previously described by Bachman et al. and Troster et al. [18,19].

2.3. Human sera

Sera were obtained from 45 patients with pSS that satisfied the specified European criteria [20], 49 patients with SLE [21], 18 patients with rheumatoid arthritis (RA) [22] and 43 normal subjects. These sera were tested for the presence of antibodies directed against the two homologous peptides as well as hMBP and rechLa. All sera had been previously screened for the presence of anti-Ro/SSA and anti-La/SSB autoantibodies by counterimmuno-electrophoresis (CIE) and immunoblot, as described previously [6].

2.4. Animal immunizations

The synthesized peptides 147–154aa of La/SSB and 139–146aa of the MBP were used for the immunizations of New Zealand White female rabbits, 6–8 weeks old with complete Freud's adjuvant. In each immunization 0.5 mg of immunogen was used according to previously described protocols [23]. The oligopeptide carrier SOC_4 alone was used for the immunization of the rabbits that were used as control rabbits for this study. Successive bleedings of the immunized animals were performed at days 0, 11, 35, 81, 132, 181, 213, 227, and 281.

2.5. ELISA assays

Specific anti-peptide ELISA assays were developed and performed in order to detect the antibodies in human sera as well as in rabbit sera, after the animal immunizations. Sera from all animals were tested against the peptides pep147–154, pep289–308, pep349–364, control, and MBP peptide. Sera from patients were tested for reactivity against the two homologous peptides and against the intact hMBP and rechLa proteins. Sera from the rabbits immunized with the MBP peptide were also tested against the two intact proteins. 96-well polystyrene plates (Costar, Corning, NY, USA) were coated with the solution of the coating peptide at a concentration 10 μg/ml in buffers with pH=7.26 for the MBP, pep349–364, and control peptides, and with pH=9.25 for the pep289–308 peptide. The concentration of the coating solution of the hMBP molecule was 4 μg/ml in buffer with pH=9.6 and the concentration of the rechLa coating solution was 5 μg/ml in buffer with pH=9.2. Non-specific binding was blocked using blocking buffer consisting of bovine serum albumin (BSA)

2% w/v in phosphate buffered saline (PBS) pH 7.4. Afterwards sera were added in a dilution of 1:200 in blocking buffer. After an incubation period for 2 h in room temperature, the ELISA plates were washed three times with PBS. Alkaline phosphatase-conjugated affinity purified anti-rabbit IgG (Jackson Immunoresearch), diluted 1:2300 for the assays with the human sera and 1:1400 for the rabbit sera assays in blocking buffer, and p-Nitrophenyl Phosphatase Disodium substrate (Sigma, St Louis, USA) was subsequently added and the absorbance was measured at 410 nm by an ELISA reader (Dynatech, UK). The optimum concentration of the reagents used was selected after preliminary experiments. The cut-off point for the assays was set as the mean OD values plus three standard deviations of sera from 43 normal individuals.

2.6. Inhibition assays

The specificity of the immune response against the immunizing peptides was assessed using inhibition assays. In homologous inhibition experiments, the serum from each immunized rabbit was tested in ELISA assays against the immunizing peptide and in cross-inhibition experiments the serum of each rabbit was tested against the other peptide than that used as immunogen. Briefly, serum from rabbits immunized with pep147–154, taken at immunization day 213, was diluted 1:200 in blocking buffer and preincubated with the peptides pep147–154, MBP, and the control peptide at concentrations 5, 50, 100, and 200 μg/ml. In all samples SOC_4 backbone, without peptide was added at a concentration of 10 μg/ml, in order to block any anti-SOC_4 antibodies that may have been produced by the animals and can potentially interfere with the assay. Subsequently, the samples were tested in the anti-peptide ELISA assays as previously described at the ELISA assays section. Inhibition experiments were also performed using the sera of animals immunized with the MBP peptide. Serum taken at immunization day 181 was preincubated with the MBP peptide and subsequently tested in assays against the hMBP molecule. Serum dilution and inhibitor concentration were the same as described above. The results from the inhibition experiments were calculated according to the formula:

$$\%\text{Inhibition} = 100 \times [1 - (\text{inhibited } OD_{410\,nm} / \text{uninhibited } OD_{410\,nm})]$$

3. Results

3.1. Prevalence of anti-pep147–154, anti-MBP peptide, and anti-hMBP antibodies in sera from patients with autoimmune diseases

