John G. Routsias*, Athanasios G. Tzioufas, Haralampos M. Moutsopoulos Η κλινική αξία των Β-επιτόπων ενδοκυττάριων αυτοαντιγόνων στις συστεμικές ρευματικές παθήσεις Clinica Chimica Acta 340 (2004) 1 –25

A hallmark of autoimmune diseases is the production of autoantibodies against intracellular autoantigens. Although their pathogenetic and their etiologic relationship are not fully understood, these autoantibodies are important tools for establishing the diagnosis, classification and prognosis of autoimmune diseases. Systemic rheumatic diseases are among the most complex disorders because their clinical presentation and constellation of findings are in part reflected by the wide spectrum of autoantibodies found in the sera of patients suffering from these disorders. These autoantibodies usually target large complexes consisting of protein antigens noncovalently associated with (ribo)-nucleic acid(s), like the spliceosome or Ro/La-RNPs. In this review, we first address the main characteristics and the clinical value of several autoantibodies, with respect to their diagnostic sensitivity and specificity. Subsequently, we provide a brief overview of the antigenic determinant types that have been identified on the corresponding autoantigens. The antibody targets of autontigens include primary, secondary, tertiary and quarternary structure epitopes, as well as cryptotopes, neoepitopes and mimotopes. We next focus on antigenic structures corresponding to B-cell epitopes with high disease specificity and sensitivity for all the major autoantigens in systemic autoimmunity including the Ro/La and U1 ribonucleoprotein complexes and the Ku70/80, ribosomal P, DNA topoisomerase I, filaggrin, Jo-1 and PM/SCl-100 autoantigens. These epitopes, defined at the peptide level, can be chemically synthesized and engineered for the development of new inexpensive and easier to perform assays and the improvement of the methods for autoantibody detection. Specific examples of newly developed assays that incorporate (i) epitopes with high disease specificity and sensitivity, (ii) modified epitopes, (iii) conformational epitopes and (iv) complementary epitopes are discussed in detail. Finally, we examine the potential of combining these synthetic epitopes for future development of multiplex diagnostic tests based on miniaturized autoantigen arrays.

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Review

The clinical value of intracellular autoantigens B-cell epitopes in systemic rheumatic diseases

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Abstract

A hallmark of autoimmune diseases is the production of autoantibodies against intracellular autoantigens. Although their pathogenetic and their etiologic relationship are not fully understood, these autoantibodies are important tools for establishing the diagnosis, classification and prognosis of autoimmune diseases. Systemic rheumatic diseases are among the most complex disorders because their clinical presentation and constellation of findings are in part reflected by the wide spectrum of autoantibodies found in the sera of patients suffering from these disorders. These autoantibodies usually target large complexes consisting of protein antigens noncovalently associated with (ribo)-nucleic acid(s), like the spliceosome or Ro/La-RNPs. In this review, we first address the main characteristics and the clinical value of several autoantibodies, with respect to their diagnostic sensitivity and specificity. Subsequently, we provide a brief overview of the antigenic determinant types that have been identified on the corresponding autoantigens. The antibody targets of autontigens include primary, secondary, tertiary and quarternary structure epitopes, as well as cryptotopes, neoepitopes and mimotopes. We next focus on antigenic structures corresponding to B-cell epitopes with high disease specificity and sensitivity for all the major autoantigens in systemic autoimmunity including the Ro/La and U1 ribonucleoprotein complexes and the Ku70/80, ribosomal P, DNA topoisomerase I, filaggrin, Jo-1 and PM/SCl-100 autoantigens. These epitopes, defined at the peptide level, can be chemically synthesized and engineered for the development of new inexpensive and easier to perform assays and the improvement of the methods for autoantibody detection. Specific examples of newly developed assays that incorporate (i) epitopes with high disease specificity and sensitivity, (ii) modified epitopes, (iii) conformational epitopes and (iv) complementary epitopes are discussed in detail. Finally, we examine the potential of combining these synthetic epitopes for future development of multiplex diagnostic tests based on miniaturized autoantigen arrays.

Keywords: Systemic rheumatic diseases; Amino acid; B-cell epitopes

Abbreviations: aa, amino acid; ANA, antinuclear antibodies; ATP, adenosine triphosphate; Bip, GRP78/Bip chaperone; CCP, cyclic citrullinated peptide; CIE, counter immunoelectrophoresis; CREST, Systemic Sclerosis characterized by Calcinosis; Raynaud's, Esophagus involvement, Sclerodactyly, and Telangiectasia; CTL/NK, cytotoxic T-lymphocytes/natural killer cells; CNS, central nervous system; DB, dot blot; DM, dermatomyositis; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; IB, immunoblotting; IIF, indirect immunofluorescence; IMD, inflammatory muscle diseases; IRES, internal ribosome entry site; JCA, juvenile chronic arthritis; MCTD, mixed connective tissue disease; NLE, neonatal lupus; PM, polymyositis; RA, rheumatoid arthritis; RF, rheumatoid factor; RIA, radioimmunoassay; pep, peptide; pSS, primary Sjogren syndrome; RNP, ribonucleoprotein; RRM, RNA recognition motif; RiboP, ribosomal P proteins; SCLE, subacute cutaneous lupus; SLE, systemic lupus erythrematosus; SSc, systemic sclerosis; tRNA, transfer RNA.

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1. Introduction

The detection of autoreactive antibodies against intracellular autoantigens is essential for establishing diagnosis, prognosis as well in monitoring the disease course of autoimmune rheumatic diseases. This is attested by (i) the inclusion of several autoantibodies in the diagnostic criteria for systemic autoimmune disorders [1–3], (ii) the correlation of certain autoantibodies with disease activity indices [4] and (iii) the association of some autoantibodies with specific clinical groups or disease manifestations [5]. Therefore, the methods for autoantibody detection should be reliable, reproducible, validated and easy-to-perform in the every day clinical practice. The first assays, which were developed and applied, were the radioimmunoassay (RIA) for the detection of anti-ds-DNA antibodies [6] and the double immunodiffusion precipitation method for the detection of autoantibodies, directed against intracellular components like Ro/SSA, La/SSB, Sm, RNP and Jo-1 [7–10]. Since then, a wide variety of methods have been developed for the identification of these autoantibodies.

The original, autoantibody detection assays were using crude cellular extracts or purified proteins as autoantigens; one of the limitations, however, was that they were available in limited quantities. Moreover, many of the detection methods relied on rather unstable complex autoantigen particles, composed by multiple proteins and/or nucleic acids. The first important step for the improvement of the autoantibody detection in clinical diagnostic laboratories was established in the early 1990s with the development of biotechnology and the production of recombinant autoantigens [11,12]. The bacterially expressed high-purity human autoantigens were utilized in a variety of methods including enzyme-linked immunosorbent assay (ELISA), double diffusion, immunoprecipitation, Western blot and dot blot assays [11–14]. Nevertheless, the extensive use of recombinant autoantigens in diagnostic tests, revealed a number of problems [15–17]. First, some recombinant antigens appeared to be less immunoreactive compared to the corresponding purified human antigens. This was most probably attributed to the absence of post-translational modifications of the bacterially expressed proteins and/or in misfolded structures adopted by the recombinant autoantigens [18]. All these may lead to the partial destruction of conformational epitopes and masking of putative linear epitopes. Second, the cost of a high purity recombinant antigen is, in some cases, relatively high. Finally, some problems on the reproducibility of the methods have been reported, mainly due to inter-assay reactivity variations, among different batches [19]. Afterwards, many investigators identified the parts of the protein that are actually seen by antibodies (B-cell epitopes), working mainly with synthetic peptides based on the amino acid sequence of the autoantigens [20,21]. Synthetic peptides have several advantages over recombinant antigens as antigenic substrates in diagnostic assays [22]. The peptide synthesis is a controlled chemical process in contrast to the in vivo production of recombinant proteins and leads to high purity, homogenous and stable antigen preparations. Moreover, the peptides can be engineered to fit as antigens into different assay systems. However, other studies indicate that the epitope peptides are not always good substitutes for larger antigenic protein fragments [23]. Their value is mostly dependent on the immunological properties of the cognate antigen.

