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A common serologic finding in systemic autoimmune diseases is the presence of autoantibodies against intracellular autoantigens. Although their pathogenesis is not fully understood, autoantibodies are important tools for establishing diagnosis, classification and prognosis of autoimmune diseases. In Systemic Lupus Erythematosus (SLE) and Sjögren's syndrome (SS) autoantibodies mainly target multicomponent ribonucleoprotein complex Ro/La RNP. The last years, the main characteristics, the clinical significance of the anti-Ro/SSA and anti-La/SSB autoantibodies, their biologic function, as well as their B-cell antigenic determinants (epitopes) have been addressed. More specifically, the structural characteristics and clinical associations of epitopes along with their utility as tools to investigate the autoimmune response have been investigated in detail. New insights for the pathogenetic role of epitopes in initiation, propagation and regulation of systemic autoimmunity have been emerged. In this regard, the role of epitope spreading in the diversification of autoimmune response and the anti-idiotypic antibodies in the regulation of autoantibodies (idiotypic) response are addressed.

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B-cell epitopes of the intracellular autoantigens Ro/SSA and La/SSB: Tools to study the regulation of the autoimmune response

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1. Introduction

In 1991, our laboratory prompted by professor Harry Moutsopoulos, focused on the detection of fine specificity of autoantibodies of intracellular autoantigens. At that time most of the autoantigens had been recently cloned and eventually, their primary structure had been resolved. The purpose of such a study was (a) to understand the structures, within the autoantigen, recognized by autoantibodies and investigate if they share in common sequences with foreign autoantigens, (b) to investigate whether certain epitopes are associated with different diseases, disease subtypes and individual manifestations or even its activity and severity and (c) to develop methods for autoantibody detection, characterized by high sensitivity and specificity.

After several years of intense research, ours and others laboratories realized that the detection and study of B-cell epitopes gave us important insights on the mechanisms involved in the perpetuation and regulation of the autoimmune response. In this review the major advances of B-cell epitopes of intracellular autoantigen, particularly those directed against Ro/SSA and La/SSB are discussed.

2. Major intracellular autoantigens-rationale for the detection of epitopes

Sera of patients with systemic autoimmune diseases often contain autoantibodies directed against intracellular complexes composed of a number of proteins that are non-covalently associated with nucleic acid components [1]. Many of these autoantibodies are essential for the clinical evaluation of patients with systemic rheumatic diseases, since: (i) they are included in the diagnostic or classification criteria of certain systemic autoimmune disorders [2,3], (ii) they are associated with disease activity indices, particularly in SLE [4] and (iii) some of them might be correlated with specific clinical manifestations in the spectrum of a given systemic disease [5]. In basic research, the most intriguing question is why these particular autoantigens, among thousands of molecules expressed in the organism, are selected as targets of the immune system [6]. To address this question, several investigators tried to define the fine specificity of autoantibodies to intracellular antigens, by identifying the antigenic determinants (or B-cell epitopes) recognized most frequently by autoantibodies. The identification of B-cell epitopes revealed useful information on the mechanisms involved in autoantibody production and their diversification in the course of the disease, such as molecular mimicry and epitope spreading [6]. For example, in patients with SLE, at least one autoantibody specificity can be detected 1–9 years before

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the clinical onset of the disease and its diagnosis. The autoimmune response is then spreads in an ordered manner to other autoantigens and the clinical onset of disease coincides with the cessation of new autoantibody specificities development [7]. The earliest autoantibodies detected in the pre-clinical period, as individuals progress toward clinical SLE were antibodies to Ro60 (mean 3.7 years before the disease onset). McClain et al. mapped the initial, pre-disease target of the anti-Ro60 autoantibody response to the region 169–180aa (TKYKQRNGWSHK) of the autoantigen [8]. This region belongs to the previously identified SLE related epitope 169–190aa by Routsias et al. [9], shares sequence homology with Ro orthologs in certain bacteria such as the region KYRQRGGWSHR from the ribonucleoprotein complex of *Mycobacterium smegmatis* and it has been reported to cross-react with a viral peptide (GGSGSGPRHRDGVRR) from the Epstein–Barr virus nuclear antigen-1 (EBNA-1) without to exhibit any sequence similarity [8].

In addition, the characterization of the epitopes of an autoantigen with high sensitivity and specificity, allows the development of immunoassays based on synthetic peptides which can be utilized as substrates for autoantibody detection. As example, fillagrin in RA is recognized by about 70 percent of the patients. Anti-fillagrin antibodies are targeting mainly post-translationally modified epitopes, containing citrulline [10]. Antibodies to cyclic citrullinated peptides (anti-CCP) are detected in RA patients' sera long before the onset of the disease and are associated with erosive disease [11]. In diagnostic grounds, when anti-CCP and RF antibodies are combined, the specificity for RA is exceeded the 95% [12].