Sera from patients with autoimmune diseases (pSS, SLE, and RA), as well as sera from normal subjects, were tested for reactivity against the two homologous peptides pep147–154 and MBP, and the whole hMBP protein. All autoimmune sera were found to be positive for anti-Ro and anti-La antibodies when tested by CIE. The results from the anti-peptide ELISA assays (pep147–154 and MBP) are depicted in Fig. 2A and B respectively. A significant proportion of human sera from patients with autoimmune diseases reacted with both peptides. More specifically, 22% of sera from patients with pSS, 27% of sera from patients with SLE and none from RA sera gave a positive reaction against the peptide pep147–154. In addition, 27% of sera from pSS patients, 22% of SLE, and 17% of the RA sera reacted with the MBP peptide. None of the sera from normal individuals reacted with the peptides. Nineteen

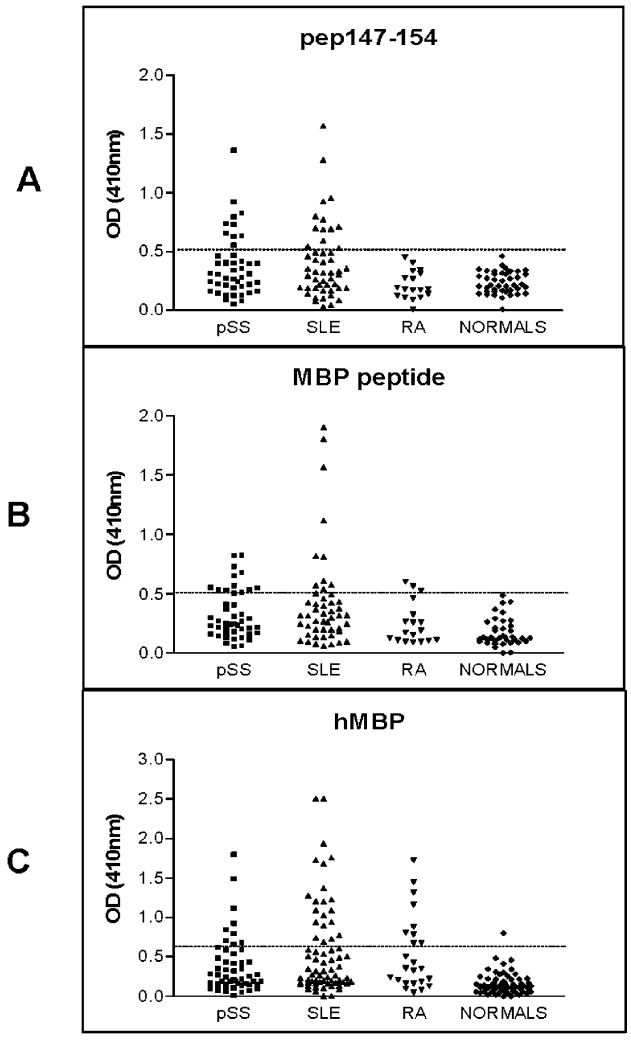

Fig. 2. Prevalence of antibodies in human sera against the (A) pep147–154, (B) MBP peptide, and (C) hMBP molecule. All autoimmune sera were found to be positive for anti-Ro/La antibodies. The dotted lines in the figures represent the cut-off values of the experiments, and were calculated from the mean optical density (OD) value of the sera from normal individuals plus three standard deviations (mean+3 × SD). All the values above the cut-off limit can be considered as positive values.