2. Intracellular autoantigens

Patients with systemic autoimmune disorders display a considerable diversity of clinical signs and symptoms. In addition, evolution from one disease to another and frequently overlaps of clinical manifestations of different diseases are observed. This heterogeneity is also reflected on the circulating autoantibody specificities, which include the reactivity towards a variety of intracellular targets (Table 1) [24,25]. In most cases, intracellular autoantigens are proteins participating in complexes with (ribo)nucleic acids, such as U1-RNP, Ro/La RNP, DNA topoisomerase I, DNA-dependent protein kinase, 60S ribosomal subunit, Histidyl-tRNA, etc. (Fig. 1). The main characteristic of the majority of those is their active involvement in protein synthesis machinery (e.g. at pre-mRNA splicing, ribosome assembly or amino acid–tRNA association). The properties of intracellular autoantigens including their biochemical characteristics, cellular localization and function, interaction with other molecules and the clinical relevance of the

Table 1
Serologic findings in connective tissue diaseases

Disease	Autoantibody target	Frequency (%)
Systemic lupus erythematosus (SLE)	ANA	90–95
	dsDNA	65–75
	Sm	20
	RNP	25–30
	Ro/SSA	30–40
	La/SSB	10–15
	Ribosomal P	10–20
	Phospholipid	30–50
	Ku	10
	calreticulin	35
Subacute cutaneous LE (SCLE)	ANA	70
	Ro/SSA	>80
Neonatal LE (NLE)	ANA	30
	Ro/SSA	100
	La/SSB	60
Drug induced LE	ANA	>90
	Histone	95–100
Sjogren's Syndrome (pSS)	ANA	55
	Ro/SSA	70
	La/SSB	60
	α-fodrin	60
Mixed connective tissue disease (MCTD)	ANA	>95
	RNP	>90
	dsDNA	10
	Ro/SSA	10
Scleroderma (Scl)	ANA	>90
	Scl-70	35
	CENP-B (in CREST)	70–90
Inflammatory muscle	ANA	80
	Jo-1	25–30
Rheumatoid Arthritis (RA) diseases (IMD)	ANA	25–35
	dsDNA	0–10
	Ro/SSA	5
	Filaggrin	70

corresponding autoantibodies are presented in Table 2 [26,27]. The prevalence of these autoantibodies in a particular disease is an important parameter that influences their diagnostic sensitivity. Sensitivity is defined as the probability of a positive test result in a patient with the disease under investigation. The other and most important parameter, which affects the diagnostic value, is the specificity of the autoantibody detection assay. Specificity is defined as the probability of a negative test result in a patient without the disease under investigation. Autoantibodies with >95% specificity for a given disease are often called disease markers, as their detection points to the disease diagnosis with 95% accuracy. Such specific autoantibodies are of particular clinical value since they offer an important clue for the diagnosis. The most specific autoantibodies are presented in Table 3 [26,28]. It should be emphasized that although anti-Sm, anti-Jo-1 and anti-Scl-70 autoantibodies are present in rather small percent of SLE, PM and SSc patients, respectively, they are significant in diagnosis of these disorders, as their presence points directly to the correct diagnosis [28]. On the other hand, other autoantibodies commonly found in different connective tissue diseases with low disease specificity are highly correlated with certain clinical disease manifestations (Table 4). Thus, for example, anti-Ro/SSA antibodies are commonly found in SS and SLE, but their detection in patients may be highly indicative for subacute cutaneous lupus or in female pregnant patients may suggest that the embryos are at risk for neonatal lupus [28]. Similarly, antihistone antibodies can be found in SLE (42%), RA (15%), Scl (10%), MCTD (15%) and SS (28%), but their absence almost excludes the diagnosis of drug induced SLE with 100% accuracy [28,29].

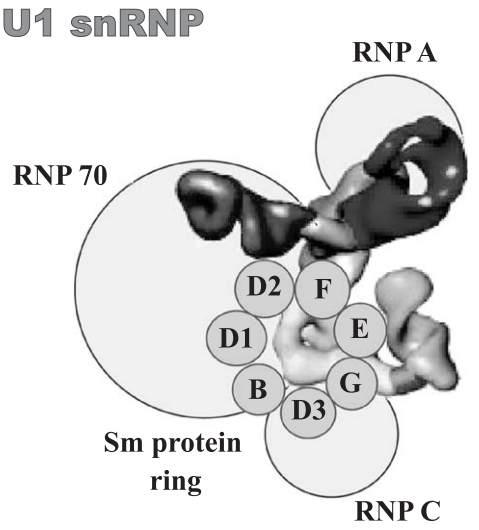


Fig. 1. Structure of U1-RNP complex. RNP-70, RNP-A, RNP-C and Sm 7-mer ring (formed by SmB, SmD1, SmD2, SmD3, SmE, SmF and SmG proteins) are noncovalently complexed with U1-snRNA (shown in sausage-like representation). This figure is based on cryo-electron microscopic pictures of the U1-snRNP particle [66].

Table 2
Major properties and clinical relevance of the autoantigens

Autoantigens' major properties and clinical relevance	
Ro 52	
Chemical properties:	p*I* = 5.9, charge = − 8.7, MW = 54.2
Cellular localization:	nucleus + cytoplasm
Cellular function(s):	Predicted role as transcription factor (based on its domain organization)
Interaction with nucleic acids:	DNA [ARGRGGG(G/C)(A/C)GRNGA motif]
Interaction with proteins:	Probably with Ro60 and calreticulin
Member of complex:	Ro/La RNP
Antibodies clinical relevance:	SS, SLE, SCLE, NLE, MCTD, RA
Ro 60	
Chemical properties:	p*I* = 7.8, charge = + 4.4, MW = 60.6
Cellular localization:	nucleus + cytoplasm
Cellular function(s):	Proposed roles in quality control of 5S rRNA biosynthesis and in ribosome assembly
Interaction with nucleic acids:	hY RNAs, 5S rRNA
Interaction with proteins:	Probably with Ro52 and calreticulin
Member of complex:	Ro/La RNP
Antibodies clinical relevance:	SS, SLE, SCLE, NLE, MCTD, RA
La/SSB	
Chemical properties:	p*I* = 6.7, charge = − 0.6, MW = 46.8
Cellular localization:	Predominantly in the nucleus
Cellular function(s):	Required for efficient and correct termination of RNA polymerase III transcription and cap-independent translation (IRES mediated). Proposed roles as ATP-dependent helicase able to melt RNA–DNA hybrids, RNA chaperone.
Interaction with nucleic acids:	hY RNAs, immature RNA polymerase III products, viral RNAs (e.g. adenovirus VA, Epstein Barr EBER), viral and human RNAs possessing IRES (internal ribosomal entry elements), RNA component of telomerase complex
Interaction with proteins:	–
Member of complex:	Ro/La RNP
Antibodies clinical relevance:	SS, SLE, NLE, SCLE
Calreticulin	
Chemical properties:	p*I* = 4.0, charge = − 58.5, MW = 46.5
Calreticulin	
Cellular localization:	endoplasmic reticulum, cell membrane, nucleus
Cellular function(s):	role in Ca^{2+} homeostasis, molecular chaperone of endoplasmic reticulum, stress protein, regulates integrin mediated adhesion (surface calreticulin), modification of gene expression by binding to the glucocorticoid receptor, component of CTL/NK granules, C1q receptor (surface calreticulin)
Interaction with nucleic acids:	rubella virus RNA, hY RNA
Interaction with proteins:	Ro 60, Ro 52, newly synthesized glycoproteins (via N-linked glycans), glucocorticoid receptor, ERp57, BiP
Member of complex:	hY1 RNP
Antibodies clinical relevance:	SLE, SS, parasitosis (e.g. oncocerciasis), celiac disease
U1-RNP 70	
Chemical properties:	p*I* = 10.3, charge = + 20.2, MW = 51.6
Cellular localization:	nucleoplasm
Cellular function(s):	Component of the spliceosome, required for the pre-mRNA splicing (removal of introns + ligation of exons → mature mRNA)
Interaction with nucleic acids:	U1 snRNA and probable other spliceosome RNAs
Interaction with proteins:	RNP C, RNP A, SmD2
Member of complex:	U1 RNP
Antibodies clinical relevance:	MCTD, SLE
U1-RNP A	
Chemical properties:	p*I* = 10.3, charge = + 12.5, MW = 31.3
Cellular localization:	see U1-RNP 70
Cellular function(s):	see U1-RNP 70
Interaction with nucleic acids:	U1 snRNA and probable other spliceosome RNAs
Interaction with proteins:	RNP 70
Member of complex:	U1 RNP
Antibodies clinical relevance:	MCTD, SLE
U1-RNP C	
Chemical properties:	p*I* = 10.0, charge = + 8.3, MW = 17.4
Cellular localization:	see U1-RNP 70
Cellular function(s):	see U1-RNP 70

Table 2 (*continued*)

Autoantigens' major properties and clinical relevance	
U1-RNP C	
Interaction with nucleic acids:	–
Interaction with proteins:	RNP 70, Sm B/B′
Member of complex:	U1 RNP
Antibodies clinical relevance:	MCTD, SLE
Sm B/B′	
Chemical properties:	p*I* = 11.7, charge = + 18, MW = 24.6
Cellular localization:	nucleoplasm
Cellular function(s):	component of the spliceosome, required for the pre-mRNA splicing
Interaction with nucleic acids:	U1, U2, U4, U5 sn RNAs
Interaction with proteins:	RNP C
Member of complex:	U1 RNP
Antibodies clinical relevance:	SLE
Sm D1	
Chemical properties:	p*I* = 12.1, charge = + 16.3, MW = 13.3
Cellular localization:	see Sm B/B′
Cellular function(s):	see Sm B/B′
Interaction with nucleic acids:	see Sm B/B′
Interaction with proteins:	Sm D2
Member of complex:	U1 RNP
Antibodies clinical relevance:	SLE
Ku 70	
Chemical properties:	p*I* = 6.2, charge = – 4.2, MW = 69.7
Cellular localization:	Nucleoplasm, nucleolus
Cellular function(s):	DNA binding factor, belongs to the regulatory subunit of DNA-dependent protein kinase. Proposed roles in DNA repair and in chromosome maintenance
Interaction with nucleic acids:	dsDNA, little affinity for ssDNA, DNA–RNA, tRNAs
Interaction with proteins:	Ku80, p350 subunit of DNA-dependent protein kinase
Member of complex:	Catalytic subunit of DNA-dependent protein kinase
Antibodies clinical relevance:	SLE, Polymyositis/Scleroderma
Ku 80	
Chemical properties:	p*I* = 5.4, charge = – 17.9, MW = 82.6