3. Structural definition of B-cell epitopes

The B-cell epitopes are diverse in structure and immunoreactivity and thereof they are classified accordingly. On the basis of the nature of the epitope within the parental protein, they are classified as: (i) linear or continuous, consisting of sequential amino acids in the primary structure of the protein, and (ii) conformational or discontinuous epitope, formed by distant regions in the protein sequence coming together in its tertiary structure. In the majority of the epitopes characterized previously as linear, not every amino acid in the sequence is essential for antibody binding. Often, there are sequence-positions that can be successfully substituted with all the 20 naturally occurred amino acids, without any loss of immunoreactivity. In this regard, linear epitopes larger than 5–6 amino acids in length, possess also features of conformational epitopes. Moreover, as the autoantigens are organized in large ribonucleoprotein complexes the term "conformational epitope" can be referred either to epitopes comprised by amino acids distributed on its secondary, tertiary or quaternary structure. Therefore, more properly the epitopes can be divided in:

- *Primary-structure epitope* (or linear epitope), consisting of sequential amino acids. Such epitopes have been identified by synthetic peptide mapping the majority of autoantigens including Ro60, Ro52, La, SmB, SmD, RNP-70 and Scl-70 etc.
- *Secondary-structure epitope*, formed by amino acids distributed in simple three-dimensional structures, such as α-helices or β-sheets. These epitopes have been identified in PM/Scl-100 autoantigen by a combination of peptide scans and mutational analyses. In these studies, epitopes were defined as a local α-helical secondary structure stretch with all amino acids relevant for antibody binding is located at one side of the helix.
- *Tertiary-structure epitopes*, are formed by distant regions of the protein sequence, which are coming together in the tertiary structure. It has been suggested that such conformational epitopes are the main target of some autoantibodies (e.g. anti-Ro60kD).
- *Quaternary-structure epitopes*, which are consisted of amino acids distributed over different subunits within a macromolecular complex, forming a structure recognized by the autoantibody. Such epitopes have been identified in Ro/La RNP complex as well as in nucleosome subunits, composed of histones and DNA elements.
- *Cryptic epitopes (cryptotopes)*. These are usually linear epitopes hidden within the native structure of the autoantigen. They become accessible to antibody binding after disruption of the three-dimensional structure (e.g. by denaturation, proteolytic degradation or chemical modification of the autoantigen). These epitopes are observed in a number of nuclear autoantigens, such as the Ro/La RNP, where the initial Ro60 epitope (for SLE) is cryptic, masked by the binding of hY RNA.
- *Modified epitopes*. Amino acids can be post-translationally modified. Examples of these modifications include: (i) Serine, Threonine, Tyrosine phosphorylation by protein kinases, (ii) Lysine acetylation or ubiquitination, (iii) Cysteine lipidation or oxidation (disulphide-bond formation), (iv) Glutamic acid methylation or γ-carboxylation, (v) Glutamine deamidation (vi) Asparagine (N-linked) and Serine/Threonine (O-linked) glycosylation (vii) Arginine citrullination or dimethylation and (viii) Proteolytic cleavage or degradation. In some instances, side chain modifications of specific amino acids, such as citrullination of arginine residues, are responsible for epitope high-affinity binding. Such modified amino acids have been reported in a variety of human nuclear proteins, including the Sm antigens D1 and D3, and nucleolin. The identification of these modified (usually linear) epitopes requires assays that provide the amino acid in its modified form. These assays are based mainly on synthetic peptides.
- *Neoepitopes*. Neoepitopes can be the post-translationally modified epitopes but also epitopes pre-translationally modified, derived by frameshift mutations or alternative splicing of mRNA. For example, Bachmann et al. [13], identified a mutated La cDNA in patients with SS that contains a frameshift mutation in a hot spot region. This mutation resulted in premature stop codon, which is located eleven amino acids downstream of the frameshift mutation. Consequently, only the sequence of the 12 amino acid La peptide (193–204aa: MKKENKIKWKLN, neoepitope) encoded by the patient's La cDNA markedly differed from the corresponding La protein sequence.
- *Apotopes*. Apotopes are epitopes that are expressed specifically on the surface of apoptotic cells. This term is not widely accepted, since it refers to epitopes that obviously belong to one of the above described categories. However, this term has been used to describe epitopes on Ro60 that differentiate Sjögren's syndrome from SLE.
- *Mimotopes*. Mimotopes are structures that mimic unknown epitopes. They are usually defined using peptide libraries. Such mimotopes can either show close homology to an antigenic sequence of a protein (linear epitope) or, alternatively, are structural homologues with a wide variety of different type epitopes (all the conformational epitope types described previously) including epitopes belonging to non-protein molecules such as polysaccharides, lipids or nucleic acids.

4. Clinical significance of antibodies to Ro/SSA and LaSSB

Anti-Ro and anti-La antibodies are found in approximately 60–90% and 30–60% of patients with primary Sjögren's syndrome, as well as in 30–40% and 10–15% of patients with SLE, respectively [14], depending on the method used for their detection. A variety of methods have been applied for their detection. Among them RNA

precipitation is considered as the gold standard method by various authors. However, this method cannot be used in the every-day clinical practice, but it is useful as reference and confirmatory assay. Agarose gel electrophoresis techniques such as counter-immunoelectrophoresis (CIE) and immunodiffusion (ID) are frequently used for the routine evaluation of sera. A small subpopulation of patient sera, contain precipitin-negative anti-La/SS-B antibodies that is believed to possess restricted epitope recognition. These are detected using anti-La ELISA or immunoblot.