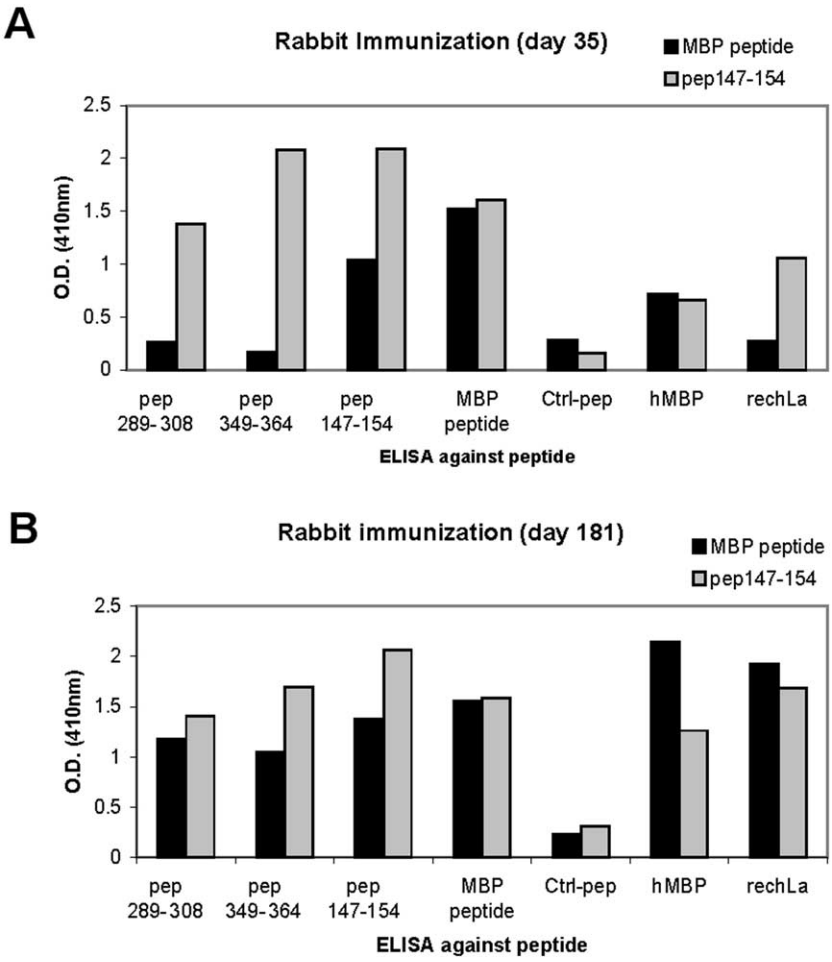

Fig. 3. Levels of antibody production of the immunized animals, against the three epitope peptide analogues of La/SSB (289–308aa, 349–364aa, and 147–154aa), the MBP peptide, the control peptide (Ctrl-pep), and the intact MBP (hMBP) and La/SSB (rechLa) proteins: (A) immunization day 35 and (B) immunization day 181. The black bars represent the antibody levels in sera from the rabbits immunized with the MBP peptide, while the gray columns represent the antibody levels in sera from the pep147–154 immunized rabbits respectively.

percent of the pSS sera, 30% of the SLE sera, and 38% of the RA sera reacted with the hMBP molecule (Fig. 2C). Only one of the sera from normal individuals gave a positive reaction against hMBP. No clinical differences between patients with and without anti-MBP antibodies were observed. The recognition of both La/SSB and MBP peptides by almost the same proportion of pSS and SLE sera, supports furthermore the hypothesis of molecular mimicry between the two peptides. La/SSB appeared to be more antigenic compared to MBP, since the sera tested are from patients with pSS or SLE, diseases characterized by prominent anti-La response.

3.2. Kinetics of the immune response to La/SSB and MBP, after rabbit immunizations with La/SSB epitope and its homologous MBP peptide

3.2.1. Immune response against the MBP and La/SSB peptides

Sera from the rabbits immunized with the epitope 147–154aa of La/SSB and the peptide corresponding to the region 139–146aa of MBP were collected after each immunization and tested for their reactivity against the two immunizing peptides and the other two B-cell epitopes of La/SSB, pep289–308 and pep349–364. Rabbit sera taken at two different immunizations, one early (day 35) and one late at the immunization period (day 181), were also examined for the presence of antibodies against the hMBP and rechLa molecules as well. After the first immunization, serum examination on day 35 showed that the animals immunized with the La/SSB epitope produced antibodies directed towards not only against the immunizing epitope, but also against the mimicking MBP peptide (Fig. 3A). Furthermore, the epitope spreading that occurred is attested by the presence of high titer antibodies against the two other epitopes of La/SSB, as well as against the rechLa and hMBP molecules. The titer of the antibodies produced was high since the OD values ranged from 1.50–2.00. At day 35 rabbits immunized with the mimicking epitope of MBP produced high titers of