Table 2 (*continued*)

Autoantigens' major properties and clinical relevance	
Ku 80	
Cellular localization:	see Ku 70
Cellular function(s):	see Ku 70
Interaction with nucleic acids:	see Ku 70
Interaction with proteins:	Ku70, p350 subunit of DNA-dependent protein kinase
Member of complex:	Catalytic subunit of DNA-dependent protein kinase
Antibodies clinical relevance:	SLE, Polymyositis/Scleroderma
Ribosomal P	
Chemical properties:	p*I* = 4.1, charge = – 9.0, MW = 11.7
Cellular localization:	cytoplasm, nucleolus (ribosome assembly site)
Cellular function(s):	Required for all three phases of protein synthesis (initiation, translocation and termination)
Interaction with nucleic acids:	28S rRNA
Interaction with proteins:	with L12 to form the functional GTPase domain and with components of the 60S subunit of the ribosome
Member of complex:	60S subunit of the ribosomal complex
Antibodies clinical relevance:	SLE, neuropsychiatric SLE
Scl 70	
Chemical properties:	p*I* = 9.7, charge = 35.8, MW = 90.1
Cellular localization:	nucleoplasm, nucleolus
Cellular function(s):	relax supercoiled DNAs by creating a transient single stranded nick in the DNA backbone
Interaction with nucleic acids:	supercoiled DNA
Interaction with proteins:	casein kinase II, PARP I [poly(ADP-ribose) polymerase]
Member of complex:	DNA topoisomerase complex
Antibodies clinical relevance:	Systemic sclerosis
Filaggrin	
Chemical properties:	p*I* = 7.7, charge = + 3.3, MW = 44.1
Cellular localization:	perinuclear (keratohyalin) granules of epidermal cells
Cellular function(s):	Involved in the aggregation of cytokeratin filaments during the epithelial hornification process
Interaction with nucleic acids:	–
Interaction with proteins:	keratin

(continued on next page)

Table 2 (*continued*)

Autoantigens' major properties and clinical relevance	
Filaggrin	
Member of complex:	–
Antibodies clinical relevance:	RA, Juvenile Chronic Arthritis
Jo 1	
Chemical properties:	p*I* = 5.5, charge = – 5.3, MW = 57.4
Cellular localization:	cytoplasm
Cellular function(s):	Catalyzes the binding of histidine to $tRNA^{His}$
Interaction with nucleic acids:	$tRNA^{His}$
Interaction with proteins:	EF-1 (elongation factor 1)
Member of complex:	Histidyl–tRNA synthetase–t $tRNA^{His}$
Antibodies clinical relevance:	IMD
PM/Scl 100	
Chemical properties:	p*I* = 8.4, charge = + 10.9, MW = 100.8
Cellular localization:	nucleolus
Cellular function(s):	role in rRNA processing, maturation and ribosome biosynthesis, component of human exosome, exoribonuclease activity
Interaction with nucleic acids:	rRNAs, pre-mRNAs
Interaction with proteins:	part of a multiprotein complex, localized in the nucleolus, consisting of 11 to 16 proteins (including PM/Sc 75)
Member of complex:	human exosome
Antibodies clinical relevance:	IMD/scleroderma overlap syndrome

Thus, the clinical value of each autoantibody specificity is different among different disease groups or subgroups and should be examined separately for each autoantigen with respect to its specific immunological and biochemical features.

Table 3
Autoantibodies with high diagnostic value

Autoantibody target	Clinical significance (disease marker)
dsDNA	SLE
Sm	SLE
Jo-1	IMD
Ribosomal P	SLE
CENP-B	CREST
Scl-70	SSc
Filaggrin	RA
RNP	MCTD
La	pSS

Table 4
Association of autoantibodies with clinical manifestations

Autoantibody target	Clinical association
dsDNA	Nephritis, active
Ro/SSA	Cutaneous lupus/neonatal lupus/congenital heart block
Ribosomal P	Neuropsychiatric SLE
Histone	Drug induced SLE
Phospholipid	Venous/arterial thrombosis

3. Mapping the autoantigenic determinants

After the definition of the autoantibodies and the initial clinical correlations, a number of investigators tried to define the fine specificity of autoantibodies (B-cell epitope or antigenic determinant mapping) [20,21], aiming to:

- The development of new, more sensitive and specific methods for autoantibody detection. In this direction, the use of chemically synthesized epitope analogues in the form of synthetic peptides can offer an alternative source of low-cost antigen of high purity and stability. In addition, synthetic peptides can be easily engineered to fit in various detection assays. Thus the peptides can be readily modified by the attachment of a biotin, aminocaproic acid or a carboxy-terminal cysteine moiety, in order to bind to streptavidin, hydrophobic or maleimide ELISA plates.
- The correlation of autoantibodies against a specific epitope with the clinical picture or certain clinical findings of a given autoimmune disease. It should be noted that the appearance of antibodies against a specific epitope may reflect: (i) an extensive epitope spreading, (ii) the existence of cross-reacting antibodies capable to interact with additional cellular components and (iii) differences in the disease pathogenetic mechanisms. These autoantibodies can be potentially correlated with specific clinical findings.
- The understanding of the antigenic structures recognized by autoantibodies. The knowledge of

the structures reacting with autoantibodies can provide valuable information for the mechanisms involved in the breakdown of immune tolerance and establishment of an autoimmune response. Detailed analysis of the epitopes structure can also reveal homologous sequences shared among antigenic epitopes and other proteins that may cross-react with the same antibody (molecular mimicry).

3.1. Types of B-cell epitopes

The B-cell epitopes are quite diverse in structure and immunoreactivity and thereof can be classified accordingly. On the basis of the protein's epitope nature, they can be 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. The term "continuous epitope" is rather misleading since although this epitope comprise from a consecutive stretch of up to 10 amino acids, not every amino acid in the sequence is essential for antibody binding. Often, there are sequence positions that can be substituted with all the 20 naturally occurring amino acids without any immunoreactivity loss. In addition, as autoantigens are clustered in large (deoxy-)ribonucleo-protein complexes, the term "conformational epitope" or "discontinuous epitope" can be referred either to epitopes comprised by amino acids distributed on its secondary, tertiary or quarternary structure [30]. Thus, a more detailed classification scheme is needed, to distinguish between these possibilities. In this scheme (Fig. 2), the epitopes are divided in:

- *Primary-structure epitope* (identified also as 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, immune response was found to be predominately directed against a local α-helical secondary structure stretch with all amino acids relevant for antibody binding located at one side of the helix.
- *Tertiary-structure epitopes*, 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 consist of amino acids distributed over different subunits within a macromolecular complex, interacting transiently or permanently to form 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. In this classification scheme, other two epitopes of special type that are not characterized by specific conformational pattern should be added.
- *Cryptic epitopes (cryptotopes).* These are usually linear epitopes, hidden in 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.
- *Modified epitopes (neoepitopes).* 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 citrulination 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 [31]. Such modified amino acids have been reported in a variety of human nuclear proteins, including the Sm antigens D1 and D3 [32], fibrillarin [33], and nucleolin [34]. The