Antibodies to Ro/SSA and La/SSB belong in the diagnostic items of primary Sjögren's syndrome (pSS) and their presence in patients with suspected pSS supports strongly the diagnosis. Furthermore, they are associated with a higher prevalence of extraglandular features, especially vasculitis and higher intensity of the lymphocytic infiltrates in the affected salivary glands [14]. However, so far, no pathogenetic role has been assigned for the disease. Pregnancy in women with anti-Ro and anti-La antibodies, may be complicated by the development of neonatal lupus syndrome in the fetus that includes also increased risk for congenital heart block, the most serious manifestation of this disorder [15]. In this rare syndrome, maternal anti-Ro and anti-La IgG autoantibodies pass through the placenta to the fetal circulation and cause tissue injury to the heart and skin. The redistribution of Ro and La autoantigens on the surface of myocardial cells is required in order to become available for binding of autoantibodies. Such autoantigen translocation can be induced either by β-estradiol, viral infection or apoptosis [16].

5. Epitopes of Ro/SSA and La/SSB

5.1. Structure of the epitopes and clinical significance

Structurally, human Ro/La RNP is composed of one of the four small, uridine rich hY RNAs (human cYtoplasmic RNAs) non-covalently associated with at least three proteins, the Ro52, La and Ro60 autoantigens. Additional components of the complex are also the proteins calreticulin and nucleolin. The localization of these complexes is mainly cytoplasmic. However, Ro60, Ro52 and La autoantigens can also be found in the nucleus but they are not associated with hY RNA. After the assembly of the Ro/La RNP in the nucleus, the complex is rapidly and quantitatively transported to the cytoplasm [17]. Under certain circumstances that include stress, UV radiation, apoptosis or viral infection the protein components of Ro/La RNP are on the cell surface.

Ro52, belongs to the tripartite motif (TRIM) or RING-B-box-coiled-coil (RBCC) protein family, thus comprising an N-terminal RING, followed by a B-box and a coiled-coil region. The RING is a cysteine-rich Zn^{2+}-binding motif of the form C_3HC_4, which binds two Zn^{2+} ions in a tetrahedral manner. The RING is predominantly a protein–protein interaction motif, which also acts as a ubiquitin-protein isopeptide (E3) ligase in the ubiquitination pathway [18]. The B-box is the second Zn^{2+}-binding motif of Ro52 and has the form CHC_3H_2. Ro52 can also homodimerize through its leucine zipper domain [19]. Several different proteomic functions have been suggested for Ro52, including DNA binding, protein interactions and Zn^{2+}-binding. Most probably, overall Ro52 functions as transcription modulator, due to its domain organization. In line with many other RING-containing proteins, Ro52 is involved in ubiquitination pathway [20]. Recent findings suggest that Ro52 autoantigen is a RING-dependent E3 ligase that is overexpressed in patients. In this regard, Ro52 may be directly involved in the reduced cellular proliferation and increased apoptotic cell death that is observed in Sjögren's syndrome and SLE [21]. Ro52-deficient mice develop uncontrolled inflammation and systemic autoimmunity as a consequence of minor tissue injury caused by ear tagging [22]. The characterization of immune cells derived from Ro52-deficient mice demonstrated that in addition to ubiquitinating the previously reported targets IRF3 and IRF8, Ro52 is required for poly-ubiquitination and degradation of IRF5. Ro52-deficient bone marrow-derived macrophages and splenocytes released more inflammatory cytokines (IL-6, TNF, type I IFN, and IL-23) upon TLR activation. In this regard the ubiquitin ligase Ro52 is induced by IFN activation of immune cells, acting as a negative regulator of IFN signaling [23].

The B-cell epitopes of Ro52 have been mapped in various studies with different methods (Table 1). The major immunoreactivity of Ro 52kD autoantigen was localized, using recombinant Ro52 fusion proteins, in the middle coiled-coil region of Ro52 [1]. The 190–245aa region of the amino acid sequence was reactive with almost all anti-Ro52 positive sera and was independent of associated diseases [24]. An epitope spanning the 200–239aa of Ro52, which contains the complete leucine zipper motif, has been also identified in the same region [25]. Autoantibodies against this epitope were associated with Neonatal Lupus and Congenital Heart Block. These autoantibodies have the potential to bind on the cell surface of cardiomyocytes in primary cultures and cause a dysregulation of the Ca^{2+}-homeostasis, which is followed by apoptosis [26]. Anti-Ro52 antibodies are also found in primary biliary cirrhosis associated with sicca syndrome. The anti-Ro52 antibodies in this setting are directed against a smaller epitope than in primary Sjögren's syndrome [24].

The Ro60 antigen is found in virtually all vertebrate cells as well as in certain bacteria (e.g. *Deinococcus radioreductans*) as well as the nematode *Caenorhabditis elegans* [27]. Its function is related with the quality control or discard pathway for nascent transcripts synthesized by RNA polymerase III (e.g. 5S rRNA precursors). Thus, Ro60 binds misfolded small RNAs (e.g. 5S RNA) leading them to degradation [27]. Recently, the structure of the *Xenopus laevis* Ro60, 78% identical to human Ro60, was solved and found to consist of two distinct domains [28]. One domain resembles the von Willebrand Factor A (vWFA) domain, which is found in extracellular matrix proteins and proteins that function in cell adhesion. The other domain consists of a series of α-helical repeats (HEAT repeats) that are arranged orbicularly around of an inner hole of 10–15 Å ("doughnut"-like structure). This hole most probably holds the 3′ ends of misfolded RNAs while the YRNAs bind to conserved residues to the outside of the "doughnut". Another conserved role for the Ro60 in facilitating cell survival after ultraviolet irradiation has recently emerged from studies in radiation-resistant eubacterium *Deinococcus radiodurans* [29] and mammalian cells lacking Ro60 [30]. Studies of mice lacking the Ro60kD protein suggest also that the normal function of Ro may be important for the prevention of autoimmune disease [30]. In these studies, mice lacking Ro were found to develop autoantibodies and membrano-proliferative glomerulonephritis.