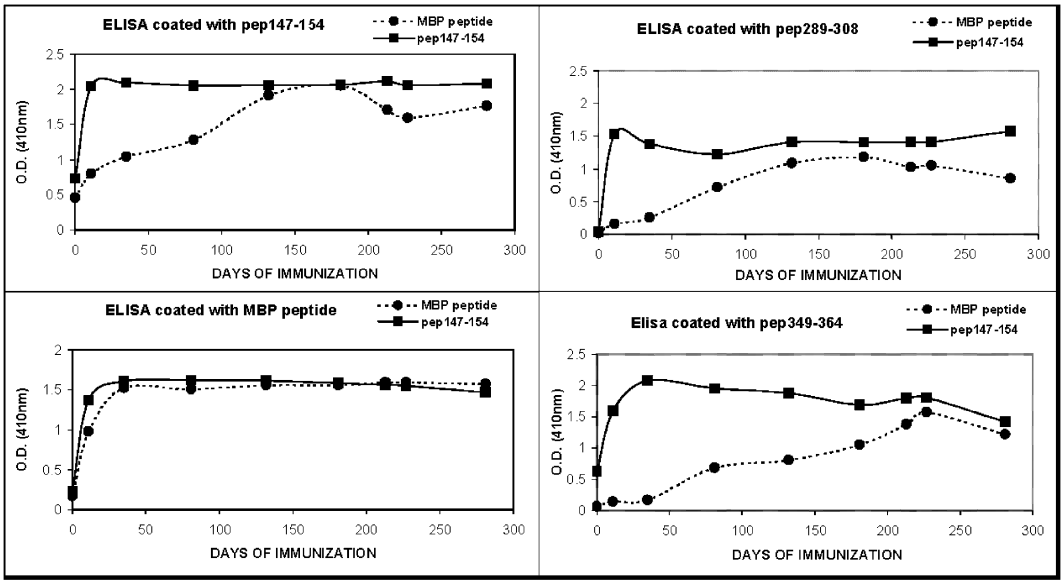

Fig. 4. Kinetics of the humoral immune responses of the immunized rabbits against the four tested peptides (289–308aa, 349–364aa, 147–154aa of La/SSB protein and 139–146aa of MBP), during the whole immunization period. The dotted line represents the immune response of the rabbits immunized with the MBP peptide (139–146aa) and the continuous line the immune response of the animals immunized with the La/SSB peptide (147–154aa).

antibodies against the immunizing peptide but low titer antibodies against the three La/SSB epitope analogues and the hMBP and rechLa proteins. Sera from the immunized animals did not react against the control peptide.

At the late immunization days (day 181), all rabbits, independently of the immunizing peptide, developed high titer of antibodies against all the four tested peptides of La/SSB and MBP, as well as against the intact hMBP and rechLa proteins (Fig. 3B).

Detailed analysis of the kinetics of antibody generation from the whole immunization period revealed that rabbits immunized with pep147–154 of La/SSB developed a sustained, high titer of antibodies against all four tested peptides, since the early immunization days, which remained high during the whole immunization period (Fig. 4). On the other hand, rabbits immunized with the MBP, demonstrated a delayed production of antibodies against the peptides corresponding to the three major B-cell epitopes of La/SSB. Again no interaction between the animal sera and the control peptide was observed.

3.2.2. Immune response of rabbits immunized with the MBP peptide against the whole hMBP and rechLa proteins

Sera from animals immunized with the MBP peptide (139–146aa of MBP) were tested for the presence of antibodies against hMBP and rechLa. At the early immunization days, low titers of antibodies to hMBP were quantified, which increased gradually during the immunization period, reaching finally an O.D. value of approximately 2.05 after the last immunization. The same animals demonstrated a more delayed antibody production against rechLa, probably representing the development of high affinity anti-La/SSB antibodies (Fig. 5).

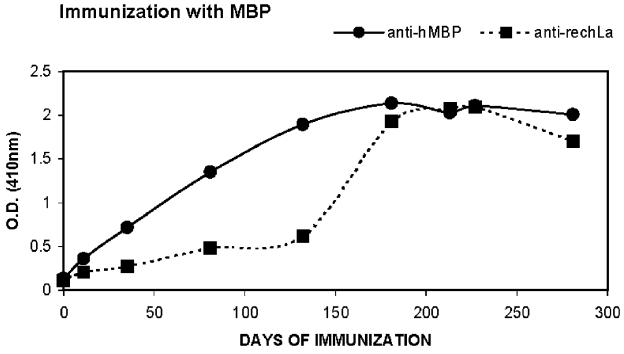

Fig. 5. Evaluation of the immune response of the rabbits immunized with the MBP peptide against the two intact proteins: the hMBP (continuous line) and the recombinant La/SSB protein (dotted line).