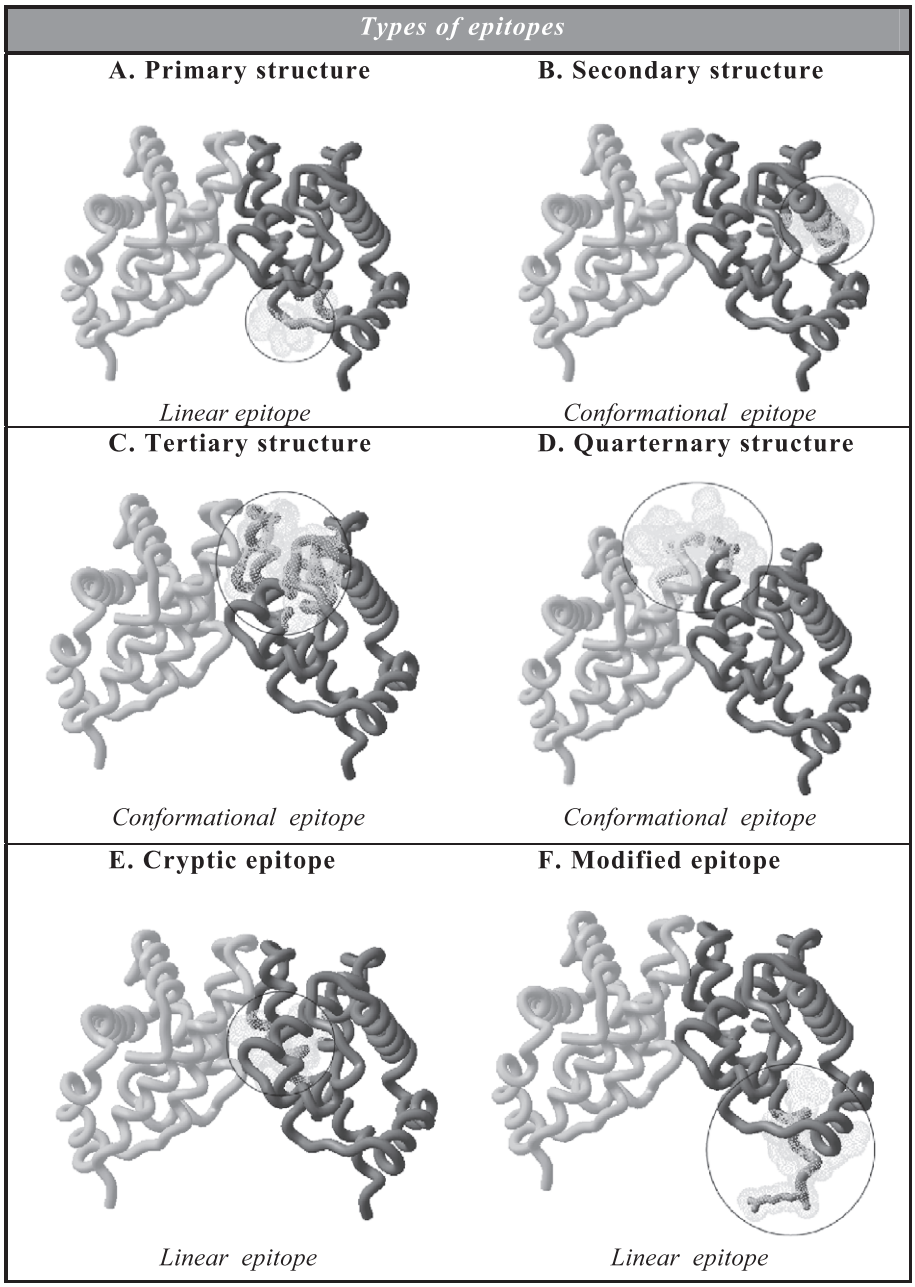


Fig. 2. Types of epitopes.

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.

Recently, epitope mapping studies using peptide libraries led to the identification of structures that mimic epitopes and are called *mimotopes* [35]. 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 nonprotein molecules such as polysaccharides, lipids or nucleic acids.

Another epitope classification, commonly used, divides the epitopes on the basis of their frequencies in a given group of patient sera. *Major epitopes* are recognized by most, if not all, autoimmune patient sera reactive with the respective autoantigen, while *minor epitopes* are recognized by a limited number of the autoimmune patient sera reactive with the corresponding autoantigen. Major epitopes are the best candidates for the improvement/replacement of the existing diagnostic assays. The clinical importance of minor epitopes depends upon their association with certain symptoms or disease subgroups. It should also be emphasized that in this classification scheme, the classification of the epitopes depends on the specific group of patients. Thus, if there is a common autoantigenic target in more than one autoimmune diseases (e.g. the Ro60 autoantigen), then a given epitope may be major for one disease (e.g. epitope 211–232aa of Ro60 in SS) and minor for another (e.g. epitope 211–232aa of Ro60 in SLE) [36].

3.2. Methods for B-cell epitope mapping

Over the past several years, a large array of epitope mapping strategies for the identification and characterization of epitopes has been used [21,30]. In early studies, autoantigen proteolytic digests were probed with sera from patients with autoimmune diseases using various assays, such as immunoblot, ELISA or immunoprecipitation. In the following years, recombinant fragments of autoantigens were used as antigens in similar assays. Afterwards methods, based on overlapping synthetic peptides, such as pepscan (peptide scan), were developed and applied for epitope mapping of numerous autoantigens. Recently, more sophisticated methods, including phage display libraries, and combinatorial peptide libraries, were successfully adapted for the mapping of autoantigens. A comparative analysis of the methods used for epitope mapping and their application for different epitopes is presented in Table 5 while the detailed procedure for the most popular epitope mapping techniques is depicted in Fig. 3.

Using these methods, several laboratories have provided conflicting results in regard to epitope mapping of intracellular autoantigens. All these studies have pointed out, however, that there is no currently available single approach that can be used for the identification of all epitopes in a given autoantigen [21,30,37]. As it is shown in Table 5 and Fig. 3, peptide scan can detect the exact location of linear epitopes and cryptotopes, but cannot identify conformational epitopes, while recombinant fragments expression can detect large antigenic fragments containing conformational and linear epitopes but cannot reveal their precise location and the cryptotopes. Similarly, synthetic peptide or phage display libraries can detect mimotopes but usually do not map the precise location of either linear or conformational epitopes in the antigenic structure. Thus, the heterogeneity of the results obtained by the epitope mapping techniques reflects the limitations of the used method.

The determination of the precise location of the autoantigens' antigenic determinants allows the use of synthetic peptides as antigen source. This results in further reduction of the antigen complexity [21,38]. Synthetic peptides can be (i) synthesized in large quantities of high purity, (ii) characterized efficiently (e.g. by mass spectra or nuclear magnetic resonance), (iii) chemically modified (e.g. covalent attachment to plastic surfaces, biotinylation, cyclization), (iv) synthesized in order to incorporate post-translational modifications (such as phosphorylation or citrullination), (v) used for the development of more specific assays, since nonspecific interactions with other serum components are significantly reduced, due to their small size and homogenous chemical form, and (vi) used for the development of more reproducible and less expensive assays since their stability is significantly higher and their cost is significantly lower compared to the recombinant autoantigens. Moreover, such test systems can be useful in defining disease subgroups and can offer information on disease prognosis. Despite the advantages of synthetic peptides utilization as antigens, their ability to improve or even replace the existing diagnostic tests and provide additional

Table 5
Comparison of epitope mapping strategies

Strategy	Advantages	Disadvantages	Epitope types
Peptide scan (pepscan)	• Fast and precise • Easy mutational analysis • Easy minimal epitope's length determination • Peptides can be modified chemically (phosphorylation, citrullination etc) • Peptide synthesis is a chemical procedure and can be easily controlled • Peptide sets commercially available	• It may not detect conformational epitopes • Small peptides (6 mer–10 mer) have high conformational freedom and may facilitate low affinity recognition by antibodies	A, B, E, F
Peptide mimotopes	• Detects both linear and conformational epitopes • Detects epitopes even if the antigen is unknown • Detects mimotops (e.g. for nonprotein antigens)	• Requires the synthesis of large number of peptides [approximately 400 + 40*(n-2) peptides, n = length of the epitope] • Initial dipeptide binding may be not specific • Affinity-purified antibodies required • High cost	A, (B), C, D, E
Peptide libraries	• Detects both linear and conformational epitopes • Detects epitopes even if the antigen is unknown • Detects mimotopes (e.g. for nonprotein antigens) • Peptide libraries commercially available	• Requires the synthesis of large number of peptides (thousands or millions of different peptides) • Not all the peptide sequences are actually synthesized • Usually libraries contain certain amino acid motifs, but not the whole peptide sequences pool • Each peptide sequence is represented by a limited number of molecules • Affinity purified antibodies required • High cost	A, B, C, D, E
Enzymatic degradation of the antigen	• Detects conformational epitopes	• Laborious, time consuming • Requires purification of the antigen • Requires sequencing of the antigenic fragments • Requires human tissue or human cell-line if the homology with other species' antigen is low • Does not detect the precise epitope	A, B, C, (E), F
Recombinant fragments expression	• Detects conformational epitopes (if the bacterially expressed antigen is correctly folded)	• Laborious, time consuming • Does not detect the precise epitope • Bacterially expressed proteins lack post-translational modifications • Usually only a few cutting sites for endonuclease exist • Cutting sites are also unevenly distributed in the antigen's length	A, B, C, (F)
Recombinant fragments expression		• In fusion proteins the protein part encoded by the vector (e.g. beta-galactosidase) usually affects proteins antigenicity	

Table 5 (*continued*)

Strategy	Advantages	Disadvantages	Epitope types
Random peptide phage display	• Identification of mimotopes (e.g. for nonprotein antigens) • Libraries commercially available • Detects conformational epitopes	• Requires the expression of large number of peptides (thousands or millions of different peptides) • Does not detect post-translationally modified epitopes • Not all the peptide sequences are actually expressed • The copy number for each peptide varies • Affinity-purified antibodies required	A, B, (C), E

The epitope types correspond to the classification presented in this table.

clinical information is clearly dependent on the specific characteristics of each autoantigen [23,38] and must be examined separately according to the clinical value of a particular autoantigen in combination with its immunological and biochemical properties.