Epitopes of Ro60kD have been described by several authors using a variety of epitope mapping procedures (Table 1). Initially, the major antigenic region of Ro60kD was identified within the central part of the molecule [1] (spanning the 181–320aa, 139–326aa and 155–295aa regions of the sequence, respectively). The exact localization of the antigenic determinants was demonstrated after the application of epitope mapping with synthetic peptides. Wahren et al. identified a major epitope in synthetic peptide 216–245aa, Scofield and associates, identified numerous epitopes covering the entire length of Ro60 [31], presumably, due to extended epitope spreading and our group located the antigenic regions of Ro60kD in the 169–190aa and 211–232aa parts of the antigen [9]. In a clinical study it was shown that the epitope 169–190aa was mainly recognized by anti-Ro positive sera from SLE patients and the epitope 211–232aa from patients with SS. The epitope 169–190aa, was found to share conformational and

Table 1
Epitopes mapped on Ro60, Ro52 and La autoantigens.

Major epitope(s)	Minor epitope(s)	Reference
Ro 60kD autoantigen		
485-492aa	numerous epitopes	R. H. Scofield, et al. Proc Natl Acad Sci U S A 88, 3343 (1991).
181-320aa		M. Wahren, et al. Autoimmun 5, 319 (1992).
139-326aa	410-538aa	D. P. McCauliffe, et al. J Rheumatol 21, 1073 (1994).
155 - 295aa		M. R. Saitta, et al. J Immunol 152, 4192 (1994).
182-236aa 237-292aa		C. H. Veldhoven et al., Clin Exp Immunol 101, 45 (1995).
169-190aa (SLE related) 211-232aa (SS related)	183-204aa, 449-470aa	J. G. Routsias, et al. Eur J Clin Invest 26, 514 (1996).
216-245aa		M. Wahren-Herlenius, et al. Immunol Today 20, 234 (1999).
169-180aa (initial epitope)		M. T. McClain et al., Nat Med 11, 85 (2005).
82-244aa (apotope)		J. H. Reed, et al. Arthritis Rheum 58, 1125 (2008).
193-236aa (SS related apotope)	82-146aa	J. H. Reed et al., Arthritis Rheum, (2010).
Ro 52kD autoantigen		
216-292aa (SLE related) 55-292aa (SS related)		B. Bozic, et al. Clin Exp Immunol 94, 227 (1993).
169-291 (leucine zipper region)		J. P. Buyon, et al. J Immunol 152, 3675 (1994).
136-227aa		I. Blange, et al. J Autoimmun 7, 263 (1994).
277-292aa (SS related)	2-11aa, 107-122aa, 107-126aa, 365-382aa	V. Ricchiuti et al., Clin Exp Immunol 95, 397 (1994).
195-209aa		M. B. Frank, et al. Clin Exp Immunol 95, 390 (1994).
211-232 (leucine zipper)		T. Kato et al., Arthritis Rheum 38, 990 (1995).
190-245aa (SS related)	228-245aa	T. Dorner et al., Hepatology 24, 1404 (1996).
197-245aa	153-196aa	T. Dorner et al., J Rheumatol 23, 462 (1996).
14-54aa (zinc finger)		N. Pourmand, et al. J Autoimmun 11, 11 (1998).
200-239 (CHB related)		L. Strandberg et al., Clin Exp Immunol 154, 30 (2008).
La 48kD autoantigen		
88-101aa, 283-338aa		H. Kohsaka et al., J Clin Invest 85, 1566 (1990).
111-187aa, 346-408aa		Y. M. Weng, et al. J Clin Invest 92, 1104 (1993).
111-187aa	149-223aa	M. Rischmueller, et al. Clin Exp Immunol 101, 39 (1995).
331-343aa		H. Troster et al., J Autoimmun 8, 825 (1995).
349-364aa 147-154aa	291-302aa 301-318aa	A. G. Tzioufas et al., Clin Exp Immunol 108, 191 (1997).
1-107aa, 111-242aa (apotopes)		J. H. Reed, et al. J Autoimmun 31, 263 (Nov, 2008).

antigenic similarity with the HLADR3 β-chain, an HLA class II allele, which has been shown to correlate with the anti-Ro60 response [32]. The same epitope was recently found to be the initial pre-disease target of autoantibodies in individuals, who developed SLE several years later [8]. This early recognized epitope has been reported to cross-react directly with a peptide from the latent viral protein Epstein–Barr virus nuclear antigen-1 (EBNA-1) [33]. Recent studies suggest also that although the exact Ro epitopes were identified as small peptide moieties, their recognition by autoantibodies is conformation-dependent and is dramatically enhanced upon interaction with the molecular chaperone calreticulin [34].