3.3. Study of the specificity and cross-reaction of antibodies, using inhibition experiments

Inhibition experiments were performed in order to investigate whether pep147–154 of La/SSB and MBP peptide could inhibit the binding of antibodies produced

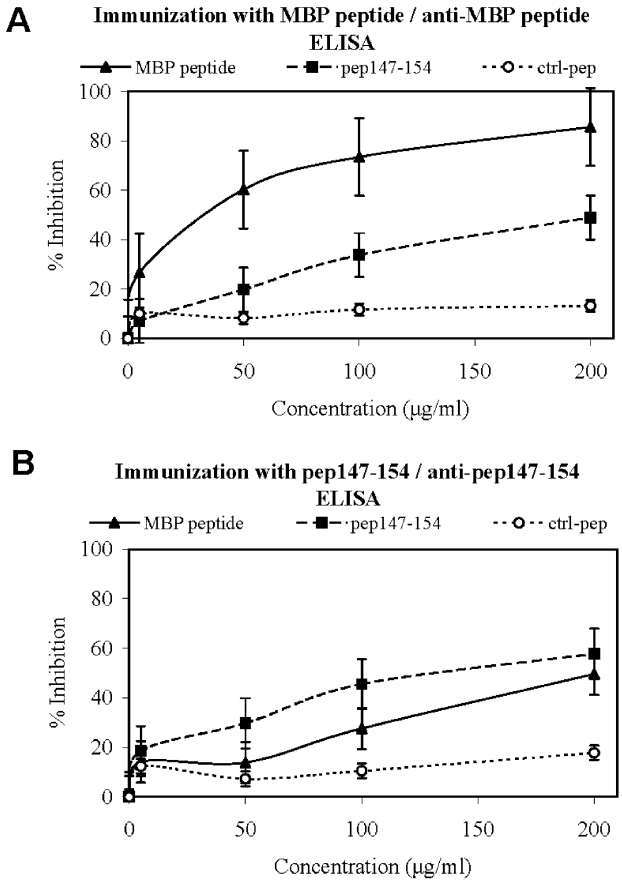

Fig. 6. Results from the homologous inhibition experiments using sera from both immunized animals: (A) inhibition anti-MBP antibodies from MBP peptide immunized animals with MBP, pep147–154, and control peptides in anti-MBP peptide ELISA assay, (B) inhibition anti-pep147–154 antibodies from the pep147–154 immunized animals with MBP, pep147–154, and control peptides in anti-pep147–154 ELISA assay.

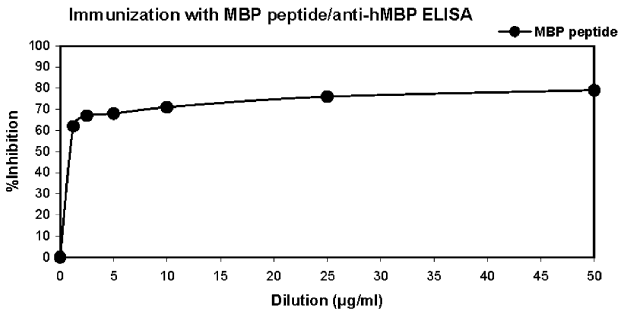

Fig. 7. Results from the homologous inhibition experiment using sera from MBP peptide immunized animals inhibited by the MBP peptide (139–146aa) in anti-hMBP ELISA experiments. The reactivity of the sera was inhibited by 80%.

from the immunized rabbits, to the homologous peptides or to the whole MBP molecule, when they were used as coating peptides in ELISA assays. When the MBP peptide was used as inhibitor in the serum of animals immunized with the MBP peptide in anti-MBP peptide ELISA, it could inhibit the binding of the antibodies produced by approximately 86%. The reactivity of sera from the animal immunized with the MBP peptide was inhibited by 50% when pep147–154 of La/SSB was used as inhibitor. The reactivity of the serum from the animal immunized with the pep147–154 from La/SSB protein was inhibited by 50% and 58% when the MBP peptide and the pep147–154 were used as inhibitors respectively. No inhibition was observed in all cases, when the control pep was used as the inhibitor (Fig. 6A and B).