4. Structures corresponding to B-cell epitopes with high disease sensitivity and specificity

To date, B-cell epitopes of almost all major autoantigens have been mapped with one or more methods. The nature of the identified epitopes ranges from

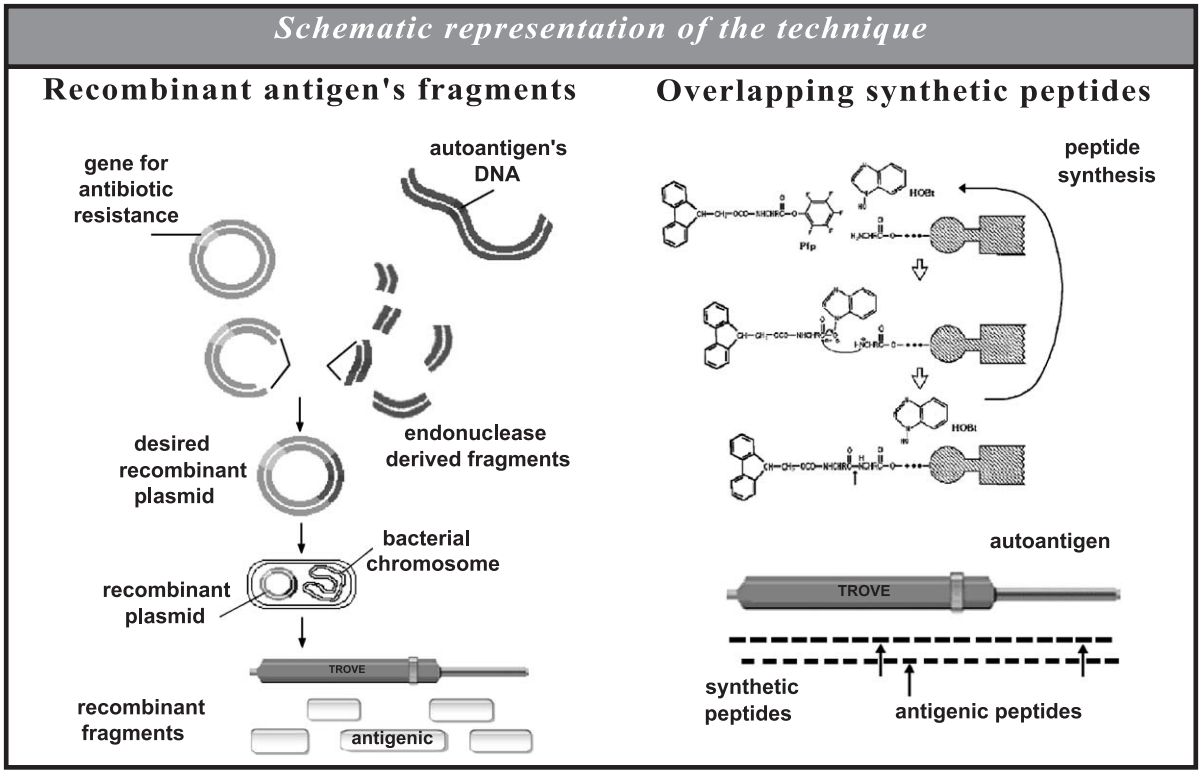


Fig. 3. Comparison of the most commonly used epitope mapping techniques.

linear to quaternary structure epitopes. Since an analysis of the extensive data derived from the numerous epitope mapping studies in all antigenic targets of systemic autoimmunity would exceed the scope of this review, some major epitope mapping studies that led to the precise identification of relatively small in length epitopes with high disease sensitivity and specificity are summarized in Table 6. These epitopes are depicted with respect of their localization into the overall autoantigen domain organization (Table 6). One general, intriguing observation is that some of the epitopes have been located in functional domains of the cognate autoantigen [30,39]. Whether autoantibodies against functional domains of autoantigens are able to penetrate within the living cell and influence in vivo their biological function of their target is yet unclear.

4.1. The Ro/La ribonucleoprotein complex

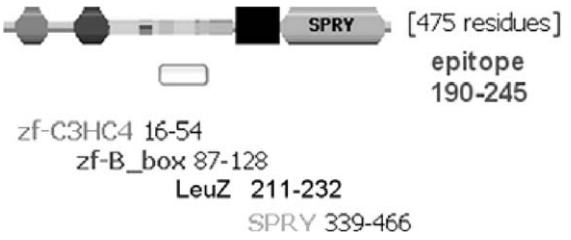
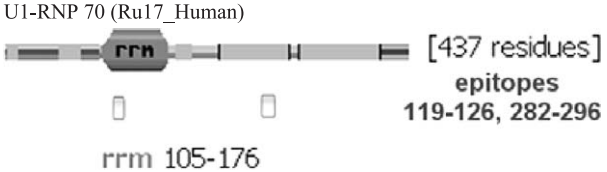
The Ro/La ribonucleoprotein complex is formed by the noncovalent association of the Ro52, La and Ro60 autoantigens with a small cytoplasmic RNA (hYRNA) [40,41]. Additional components of the complex have been recently identified as the proteins calreticulin [42] and nucleolin [43].

The major immunoreactivity of Ro52kD autoantigen was localized, using various recombinant Ro52 fusion proteins, near its leukine zipper domain [44–46]. The 190–245aa region of the amino acid sequence was reactive with almost all anti-Ro52 positive sera and was independent of associated diseases [44].

Autoepitopes of Ro60kD have been described by several authors using a variety of epitope mapping procedures [47,48]. Three major studies [49–51] using recombinant fragments of Ro60kD identified a major epitope within the central part of the molecule (within 181–320aa, 139–326aa and 155–295aa regions of the sequence, respectively). Epitope mapping with synthetic peptides, in our laboratory, revealed the precise antigenic regions of Ro60kD in 169–190 and 211–232 parts of the antigen [36]. One of them, the 169–190 epitope, was found to share conformational and antigenic similarity with HLA-DR3 β-chain, an HLA class II allele, which was described to be highly associated with the anti-Ro60 response [52]. Our recent results indicate that although these epitopes were identified as small peptidic moieties (22 aa in length), their recognition by autoantibodies is conformation-dependent and their antigenicity is dramatically enhanced upon interaction with the molecular chaperone calreticulin [53]. Using complexes of highly purified human calreticulin with the linear epitopes of Ro60Kd, it was found that almost all positive anti-Ro60Kd sera bound strongly onto the newly formed conformation of the epitopes [53]. When calreticulin or the linear epitopes of Ro60Kd were tested individually with the same sera, the prevalence of positive reactions was much lower. In addition, sera from pSS or SLE patients without anti-Ro/SSA antibodies did not react with the calreticulin–linear epitope complexes of Ro60Kd [53]. These observations suggest conformation-dependent enhancement of antigenicity of the Ro60kD epitopes upon interaction with the chaperone protein calreticulin and such kind of complexes can potentially be used as substrates for the efficient detection of autoantibodies.

Recent studies in our laboratory have been also focused on the zinc finger motif of Ro60Kd protein. The zinc fingers are secondary structure elements, responsible for protein–DNA and protein–protein interactions [54,55]. They can also hold putative conformational B-cell epitopes, since their structure is affected by zinc binding and redox conditions. Using synthetic peptide analogues corresponding to (i) to the zinc finger motif of Ro60Kd, spanning the region 301–327aa (Zif-1), (ii) a truncated form of the zinc finger motif, without the intermediate loop (310–319aa) of the molecule (Zif-2), and (iii) the intermediate loop of the zinc finger motif (Zif-3). It was found that the majority of anti-Ro/SSA and La/SSB positive sera from patients with pSS bound in the full-length peptide, in the absence of zinc ions. In contrast, the native form of the zinc finger domain, in the presence of zinc ions, could bind to Ro52Kd, but not to autoantibodies [56]. Thus, the zinc finger domain of Ro60kD contains a B-cell epitope with high specificity for pSS. This epitope is located in the intermediate loop, as assessed by the enhanced reactivity of the Zif-3 peptide and the limited reactivity of the truncated peptide lacking the intermediate loop (Zif-2) [56]. Thus, autoantibodies were found to target the disrupted conformation of the intermediate loop of this functional domain, which is presumably

Table 6
Localization of the epitopes in the domain organization of autoantigens

Schematic representation	*Biochemical features*
Ro 52 (Ro52_Human) 	zf-C3HC4: RING-type zinc-finger, a specialized type of Zn-finger that binds two atoms of zinc, and is probably involved in mediating protein-protein interactions. zf-B_box: B-box zinc-finger, is generally associated with a ring finger and a coiled coil motif to form the so-called tripartite motif. It is found essentially in transcription factors, ribonucleoproteins and protooncoproteins. SPRY Domain: is named from SPla and the RYanodine Receptor, unknown function LeuZ: Leucine zipper, a motif involved in binding to DNA or proteins. TROVE Domain: This presumed domain is found in TEP1 and Ro60 proteins, that are RNA-binding components of Telomerase, Ro and Vault RNPs. This domain has been named TROVE, (after Telomerase, Ro and Vault). This domain is probably RNA-binding. Zinc-finger: a domain that binds one atom of zinc, and is probably involved in mediating protein-protein or protein-RNA interactions. La domain: This presumed domain is found at the N-terminus of La RNA-binding proteins. RRM: Many eukaryotic proteins that are known or supposed to bind single-stranded RNA contain one or more copies of this putative RNA-binding domain.
Ro 60 (Ro60_Human) 	
La/SSB (La_Human) 	
Calreticulin (Crtc_human) 	Calreticulin: is a calcium-binding protein organized in of three domains: An N-terminal, probably globular, domain of about 180 amino acid residues (N-domain). A central domain of about 70 residues (P-domain) which contains three repeats of an acidic 17 amino acid motif. This region binds calcium with a low-capacity, but a high-affinity. A C-terminal domain rich in acidic residues and in lysine (C-domain). This region binds calcium with a high-capacity but a low-affinity. RRM: see La/SSB autoantigen
U1-RNP 70 (Ru17_Human) 	