The La antigen is a phosphoprotein binding with a variety of small RNAs, including 5S cellular RNA, tRNA, 7S RNA, and hY RNAs,

all transcribed by RNA polymerase III [35]. In molecular level, it binds a short polyuridylate sequence (poly-U) that exists at the 3-end of almost all nascent pol III transcripts. Moreover, La binds viral RNAs including adenovirus VA, Epstein–Barr EBER, viral and human RNAs possessing internal ribosomal entry elements (IRES) and RNA component of telomerase complex [36]. Structurally the human La contains the La motif in its N-terminal region, a typical RNA recognition motif (RRM) in its central part and an unusual RRM, encompassing residues 229–326aa. The latter is followed by a long, flexible polypeptide that contains a short basic motif (SBM), a regulatory phosphorylation site on Ser366 and a nuclear localization signal (NLS). The three-dimensional structure of the La motif, the central RRM and the carboxyl-terminal RNA recognition domain of the autoantigen were solved. The La motif folds into a winged-helix motif elaborated by the insertion of three helices. The central RRM consists of a four-strand β-sheet attached to two α-helices while the C-terminal domain folds to generate a five-stranded, antiparallel β-sheet surface that is terminated by a long α helix. It appears that both the La motif and the adjacent central RRM are required for high-affinity poly-U RNA binding, and that the C-terminal RRM, in conjunction with the SBM downstream, contributes to La interactions with non-poly-U RNA targets such as viral RNAs and TOP (define) mRNAs [37]. The specific binding of La to precursor RNA molecules protects them from exonuclease digestion thereby regulating their downstream processing. Other cellular functions of La/SS-B autoantigen include the retaining of precursor RNA molecules in the nucleus, an ATP-dependent helicase activity that melts RNA–DNA hybrids, unwinding ability of double-stranded RNA, association with telomerase, hence influencing telomere homeostasis, an RNA chaperone activity performed by transient bipartite (5′- and 3′-end) binding of nascent transcripts synthesized by polymerase III (e.g. tRNA precursors) and the induction of cap-independent translation (La binds IRES to elements promoting the internal, cap-independent initiation of translation). La protein is vital for mouse development and the establishment of embryonic stem cells. Thus, La−/− offspring is not viable and La−/− blastocyst outgrowths revealed loss of the inner cell mass [38].

During the last decade, the target B-cell epitopes of anti-La/SS-B autoantibodies have been mapped (Table 1) [6]. Some of the La epitopes were found to reside in functional regions of the autoantigen, including the central RNA recognition motif (RRM) and the ATP binding site. However, the interaction of hY RNA with the RRM motif did not affect the autoantibody binding in the same region. In contrast, the interaction of the ATP binding site with ATP abolished the autoantibody binding at the same part of the protein. Highly antigenic peptides were identified in the sequences: ^{147}HKAFKGSI^{154} (147–154aa) (located within the central RRM motif), ^{291}NGNLQLRNKEVT^{302} (291–302aa), ^{301}VTWEVLEGEVEKEA-LKKI^{318} (301–318aa) and ^{349}GSGKGKVQFQGKKTKF^{364} (349–364aa) [39]. The most sensitive and specific epitope in detecting anti-La antibodies was the sequence spanning the sequence 349–364aa, which showed a sensitivity and specificity higher than 90%. The presence of antibodies against this epitope was also associated with the HLA-DQ0501* (Tzioufas Wassmuth, Ann Rheuma Dis). Other epitopes have also been identified in other parts of the molecule using recombinant fragments of La/SS-B or synthetic peptides [1]. Their existence is believed to be correlated with extended intramolecular spreading of epitopes to the entire La/SS-B molecule.

Recently, we constructed *in silico* the overall structure of Ro/La RNP and studied its structural, antigenic and functional aspects (unpublished observations). Human Ro60 protein was built by homology modelling from *Xenopus* Ro (79% similarity), while 81% of human La (spanning amino acids 5–334) was constructed by assembly of 3 parts: (i) 5–202 aa (La motif +RRM1), (ii) 203–224aa (linker region), (iii) 225–334 aa (RRM2 + NRE). hY1 RNA model was built on the basis of its known secondary structure by a stepwise fashion incorporating stem 1 and its terminal uracils from the structures of Ro60 and La that were already solved in complex with RNA. It was found that hY1-RNA completely masks epitopes 169–190aa of Ro60 and 145–164aa of La that were previously associated with SLE but not the SS related epitopes 211–232aa of Ro60 and 349–364aa of La. ELISA experiments confirmed this prediction and it was found that Ro60–hY1-RNA interaction was inhibited by anti-Ro169–190 antibodies, but not by autoantibodies targeting other regions of the molecule as anti-Ro 443–454 or anti-Ro211–232. La–hY1-RNA interaction was inhibited by purified anti-La145–164 antibodies but not by anti-La349–364 and anti-La301–32. Furthermore, electrophoric mobility shift assays (EMSA) demonstrated that Ro60 and La interactions with hY1 RNA can be inhibited by peptides corresponding to epitopes Ro169–190 and La145–164. Therefore, the SLE related epitopes on Ro and La autoantigens appear to be cryptotopes in Ro/La RNP three-dimensional structure, masked by hY1 RNA. On the other hand, SS related epitopes are directly accessible by autoantibodies in Ro/La RNP structure.