Sera from the animals immunized with the MBP peptide were also preincubated with the same (immunogenic) peptide and tested in ELISA against hMBP. This experiment showed an inhibition of approximately 80%, pointing to the fact that the majority of the antibodies against hMBP are those directed against the epitope 139–146aa of MBP (Fig. 7).

Sera from immunized rabbits were also tested in cross-inhibition experiments. In these assays the serum reactivity of animals immunized with the MBP peptide, was inhibited by 67% and 83% in pep147–154 ELISA, when the MBP and pep147–154 peptides were used as inhibitors respectively (Fig. 8A). Similarly, the reactivity of the serum obtained from the animals immunized with the pep147–154 of La/SSB protein, was abolished by 66% and 48% when the peptides MBP and pep147–154 were used as inhibitors in MBP peptide ELISA respectively (Fig. 8B). This experiment suggested that antibodies against the peptides pep147–154 of La and pep 139–146aa of MBP cross-reacted.

4. Discussion

Despite the progress of molecular immunology and the large body of knowledge, achieved over the past several years, the origin of autoimmune diseases remains poorly understood. The key element in autoimmune diseases is the production of autoreactive B- and T-lymphocytes, directed against different organ or non-organ specific autoantigens. The latter are, in their vast majority, large subcellular complexes composed of a number of proteins, non-covalently associated with nucleic acids. Among these diseases, antibodies to cytoplasmic ribonucleoproteins (named also hy or Ro RNPs) are usually detected in primary Sjogren's syndrome and SLE. In the last years, a number of investigators have attempted to define the fine specificity of autoantibodies (B cell epitopes) and to a lesser extent of autoreactive T cells (T cell epitopes). The identification of major epitope(s) allows: (a) to define more homogenous disease subgroups, (b) study in depth the structure and the biologic properties of a given epitope, (c) develop assays with higher sensitivity and specificity for

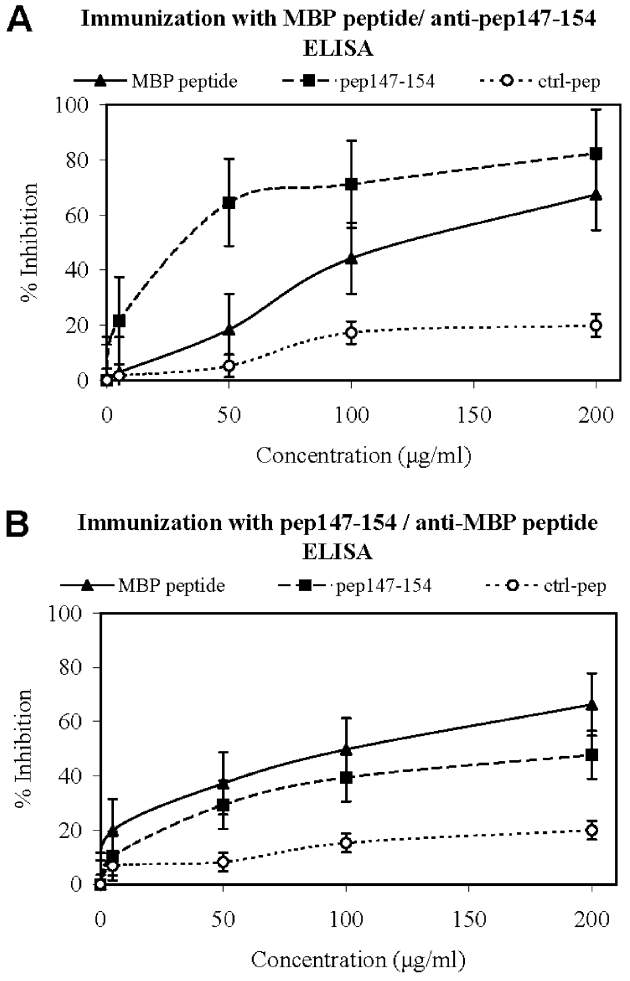

Fig. 8. Cross-inhibition of sera reactivity against the immunizing peptides: (A) Inhibition of the reactivity of sera from rabbits immunized with the MBP peptide by soluble MBP, pep147–154, and control peptides in anti-pep147–154 ELISA assay, (B) Inhibition of the reactivity of sera from rabbits immunized with the pep147–154 by soluble MBP, pep147–154, and control peptides in anti-MBP peptide ELISA assay.

autoantibody detection, and (d) create therapeutic tools targeting highly specific structures within the moiety of the autoantigens. The B-cell epitopes have been studied more extensively, since the handling of autoantibodies is easier, compared to autoreactive T cells. B cell epitopes can be either linear, formed by adjacent aminoacid residues in the primary structure of a protein, or conformational, consisting of aminoacid residues from distant regions in the sequence of a given protein, that are spatially juxtaposed upon folding. Ro and La proteins contain multiple linear and conformational epitopes. Though, the initiation of the autoimmune response is not well understood.