(continued on next page)

Table 6 (*continued*)

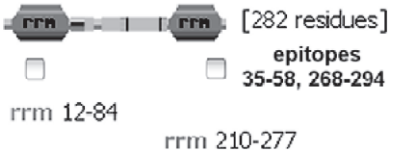
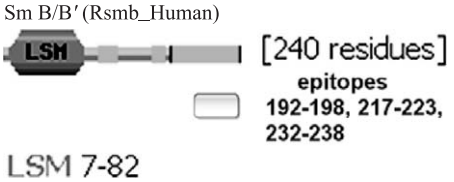
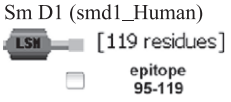
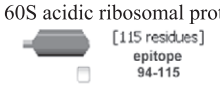
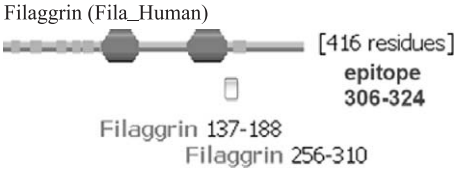
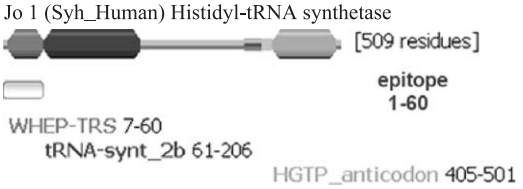
Schematic representation	*Biochemical features*
U1-RNP A (Ru1a_Human) 	RRM: see La/SSB autoantigen.
U1-RNP C (Ru1c_Human) 	ZnF_U1: Matrin type zinf-finger, a family of C2H2-type zinc fingers, present in matrin, U1 small nuclear ribonucleoprotein C and other RNA-binding proteins.
Sm B/B′ (Rsmb_Human) 	LSM domain: The LSM domain contains Sm proteins as well as other related LSM (Like Sm) proteins. The U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs) involved in pre-mRNA splicing contain seven Sm proteins (B/B', D1, D2, D3, E, F and G) in common, which assemble around the Sm site present in four of the major spliceosomal small nuclear RNAs. The U6 snRNP binds to the LSM (Like Sm) proteins.
Sm D1 (smd1_Human) 	LSM domain: see Sm B/B′ autoantigen.
Ku 70 (Ku70_Human) ATP-dependent DNA helicase II, 70kD subunit 	Ku_N (N-terminal alpha/beta), Ku (beta-barrel), Ku_C (C-terminal arm): The Ku heterodimer (composed of Ku70 and Ku80) contributes to genomic integrity through its ability to bind DNA double-strand breaks and facilitate repair by the non-homologous end-joining pathway. This is the amino terminal alpha/beta domain. This domain only makes a small contribution to the dimer interface. The domain comprises a six stranded beta sheet of the Rossman fold. SAP domain: The SAP (after SAF-A/B, Acinus and PIAS) motif is a putative DNA binding domain found in diverse nuclear proteins.
Ku 80 (Ku86_Human) ATP-dependent DNA helicase II, 80kD subunit 	Ku_N (N-terminal alpha/beta), Ku (beta-barrel), Ku_C (C-terminal arm): see Ku 70 autoantigen. LeuZ: Leukine zipper, a motif involved in binding to DNA or proteins.
Ribosomal P (Rla2_Human) 60S acidic ribosomal protein P2 	60s_ribosomal: component of ribosomes, the particles that catalyze mRNA-directed protein synthesis in all organisms.

Table 6 (*continued*)

Schematic representation	*Biochemical features*
dsDNA (double stranded DNA)  Asp/Glu-Trp-Asp/Glu-Tyr-Ser/Gly (molecular mimic)	This pentapeptide is a molecular mimic of double-stranded DNA. This sequence is also present in the extracellular domain of human NMDA (*N*-methyl-D-aspartate) receptor subunits NR2a and NR2b.
Scl 70 (Top1_Human)  DNA Topoisomerase I	Topoisomer_I_N: Eukaryotic DNA topoisomerase I motif. DNA topoisomerase I promotes the relaxation of DNA superhelical tension by introducing a transient single-stranded break in duplex DNA and are vital for the processes of replication, transcription, and recombination. Topoisomerase_I:Eukaryotic DNA topoisomerase I, catalytic core. TOPEUc: DNA Topoisomerase I (eukaryota) motif.
Filaggrin (Fila_Human) 	Filaggrins are filament-associated proteins that interact with keratin intermediate filaments of terminally differentiating mammalian epidermis via disulphide bond formation The proteins are synthesised as large, insoluble, highly-phosphorylated precursors, containing multiple tandem repeats of 324 amino acids.
Jo 1 (Syh_Human) Histidyl-tRNA synthetase 	WHEP-TRS: A conserved domain of 46 amino acids that exist in a number of higher eukaryote aminoacyl-transfer RNA synthetases. tRNA_synt: This domain is the core catalytic domain of tRNA synthetases and includes glycyl, histidyl, prolyl, seryl and threonyl tRNA synthetases. Anticodon binding domain: This domain is found in histidyl, glycyl, threonyl and prolyl tRNA synthetases It is probably the anticodon binding domain.
PM/Scl 100 (Pmc2_Human) 	3'-5' exonuclease: This domain is responsible for the 3'-5' exonuclease proofreading activity of DNA polymerase I and other enzymes. HRDC domain: The HRDC (Helicase and RNase D C-terminal) domain has a putative role in nucleic acid binding. Mutations in the HRDC domain cause human disease. 35EXOc: 3\' -5' exonuclease proofreading domain present in DNA polymerase I, Werner syndrome helicase, RNase D and other enzymes.

involved in the interaction of Ro60kD with the Ro52kD polypeptide.

La/SSB contains also B-cell epitopes suitable for substrates for autoantibody detection. Some of the La epitopes were found to reside in functional regions of the autoantigen, like the RNA recognition motif (RRM) and the ATP binding site [57–59]. The interaction of hYRNA with the RRM motif, however, did not affect the autoantibody binding in the same region [57]. In contrast, the interaction of the ATP binding site with ATP abolishes the autoantibody binding at the same part

of the protein [59]. B-cell epitope mapping of La/SSB was performed also in our laboratory using 20-mer synthetic peptides overlapping by eight amino acids covering the whole sequence of the protein. Peptides highly antigenic were those spanning the sequences: ^{147}HKAFKGSI^{154} (located within RRM motif: 113–182aa), ^{291}NGNLQLRNKEVT^{302}, ^{301}VTWEVLEGEVEKEALKKI^{318} and ^{349}GSGKGKVQFQGKKTKF^{364} [60]. In order to investigate the value of the previously defined synthetic epitope analogues of the La/SSB autoantigen as diagnostic tools, a total number of 122 sera with anti-La/SSB activity, from patients with pSS or SLE, were tested against the four La/SSB epitopes [61]. The peptide-based ELISA assays presented sensitivities ranging from 78% to 90% and specificities from 69% to 94%. The most sensitive and specific peptide ^{349}GSGKGKVQFQGKKTKF^{364} (>90% sensitivity and specificity) was synthesized in attachment with a tetramer sequential oligopeptide carrier SOC_4 and used for immunoassay development. Ninety percent of anti-La positive sera were reactive with both the synthetic peptide 349–364aa and the recombinant La protein [61]. Thus, this epitope analogue exhibited comparable with the recombinant La/SSB value for the detection of anti-La/SSB antibodies. Clinical aspects of antibodies to linear B-cell epitopes of La/SSB in pSS were also studied by our group [62]. It was found that autoantibodies to the La/SSB epitope, p349–364aa, were significantly positively associated with longer disease duration ($p<0.05$), recurrent or permanent parotid gland enlargement ($p<0.005$), and a higher proportion of non-exocrine manifestations ($p<0.005$), compared to patients without autoantibodies [62].

Sequence similarity searches revealed that the La epitope ^{147}HKAFKGSI^{154} possess 83.3% similarity with the ^{139}HKGFKGVD^{146} region of human myelin basic protein (MBP), an autoantigen of the organ-specific autoimmune disease, multiple sclerosis. The homologous MBP peptide was synthesized and found reactive with a significant proportion of anti-La/SSB positive sera. In addition, immunization of rabbits with the MBP peptide resulted in the formation of antibodies against both La and MBP peptides as well as against recombinant La and MBP proteins, indicating a possible cross-reaction between these autoantigens [63].

4.2. Calreticulin

The protein calreticulin is an exemplary multifunctional molecule capable of interacting with proteins, peptides, sugars and nucleic acids. The exact mode of interaction with other Ro/La RNP complex components is controversial, since it has been found to interact with either Ro52, hYRNA or the epitopes of Ro60kD autoantigen [42,53]. Its immunoreactivity is rather limited, involving antigenic regions in the N-terminus and the central part of the molecule [64,65]. However, if Ro epitopes are complexed together with calreticulin, the antigenicity of the complex is increased in a greater extent than the calreticulin or the Ro epitopes alone [53].