6. Epitope spreading

The initiation of a vigorous autoimmune response occurs when tolerance to self-antigens is broken, a phenomenon that has fascinated researchers for over a century. In SLE, the onset and progression of autoantibody development before the clinical diagnosis has been studied in detail. Arbuckle et al., evaluated serum samples obtained from 130 persons before they received a diagnosis of SLE [7]. They found that in 88% of the patients with SLE, at least one SLE autoantibody tested was present before the diagnosis (up to 9.4 years earlier; mean, 3.3 years). Autoantibodies targeting Ro/La RNP appear earlier than anti-Sm and anti-nuclear RNP antibodies (a mean of 3.4 years vs. 1.2 years) [7]. The proportion of SLE patients with anti-Sm or anti-nuclear RNP antibodies increases dramatically in the year before diagnosis, indicating that appearance of these autoantibodies herald the clinical onset of the disease [7]. In this regard, the rate of appearance of new autoantibody specificities has been found to gradually increase until the diagnosis of SLE and to be halted afterwards [7]. The earliest autoantibodies detected in the pre-clinical period, as individuals progress toward clinical SLE were antibodies to Ro60. McClain et al. mapped the initial, pre-clinical disease target of the anti-Ro60 autoantibody response within the domain 169–180aa (TKYKQRNGWSHK) of the autoantigen [8]. This region belongs to the previously identified SLE related epitope 169–190aa by Routsias et al. [9].

After the initial response against Ro60 autoantigen, the autoantibody targets can be expanded to the entire Ro60 molecule by a procedure known as epitope spreading. The term epitope spreading was introduced in the early 1990s to describe the ability of the B and T cell immune response to diversify, at the level of specificity, from a single determinant to many different sites on a given autoantigen [40]. This procedure is not a feature restricted to systemic autoimmune diseases, but is a common characteristic of the adaptive immune responses mounted against different pathogens. Two types of epitope spreading have been described, (i) the intramolecular spreading, in which the autoimmune response spreads in epitopes within the same protein and (ii) the intermolecular spreading that involves also other protein components, physically associated within the same antigenic complex, such as the spliceosome, and the Ro/La particles (Fig. 1). In this regard, McClain et al. showed that following immunization of rabbits with an antigenic peptide of Ro60 autoantigen (274–289aa) led to the

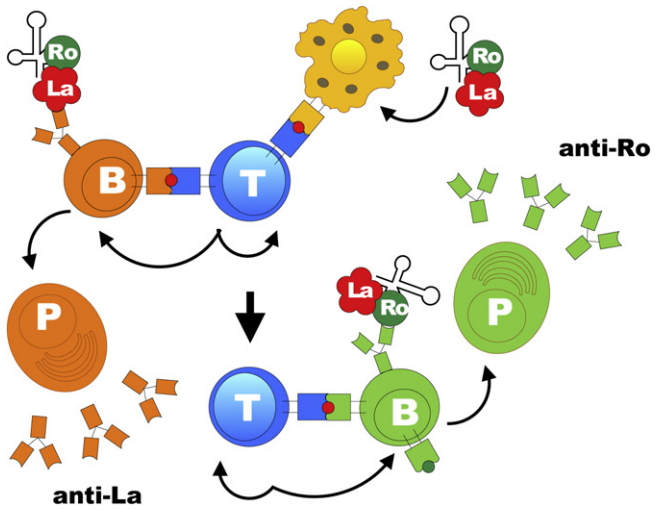

Fig. 1. Model showing mechanisms of B-cell epitope spreading. Antigen-presenting cells (APCs) (macrophages or dendritic cells) capture Ro/La complexes (derived from apoptotic or necrotic material) and present La peptides to Th cells, which become activated and in turn, provide help to autoreactive B cells with anti-La specificity. Clonal expansion of anti-La B cells occurs, following by their diversification to plasma cells, capable of producing anti-La autoantibodies. The same Th cells can activate also B cells with anti-Ro specificity, since these cells, acting as antigen-presenting cells, can capture Ro/La complexes and present La peptides within their MHCII molecules. This results to clonal expansion of anti-Ro B cells, following by diversification to plasma cells, capable of producing anti-Ro autoantibodies.

production of antibodies against multiple epitopes of Ro60 and La. In addition, antibodies to the common spliceosomal proteins Sm-B′, Sm-D1, RNP-A and RNP-C were also produced [41]. These results demonstrated that loss of tolerance to a single antigenic determinant of the autoantigen can initiate and perpetuate an autoimmune response which virtually recreates the humoral autoimmune specificity seen in human SLE. However epitope spreading cannot explain how the autoimmune response "jumps" from one particle (e.g. hy1 Ro/La RNP) to another (e.g. spliceosome). A clue to the mechanism involved in the aforementioned production of antibodies to the common spliceosomal proteins has been recently reported by our group and others [42,43]. Since, most of nuclear autoantigens in lupus are RNA binding proteins and the major epitopes were previously mapped within their RRM motifs [1], molecular similarity of conserved sequences within the RRM could be involved in the intermolecular and inter-particle diversification process of autoimmune response. According to this model, a consensus sequence as the RNP motif, conserved in many nuclear, nucleolar and cytoplasmic antigens, plays the role of a 'driver' epitope. Cross-reactive autoantibodies targeting this epitope have the potential to spread the autoimmune response to other RNA binding proteins through molecular mimicry. Subsequently, intramolecular spreading to these specific proteins can occur. This hypothesis is further supported by the observation that the 'driving' epitope sequence in RNP motif is a CD4+ T cell epitope in lupus mice and is often targeted by autoantibodies, very early during the course of the disease [44]. Remarkably, this sequence is present in components of Ro/La RNP such as Ro60 (aa119–131), La (aa146–158) and nucleolin (aa346–358, aa517–529) as well as in spliceosomal proteins such as RNP-70 (aa139–151) and RNP A (aa47–59, aa239–251). Our recent work suggested that the RRM region of La/SSB can trigger inter-particle B-cell diversification to U1-RNP-70 autoantigen via molecular mimicry [43], confirming the above model. The proposed model can be applied also for other epitopes such as the proline rich region PPPGMRPP that holds a cross-reactive epitope, shared in common by several spliceosomal autoantigens and recently identified as early target of RNP humoral autoimmunity in SLE [45]. Thus, besides intramolecular and intermolecular spreading, the autoimmune response can also diversify and perpetuate via molecular mimicry of "key driving sequences", common in autoantigens targeted in SLE.