Among the major mechanisms, thought to be involved in the initiation of autoimmune response is molecular mimicry. In order for molecular mimicry to take place, two molecules should share homology in their linear primary structure, leading eventually to a conformational fit. The homology between a foreign molecule (xenoantigen) and a self molecule can mislead the immune system, and induce an immune response against the self molecule [24]. Viral infections can induce autoimmune responses by cross-reaction of specific viral antigens with self-proteins. [24–27]. On the other hand, the contribution of molecular mimicry towards the pathogenesis of autoimmune diseases is still not clear.

In a previous study it was shown that the B-cell epitope 147–154 of La/SSB presents sequence similarity with the region 139–146aa of MBP [6]. Prompted by this observation we have investigated the immunologic response against these two regions in human autoimmune sera as well as in rabbits, after immunization. Twenty-two to twenty-seven percent of sera from patients with SLE and pSS patients contained antibodies against the peptides derived from La/SSB and MBP. Furthermore, 18–30% reacted also with affinity purified hMBP. However, the presence of anti-MBP antibodies in sera from patients with SLE and pSS is not reported in the literature so far. In the present study it was shown for the first time that sera from patients with systemic autoimmune diseases contain antibodies that react with the MBP molecule, which possess a homologous region with the La/SSB autoantigen [28,29]. Previous studies have demonstrated that a proportion of patients with SLE could present with manifestations from the central nervous system (CNS), including multiple sclerosis [29–31]. Despite the fact that a significant proportion of the patients tested had anti-MBP antibodies, none of them presented with CNS disease.

In the present study it is demonstrated for the first time that highly homologous regions derived from different autoantigens can induce different immune responses, with regards to the kinetics and the levels of antibody production. In fact, rabbits immunized with the peptide analogue of the La/SSB epitope, mounted an early immune response, not only against the immunizing peptide but also against the other two B-cell epitopes of La/SSB, as well as the rechLa. These animals produced also antibodies that reacted with the MBP derived peptide as well as with whole hMBP protein. The same animals did not develop any antibodies against the irrelevant peptide that was used as the control peptide. On the other hand, animals immunized with the MBP peptide had different immune responses against the same antigens, compared with the La peptide immunized animals. Although these animals produced antibodies against both the immunizing peptide of MBP and the pep147–154 of La/SSB, shortly after the immunizations, they developed an immune response against the other B-cell epitopes of La and against the two full-length proteins (rechLa and hMBP) only at the very late stages of the immunization course (day 181).

With both immunogens the animals produced first antibodies against the small B-cell epitopes, followed later by antibodies against the intact molecules. Thus, it appears that shortly after the immunizations, the rabbits developed low affinity antibodies for the peptide targets. Later the anti-peptide antibodies produced were of high-affinity for their targets through the process of affinity maturation [32]. This process is antigen-driven involving somatic mutations of the produced antibodies. Small peptides, such as the pep145–164 of La/SSB and 139–146aa of MBP, are more flexible than full-length proteins. As a result, they can exist in more than one different conformations, fitting the antigen binding site of the low-affinity antibodies that are produced early at the immunization period. On the other hand, the corresponding linear B-cell epitopes within the moiety of large proteins have more rigid conformations, only allowing the interaction with antibodies of high affinity. These antibodies are usually produced at the final stages of the immunizations [33,34].