4.3. The U1-RNP ribonucleoprotein complex

U1-RNP is a multi-component complex composed of several antigenic polypeptides (RNP-70, RNP-A, RNP-C, SmB, SmD1, SmD2, SmD3, SmE, SmF, SmG) noncovalently complexed with U1-RNA. U1-RNP is a major component of the spliceosome, a dynamic molecular machine, used for pre-mRNA splicing, whose 3D architecture was recently defined with cryo-electron microscopy (Fig. 1) [66]. The antigenic properties of its polypeptides have also been studied. Similar to the described 147–154 epitope of La/SSB, the major B-cell epitope of both U1-RNP-70 and U1-RNP-A autoantigens is located within their RNA binding domain (RRM) [67–73]. On U1-RNP-70, multiple and most probably discontinuous epitopes are located in this region of the autoantigen [67–71]. Henriksson and Pettersson [72] using human-Drosophila chimeric recombinant proteins defined that the essential for antibody recognition area resides in the part 99–128 of the sequence. Subsequently, the same group of investigators mapped the major antigenic region of the autoantigen in a smaller fragment comprised from residues 119–126 located at an easily accessible region in the tertiary structure of the RNA binding domain of U1-RNP-70 [73]. Within this region, valine at position 125 was also identified as the crucial residue for antibody binding [73]. Other investigators reported an additional conformational epitope that can be generated in the RRM domain upon its binding to U1 RNA [74].

This quaternary-structure epitope has been found to be reactive with sera from patients with MCTD. On RNP-A antigen, the epitope in its RRM motif (35–58 region) possesses characteristics of a disease-specific epitope, reacting with 94% of MCTD and only 19% of SLE patients [75]. U1-RNP-C lacks a RRM motif and does not bind to U1-RNA but is indirectly associated with it via its interaction with U1-RNP-70. The major epitope of U1-RNP-C antigen is located in its 102–125 region, a region containing the APGMRPP (119–125aa) segment [76–78]. This proline-rich sequence is highly homologous with regions (i) on U1-RNP-A (PPGMIPP: 166–172aa) [79], (ii) on Sm-B/B′ (PPGMRPP: present several times in the carboxy-terminal part) [80] and (iii) SmN (PPGMRPP: present several times in the carboxy-terminal sequence of the antigen) [80]. It is intriguing that these "repetitive epitopes" have been found to react with the same autoantibodies (immunological cross-reactivity). Thus, a specific subset of autoantibodies, cross-recognizing different components of the same macromolecular complex, can be potentially detected by a single peptide [81]. A sensitive, highly reproducible ELISA, was developed in our laboratory to investigate whether the synthetic heptapeptide PPGMRPP anchored in five copies to a sequential oligopeptide carrier (SOC), $[(PPGMRPP)_5–SOC_5]$ is a suitable antigenic substrate to identify this autoantibodies subgroup [82]. The sensitivity of the method was 98% and the specificity was 68% for the determination of anti-Sm antibodies, while for the determination of anti-Sm and/or anti-U1RNP reactivity (antibodies to snRNPs), the corresponding values were 82% and 86%, respectively. The intra-assay coefficient of variation (CV%) ranged from 2.7 to 6 and the inter-assay CV% ranged from 9 to 14.5. These results suggest that the PPGMRPP peptide anchored to a pentameric SOC as a carrier is a suitable antigenic substrate for the detection of autoantibodies.

4.4. The Ku antigenic complex

The Ku autoantigenic system is DNA binding heterodimer, associated with the DNA-dependent protein kinase (DNA-PK). DNA-PK phosphorylates chromatin-bound proteins and is involved in dsDNA break repair, V(D)J recombination and isotype switching. The kinase's Ku antigenic component is composed of two proteins with molecular weight 70 and 80 kDa named Ku70 and Ku80, respectively. Assembly of the Ku heterodimer is required to obtain DNA binding activity and association with the DNA-PK. The heterodimerization of Ku antigen involves the 1–115aa and 430–482aa regions of Ku70 and the central part (371–510aa) of Ku80 autoantigen. Its immunoreactivity is localized mainly in the extreme carboxyl-terminus region of each of the K70 and Ku80 autoantigens [83–87]. In the case of K-70, the major epitope resides also within the DNA binding (SAP) domain of the autoantigen [86].

4.5. Ribosomal P protein

Ribosomal P antibodies are detected in the sera of 10–20% of patients with SLE. Although there is a lot of controversy over the clinical correlation of the autoantibodies in SLE, these are considered to be a marker for SLE since they are rarely identified in other connective tissue diseases. The frequencies, clinical and immunogenetic associations of antiribosomal P antibodies were evaluated in a large multiethnic cohort of 394 patients with SLE. It was found that the antiribosomal P response observed in approximately 15% of patients was strongly influenced by certain MHC class II alleles (the HLA-DR2, DQ6 haplotypes DRB1*1501 or *1503, DQA1*0102 and DQB1*0602) and was correlated with diffuse neuropsychiatric dysfunction [88]. The major epitope is located within the common C-terminal part of the three ribosomal proteins, which consists of 22-amino-acid residues (Lys-Lys-Glu-Glu-Lys-Lys-Glu-Glu-Lys-Ser-Glu-Glu-Glu-Asp-Glu-Asp-Met-Gly-Phe-Gly-Leu-Phe-Asp) [89]. The immunodominance of this epitope was shown by the ability of an epitope peptide analogue to completely adsorb autoantibody binding to ribosomal P proteins. This observation indicates that patient sera recognize mainly a linear (primary structure) epitope on the P proteins, although immunoprecipitation studies provide evidence for recognition of additional conformational epitopes as well. This epitope has been also associated with SLE and especially with CNS involvement [90]. Using as substrate a synthetic 22-amino-acid peptide, which corresponds to the ribosomal P0, P1

and P2 common epitope, we studied the specificity and sensitivity of the method and evaluated the frequency and clinical associations of anti-P antibodies in two groups of SLE patients: (a) unselected patients and (b) patients with central nervous system (CNS) involvement. The overall prevalence of anti-ribosomal P antibodies in SLE patients with active CNS disease was statistically significantly higher, as compared to unselected SLE patients ($x^2 = 6.04$, $p < 0.05$). These antibodies were found in a high proportion of patients without anti-cardiolipin antibodies (52.4%) and they were associated with diffuse CNS involvement [psychiatric disorders (71%) and epilepsy (75%)] [91].

4.6. Antibodies to double-stranded DNA

Anti-dsDNA antibodies are an important diagnostic marker and pathogenetic factor for SLE, since many clinical manifestations of SLE appear to be mediated by them, such as kidney damage and disease activity [92,93]. Several different cross-reactivities have been identified for anti-DNA antibodies, including bacterial polysaccharide, cell membranes and phospholipids, microbial protein antigens and extracellular matrix components [94]. It remains unclear if dsDNA elicits the production of anti-dsDNA autoantibodies or if another antigen triggers their production, due to structural similarity. Several efforts have been reported for identification of dsDNA mimotopes in order to discover new diagnostic candidates for anti-dsDNA antibodies [95,96]. In these studies, monoclonal or affinity-purified anti-dsDNA antibodies were used to screen phage peptide libraries and to identify the candidate peptides. The Asp/GLu-Trp-Asp/Glu-Tyr-Ser/Gly peptide, determined by this approach, was additionally found to induce anti-dsDNA autoantibodies in mouse immunizations and to inhibit specifically renal deposition of anti-dsDNA antibodies, underlying its value as dsDNA mimotope and a candidate for the development of new diagnostic assays [97–99].

4.7. DNA topoisomerase I

Autoantibodies to topoisomerase I or scl-70 are markers for systemic sclerosis. Their presence is associated with pulmonary fibrosis, a major clinical feature which increases the morbidity and mortality in these patients. Epitope mapping studies based on topoisomerase I recombinant fragments revealed the major antigenic sites on the 405–484 [100], 453–560 [101] and 512–563 [102] regions of the autoantigen. B-cell epitope mapping with synthetic 20-mer peptides (overlapping by eight residues) allowed the identification of the epitopes at the peptide level [103]. Four major epitopes were found to react with anti-Topo I sera, but not with the control sera: ^{205}WWEEERYPE-GIKWKFLEHKG^{224} (epitope I), ^{349}RIANFKIEPPG-LFRGRGNHP^{368} (epitope II), ^{397}PGHKWKEVRHDNKVTWLVSW^{416} (epitope III) and ^{517}ELDGQEYVVEFDFLGKDSIR^{536} (epitope IV). Epitopes II to IV are localized at highly exposed sites of the Topo I tertiary structure, whereas epitope I is localized at a less accessible site. Epitope IV was also found to reside within the antigenic region previously defined in two out of three studies with recombinant fragments. In a cohort of 81 SSc patients with clinical data on the evolution of their disease, patients with antibodies recognizing at least three of the four epitopes had 3.1 times ($P = 0.02$) the risk of developing pulmonary fibrosis compared with patients whose sera recognized no epitopes or only one or two of the four epitopes [103].