7. Complementary epitopes

7.1. Definition and molecular design

In the 1970s Jerne proposed the network hypothesis, in which complementary interactions involving idiotypes and anti-idiotypes of antibodies contributed to the homeostasis of the adaptive immune response [46]. Antibodies produced against an infectious agent can elicit anti-idiotypic antibodies that may have the incidental property of being antibodies to the host structure. Anti-idiotypic antibodies can either "neutralize" the idiotypic antibodies or elicit, upon immunization, antibodies with the parental antigenic specificity. These functions are referred as the idiotypic–anti-idiotypic network serving as intrinsic regulatory mechanism of the adaptive humoral immune responses. Recently, it has been shown that anti-idiotypic antibodies might also act as regulators of the autoimmune response in SLE [47]. Despite the attractive theory, presenting in the opening lines of this section, the detection of anti-idiotypic antibodies in clinical specimens is often challenging, since most autoimmune diseases involve polyclonal responses to self antigen, as a result of the previously mentioned intra- and intermolecular spreading. Moreover, some idiotypes are unique to each patient, and therefore the performance of general studies and assumptions is often difficult or impossible. The isolation of a non-homogenous population of antibodies and the possibility of cross-reactions, mainly due to polyreactive antibodies [48].

Based on the detailed knowledge of the antigenic structures that are recognized by autoantibodies, one can design complementary epitopes, anticipated to be recognized by anti-idiotypic antibodies, as suggested by the "molecular recognition" theory [49]. According to this theory, a sense peptide, transcribed and translated from a nucleotide sequence read in the 5′ → 3′ direction binds to its complementary peptide counterpart, transcribed and translated in frame with that of its sense peptide from a nucleotide sequence read in the 5′ → 3′ direction on the opposite DNA strand. Previous experimental data, utilizing mainly monoclonal antibodies suggest that these interacting complementary peptides have the ability of generating and eventually detecting interacting pairs of idiotypic and anti-idiotypic antibodies [50].

8. Antibodies to complementary epitopes are anti-idiotypic antibodies to autoantibodies

Studies in our laboratory, using peptides and complementary peptides, corresponding to major B-cell epitopes of La/SSB, have demonstrated that in SLE and SS there is an active idiotypic–anti-idiotypic network [51]. The anti-idiotypic antibodies were isolated using the complementary epitopes and found to bind anti-La/SSB antibodies, competing with La/SSB epitopes for their antigen binding site. In some cases the anti-idiotypic antibodies were capable of completely masking the anti-La/SSB antibodies, inhibiting their anti-La/SSB reactivity. A specific procedure was developed with the use of complementary peptides for the release of anti-La/SSB antibodies from their anti-idiotypic counterpart [51]. This procedure applied in anti-La (−), anti-Ro/ANA (+) sera from patients with SLE and pSS. Ninety-four percent of pSS sera and 80% of SLE sera were found negative for anti-epitope 349–364 antibodies in ELISA prior to the treatment with complementary epitope. After unmasking the anti-La antibodies, all SS and SLE sera became

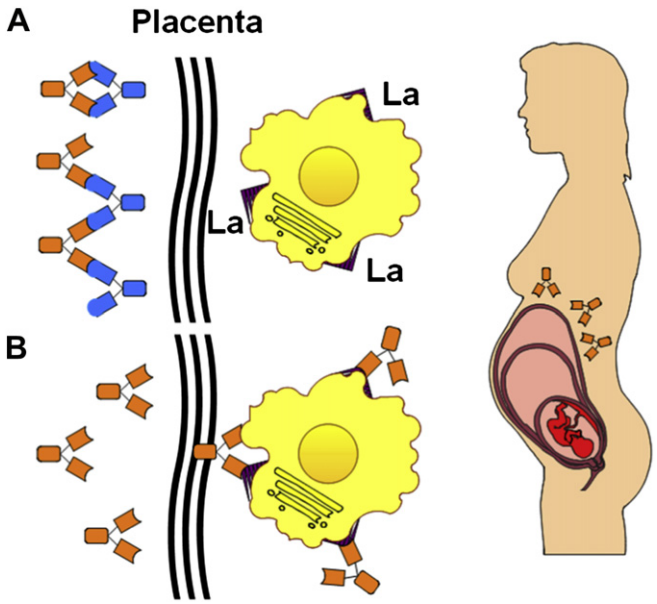

Fig. 2. (A) In neonatal lupus, the presence of anti-idiotypic antibodies to autoantibodies against La/SSB may protect the fetus by blocking pathogenic maternal autoantibodies. In this regard, large immunocomplexes are formed that cannot cross the placenta. (B) If anti-Id antibodies do not exist, maternal anti-La IgG autoantibodies pass through the placenta to the fetal circulation and cause tissue injury to the heart and skin.