In order to investigate the specificity of the immune response in immunized animals, inhibition assays using the peptides as inhibitors were performed. These experiments showed that in sera from animals immunized with the MBP peptide, the same peptide could inhibit almost completely (86%) the binding of antibodies, while pep147–154 inhibited this reaction only partially (49%). The same was observed in inhibition experiments, using sera from rabbits immunized with the epitope of La/SSB. As previously, the immunizing peptide inhibited more efficiently the binding of the anti-peptide antibodies in the solid phase, than the mimicking MBP peptide. These data revealed that in either case the immunized animals developed antibodies, bound in both peptides, albeit much more specifically with the immunizing peptide. This observation reflects most probably differences in the affinity of the anti-peptide antibodies for their targets. The relation between the two antibody populations was confirmed with cross-inhibition assays, where the peptides 147–154aa of La/SSB and 139–146aa of MBP were equally capable of inhibiting the reaction of the antibodies that were produced against the peptides. When sera from rabbits immunized with the MBP epitope were preincubated with the immunogenic peptide and subsequently tested against the whole hMBP, an inhibition of antibody binding by more than 80% was observed. This experiment indicates that the majority of anti-hMBP antibodies, produced after rabbit immunization with pep139–146 of MBP, are directed specifically against this particular peptide.

The knowledge of the structure of autoepitopes is important for the understanding of the mechanism(s) through which autoimmune responses are triggered and autoantibodies are generated. Studies in sequential human sera from patients with SLE and immunization of experimental animals with the epitopes showed that: (a) early in the course of the disease or the immunization dates, the autoantibody response is limited and directed against particular epitopes, while with time, it expands, involving neighboring or even distant epitopes within the complex of the autoantigen; this phenomenon is called epitope spreading [1,35] and (b) revealed putative T cell epitopes on the autoantigens [7,8] which are mainly responsible for the initiation, augmentation and perpetuation of the aberrant autoimmune reactivity. Thus, it appears that the autoimmune response is an antigen driven dynamic process, very similar to that observed in a specific immune response against foreign antigens. Spreading of the immune response after animal immunizations with peptides derived from either MBP or La/SSB has been described in the literature. In fact, a number of previous studies have shown that immunizations of mice with the major T-cell epitope 84–104aa of MBP can induce antibodies and T-cell responses against other epitopes of MBP [36–39]. In addition, immunizations of rabbits or mice with peptides corresponding to B- or T-cell epitopes of La induce the development of antibodies, not only against the specific epitope but also against other epitopes of La/SSB [8,40].

The major mechanism that has been proposed for the induction of epitope spreading, involves the crucial role of the T-helper cells. The administration of exogenous peptides of human La/SSB protein in the animals increases the concentration of this protein, that is responsible for the priming of T-helper cells. In turn, B-lymphocytes presenting in their MHC-II molecules the immunizing peptide, are activated by these primed T-cells, and induce the humoral immune response against this epitope. Furthermore, the primed T-helper cells can activate other B-cells specific for different protein components of the RoRNP complex. There is evidence that these B-cells have the ability to bind and internalize the whole RNP complex, via their surface immunoglobulins, and then to present different protein parts through their MHC-II peptides. Thus, the activated B-cells produce antibodies of different specificities leading to a full blown autoimmune response [7,41,42].

This mechanism requires that the immunogenic peptide shares a high degree of homology with the corresponding endogenous region, since the induction of immune response against the endogenous peptide is decisive for the development of epitope spreading. Scofield et al. [43] used three peptides corresponding to the B-epitopes of human Ro-60kD autoantigen for the immunizations of BALB/c mice. They found that only immunizations with the peptides that shared high degree of homology with the corresponding endogenous mouse Ro-60kD could induce spreading of the immune response against the other epitopes of Ro60kD. Taken together it appears that the different kinetics of the immune responses in rabbits, following the immunizations with the two homologous peptides of La/SSB and

MBP is probably due to the difference in homology between these sequences with the corresponding sequence of rabbit La/SSB. Rabbit La/SSB protein has not yet been completely sequenced. A small fragment of the whole rabbit protein has been identified (Swissprot accession number Q04504), and this fragment does not include the epitope that corresponds to the examined epitope pep147–154 of human La/SSB.

In conclusion we found that one of the major B-cell epitopes of human La/SSB protein shares 83% of sequence similarity with the region 139–146aa of MBP. A significant proportion of the patients with systemic autoimmune diseases, such as SLE, pSS, and RA, contain antibodies against not only the La protein, but also against the mimicking MBP peptide and the intact hMBP molecule. Animals immunized with the epitope of La/SSB protein developed an early immune response against the two homologous epitopes, as well as against the other B-cell epitopes of La/SSB, while those animals immunized with the MBP peptide produced early antibodies against the two peptides with the molecular similarity, delayed antibodies against the other epitopes of La. Thus, despite the fact that these two regions (pep147–154 and MBP peptide) share high homology, they can induce different immune responses.

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