4.8. Filaggrin

Filaggrin, a cytokeratin filament aggregating protein of the epidermis, is the common antigenic target for anti-perinuclear factor (APF) and anti-keratin antibodies (AKA), which were considered for many years to be the different autoantibody specificities in the sera of RA patients [104,105]. The main target structures in filaggrin have been identified in citrulline-containing sequences, with a major epitope located on amino acids 306–324. Citrulline residues arise from post-translational modification of arginine residues by the enzyme peptidylarginine deiminase [106,107]. These modified residues have been shown to form the central position of epitopes targeted by anti-filaggrin antibodies. In addition, recombinant filaggrin fragments were recognized by RA autoantibodies only after in vitro enzymatic deimination [107]. The antibodies bound to the citrullinated substrates were detected in over 80% of RA sera with a high disease specificity using several synthetic

peptides containing citrulline. Using RA and non-RA sera, the anti-CCP ELISA proved to be highly specific (98%), with a reasonable sensitivity (68%) [108]. In comparison with the IgM rheumatoid factor (IgM-RF) ELISA, the anti-CCP ELISA had a significantly higher specificity (96% for CCP versus 91% for IgM-RF). The sensitivity of both tests for RA was moderate: 48% and 54% for the anti-CCP ELISA and the IgM-RF ELISA, respectively [109]. Combination of the anti-CCP and the IgM-RF ELISAs resulted in a significantly higher positive predictive value of 91% and a slightly lower negative predictive value of 78% as compared to the IgM-RF ELISA alone [109]. Similarly, in another study, when anti-CCP and RF antibodies were combined, the specificity reached the 99.6% [110]. Finally, follow-up studies revealed that anti-CCP antibodies can be detected in early stages of RA and these antibodies were associated with more severe radiological damage [109,111,112]. Thus, the detection of anti-CCP antibodies have several advantages over the classical APF and AKA assays and may replace them in the near future.

4.9. Histidyl-tRNA synthetase (Jo-1)

The existence of autoantibodies targeting the Jo-1 autoantigen is a diagnostic marker for autoimmune myositis (polymyositis/dermatomyositis) and a prognostic marker for more severe clinical course of the disease. Their target epitope is found in the amino-terminal 60 amino acid within the autoantigen coiled-coil WHEP-TRS domain, which is a domain found in a number of eukaryotic amino-transfer RNA synthetases [113,114].

4.10. PM/Scl-100

The PM/Scl particle is the human analogue of the yeast exosome, a complex consisting of 11–16 polypeptides, with exoribonuclease activity during the RNA processing [115]. The PM/Scl-100 protein is the prime target of autoimmune response against the PM/Scl complex, in patients with Polymyositis/Scleroderma overlap syndrome. Immune response against anti-PM/Scl-100 was shown to be predominately directed against a 15-amino-acid region with α-helical secondary structure [116].

5. Future directions

The definition of B-cell epitopes of autoantigens provided a useful aid in the diagnostic armamentarium of systemic autoimmune diseases. The successful development of new diagnostic assays is hindered, however, by a number of factors concerning autoantibody cross-reactivity, autoantibody masking and intermolecular epitope spreading as well as by issues concerning the total number of autoantibody specificities to be tested and the amount of serum required. Recently, new advances in the diagnostic assay development provide clues to overcome these problems:

5.1. Complementary epitopes: a tool to neutralize the anti-idiotypic antibodies

Anti-idiotypic antibodies, reactive with idiotypes of autoantibodies, are capable of regulating the autoimmune response [117]. The same antibodies may also interfere in autoantibody detection by competing with antigen for binding in the same paratopic site (antigen inhibitable or Ab2β anti-idiotypic antibodies, according to Jerne's classification). In order to derive peptides capable of neutralizing anti-idiotypic antibodies, we have taken advantage of the antisense-complementary peptide approach [118]. This approach is based on the molecular recognition theory. According to this theory, the translation of two complementary mRNAs produces a pair of peptides with inverted hydrophobicity profiles. This complementarity in hydrophobicity may lead, under certain conditions, to strong interaction between these two (sense and antisense) peptides [119,120]. Many different systems of sense and antisense peptides that bind one to another with specificity and varied affinity have been described in the literature [121]. Interestingly, these peptides have the ability to generate interacting pairs of idiotypic and anti-idiotypic antibodies upon their application in animal immunizations [122].

Aiming to study the idiotypic–anti-idiotypic network in anti-La/SSB positive sera, we prepared complementary peptides corresponding to major epitopes of La/SSB [118]. The synthetic complementary peptides of La/SSB deduced from the sequence of RNA antisense to the mRNA encoding the epitopes 289–308aa and 349–364aa of La/SSB. These peptides

reacted with a significant proportion of patient sera with anti-La specificity. From these patients sera, anti-complementary epitope and anti-epitope antibodies were purified and digested with pepsin in order to produce $F(ab')_2$ fragments. The antibodies against epitopes found to specifically interact with the $F(ab')_2$ fragments of antibodies recognizing complementary epitopes and vice versa, suggesting their idiotype–anti-idiotype relation. Inhibition experiments demonstrated that anti-idiotypic antibodies compete with the antigen for the binding site (paratope) of antibodies against La/SSB epitopes. In autoimmune sera, which were negative by the conventional methods for anti-La/SSB antibodies, the anti-idiotypic antibodies were found to bind and mask the idiotypic (anti-La/SSB) antibodies [117]. Using the complementary epitopes as inhibitors of the anti-idiotypic antibodies, we were able to recover the hidden anti-La/SSB reactivity. The procedure that was developed for the release of anti-La/SSB antibodies from idiotypic antibodies includes the following steps: (i) sera were heated at 55 °C for the dissociation of Id–anti-Id complexes, (ii) complementary epitope 349–364 was added as anti-Id blocking agent and the mixture was submitted to slow cooling (55 °C → 25 °C in 3 h) to favour the establishment of a new equilibrium with the participation of cpep as anti-Id antibody binder in lieu of idiotypic (anti-La/SSB) antibodies, and (iii) the procedure was followed by an ordinary anti-La/SSB epitope ELISA. To investigate the prevalence of masked anti-La/SSB antibodies, the heat + complementary peptide procedure was applied in 44 anti-La (−), anti-Ro/ANA (+) sera from patients with SLE and Sjogren's syndrome. Ninety-four percent of Sjogren's syndrome sera and 80% of SLE sera were found negative for anti-pep 349–364 antibodies in ELISA prior to the treatment. After the heat + complementary epitope treatment, all SS and SLE sera became positive for anti-epitope 349–364 antibodies, while none of the normal sera exhibited a positive reaction. Heating without addition of cpep 349–364 had no effect in patient sera reactivity. Thus, virtually all anti-Ro/ANA (+) sera possess also hidden anti-La/SSB antibodies that can be unmasked by treatment with the complementary epitope [117]. This procedure was also tested in animals immunized with La epitope 289–308 or complementary epitope 289–308 and found to efficiently block the anti-idiotypic antibodies that interfere in anti-La/SSB detection [123]. This methodology provides new advantages for the improvement of the assays employed in the detection of anti-La/SSB antibodies.

5.2. Autoantigen arrays: a novel method for multiplex autoantibody detection

Apart from the anti-idiotypic interference, the existing autoantibody detection methods are hindered by the total number of potential autoantigens and the requirement for large quantities of human sample and antigen. To circumvent these limitations, some groups recently developed miniaturized autoantigen array technology [124] to perform multiplex characterization of human autoantibody responses [125,126]. Joos et al. [125] described the construction of autoantigen microarrays containing 18 prominent autoantigens spotted onto surfaces including silane-treated glass slides and nitrocellulose. These arrays proved to be sensitive and specific for detection of autoantibodies to many of the spotted antigens, with as little as 40 fg spotted protein still detectable for one of the protein standards used. Bound antibodies were visualized using a chemiluminescent based system. Similarly, Robinson et al. [126] constructed autoantigen arrays to perform large-scale multiplex characterization of autoantibody responses against structurally diverse autoantigens. These arrays were produced by attaching the autoantigens to derivatized glass slides using a robotic array. The 196 distinct autoantigens that were used covered eight human systemic autoimmune diseases such as SLE, SS and RA. The autoantigens included a wide variety of biomolecules: (i) peptides derived from Sm-D, U1 RNP-70 and histones, (ii) recombinant Ro52, La, Jo-1, Sm-B, U1 RNP-C and topoisomerase I proteins, (iii) nucleic acids such as dsDNA and rRNA, (iv) lipids like cardiolipin and (v) ribonucleoprotein complexes such as Sm/RNP. The arrays were probed with mixtures of highly characterized autoimmune disease serum samples. The results obtained correlated precisely with those obtained by conventional detection methods, including ELISA, immunoprecipitation and WB [126].

Generally, autoantibody profiling may serve purposes including: (i) classification of individual patients and subsets of patients based on their 'autoantibody fingerprint', (ii) examination of epi-

tope spreading and antibody isotype usage, (iii) discovery and characterization of candidate autoantigens, and (iv) tailoring antigen-specific therapy. Multiplex assays have considerable potential as methods to characterize autoepitopes of virtually all possible types for a number of autoantigens at the same time. Once all epitopes with diagnostic value have been identified at the peptide level (for a given group of autoantigens), they could then be combined into a miniaturized array. This peptide array can incorporate a variety of peptide analogues (including modified epitopes, conformational epitopes and mimotopes) and can be used as diagnostic reagent. Thus, possible correlations of autoantibodies targeting specific epitopes with clinical manifestations and disease subsets can be readily determined. In the coming years, proteomics technologies will broaden our understanding of the underlying mechanisms and will considerably ameliorate the diagnostic approach in autoimmune disease.

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