positive for antibodies against the epitope 349–364aa, while none of the normal sera exhibited a positive reaction. Animal studies, also demonstrated that Balb/c mice immunized with complementary epitopes of La/SSB develop anti-human La/SSB antibodies [52], suggesting that the complementary epitopes of La/SSB have the potential of inducing an autoimmune response against La/SSB autoantigen. Recent findings indicate that autoimmunity can be initiated through an immune response against a peptide that is complementary to the autoantigen [53]. Pedergraft III and co-workers demonstrated that a subset of PR3-ANCA positive patients with necrotizing vasculitis harbors also antibodies directed against the translated protein product of the middle fragment (105–201aa) of the antisense RNA of PR3, termed complementary PR3 or cPR3 [54]. These antibodies were not present in patients with vasculitis and anti-myeloperoxidase (MPO) autoantibodies (MPO-ANCA), patients with SLE, or healthy individuals. It was also demonstrated that human anti-cPR3 and anti-PR3 antibodies are in fact an idiotypic–anti-idiotypic pair, since mice immunized with cPR3 develop both anti-cPR3 and anti-human PR3 antibodies while complementary PR3 transcripts are present in the peripheral leukocyte RNA from a subset of ANCA patients [53,54].

9. Anti-idiotypic antibodies and disease. The examples of NLS and type 1 diabetes

Neonatal lupus syndrome (NLS) is considered a model of passively acquired systemic autoimmune disease. Placentally transported maternal IgG autoantibodies against Ro/SSA and/or La/SSB are strongly implicated in the pathogenesis of the disease. NLS is characterized by two dominant manifestations, cutaneous rash and congenital heart block (CHB), the latter being most often of third-degree severity in a structurally normal heart. The true incidence of NLS is unknown, but CHB is estimated to occur in about 2% of anti-Ro/SSA-positive mothers. The presence of anti-La/SSB antibodies may increase the risk of CHB in the fetus to 5% as compared with the presence of anti-Ro/SSA alone [55]. A putative role for the candidate antibodies in the pathogenesis of this disease derives from in vitro and in vivo data, demonstrating that maternal anti-Ro/SSA and/or anti-La/SSB antibodies opsonize fetal apoptotic cardiomyocytes, which in turn induces a proinflammatory/profibrotic response by phagocytosing macrophages, ultimately leading to tissue injury. Since, complementary peptides have the potential to adopt structures that are complementary to B-cell epitopes and mimic the shape of the paratopes of the antibodies recognizing these epitopes, they can be efficiently used for the detection of anti-idiotypic antibodies. Among the systemic autoimmune diseases, NLS is the ideal model for studying anti-idiotypic antibodies, since pathogenetic autoantibodies to Ro/SSA and/or La/SSB may be directly involved in tissue injury.

In a recent work of our laboratory we evaluated the idiotypic/anti-idiotypic network of antibodies targeting the dominant epitopes of La/SSB in mothers positive for anti-Ro and/or anti-La/SSB antibodies, aiming to define the role of this network in the development of NLS [56]. To accomplish this task, peptides and complementary peptides deduced from the sequences 289–308aa and 349–364aa of La/SSB were synthesized and tested against maternal sera. It was found that sera from mothers giving birth to a healthy child and having no history of a child with NLS exhibited higher anti-idiotypic antibody activity compared with mothers carrying a child with NLS or mothers giving birth to a healthy child but who previously gave birth to a child with NLS. Sera from mothers of healthy children, which exhibited no apparent epitope activity against amino acids 349–364, revealed a significantly greater frequency of hidden anti-349–364aa epitope responses, blocked by anti-idiotypic antibodies, as compared with sera from women pregnant with an affected child. Therefore, the presence of anti-idiotypic antibodies to autoantibodies against La/SSB may protect the fetus by blocking pathogenic maternal autoantibodies (Fig. 2) and testing for these anti-idiotypic responses may be useful in predicting a decreased risk of NLS.

Type 1 diabetes (T1D) is an autoimmune disease characterized by the presence of autoantibodies to multiple islet cell autoantigens. Autoantibodies to glutamate decarboxylase 65 (GAD65Ab) can be detected in the majority of new-onset T1D patients [57], in patients with latent autoimmune diabetes in adults and in some rare neurologic diseases, notably Stiff Person Syndrome (SPS) [58], but rarely in the general population. GAD65Ab often herald the onset of T1D by months or years and are used to predict disease together with other autoantibodies to islet cell [59]. In a recent work, Oak et al. demonstrated that masked GAD65Ab are present in the healthy population and that a lack of particular anti-Ids, rather than GAD65Ab *per se*, is a characteristic of T1D [60]. Therefore, anti-Ids may play a protective role in the immune response, by preventing GAD65Ab to bind to their antigen and potentially modulate T cell responses to GAD65. Lastly, we are very pleased to contribute this paper to this special issue to recognize the contributions of Professor Harry Moutsopoulos, the Chair of our Department as part of the special series of distinguished autoimmunologists [61–64].

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