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Serum SmD autoantibody proteomes are clonally restricted and share variable-region peptides

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Recent advances in mass spectrometry-based proteomic methods have allowed variable (V)-region peptide signatures to be derived from human autoantibodies present in complex serum mixtures. Here, we analysed the clonality and V-region composition of immunoglobulin (Ig) proteomes specific for the immunodominant SmD protein subunit of the lupus-specific Sm autoantigen. Precipitating SmD-specific IgGs were eluted from native SmD-coated ELISA plates preincubated with sera from six patients with systemic lupus erythematosus (SLE) positive for anti-Sm/RNP. Heavy (H)- and light (L)-chain clonality and V-region sequences were analysed by 2-dimensional gel electrophoresis and combined de novo database mass spectrometric sequencing. SmD autoantibody proteomes from all six patients with SLE expressed IgG1 kappa restricted clonotypes specified by IGHV3-7 and IGHV1-69 H-chains and IGKV3-20 and IGKV2-28 L-chains, with shared and individual V-region amino acid replacement mutations. Clonotypic sharing and restricted V-region diversity of systemic autoimmunity can now be extended from the Ro/La cluster to Sm autoantigen and implies a common pathway of anti-Sm autoantibody production in unrelated patients with SLE.

1. Introduction

Anti-Smith (Sm) autoantibodies, discovered by Tan and Kunkel in 1966 as serum precipitins specific for patients with systemic lupus erythematosus (SLE), hold pride of place as founding members of the family of systemic autoantibodies targeting non-histone extractable nuclear antigens [1, 2]. Anti-Sm responses are present in 10% to 30% of SLE patients and form part of the American College of Rheumatology criteria (ACR) for the classification of SLE [3,4]. The pathogenic significance of these autoantibodies is underlined by their appearance shortly before the clinical onset of SLE and the reported clinical associations with lupus nephritis and neuropsychiatric disease [5-7]. Sm autoantigens comprise a ring of 7 small nuclear ribonucleoprotein (snRNP) common core proteins (B, D1, D2, D3, E, F & G) with the snRNA passing through ring [8]. While SmB and SmD are the major targets of anti-Sm humoral autoimmunity, anti-SmD is considered to have the highest specificity for SLE [9]. Immune complexes of anti-Sm and snRNP particles are thought to perpetuate systemic autoimmunity by inducing type I interferon in plasmacytoid dendritic cells via combined Fc receptor and TOLL-like receptor 7 engagement [10,11].

Although the Sm protein-RNA antigenic complexes have been characterised in detail, the clonality and variable (V)-region composition of secreted (serum) human anti-Sm autoantibody proteomes have not been studied. Advances in mass spectrometric-based protein sequencing now allow heavy (H)- and (L)-chain V-region peptide maps to be generated for the first time from microgram quantities of autoantibodies derived from complex patient sera [12]. Recently, we have combined 2-dimensional gel electrophoresis (2-DE) of anti-Ro52/Ro60/La autoantibodies with de novo and database-driven V-region protein sequencing to show that humoral responses against these protein-RNA complexes are mediated by public (shared) autoreactive B cell clonotypes [13-15]. An early study reported oligoclonality and restricted V-region gene usage by anti-Sm hybridomas derived from

MRL/*lpr* mice, raising the possibility of restricted anti-Sm clonality in human SLE [16]. In the present study, we characterise serum SmD autoantibody proteomes by high resolution mass spectrometric sequencing for the first time and find that the expression of these clinically important autoantibodies is dominated by unique sets of public clonotypes.

2. Patients and methods

2.1. Patients

Sera were collected from six patients with SLE who were positive for anti-Sm autoantibodies by commercial line blot immunoassay (Euroline ANA profile 5, Euroimmun, Lubeck, Germany). Demographic characteristics and serologic findings in the patients are shown in Table 1. Control sera were taken from four healthy controls and three anti-SmD antibody-negative SLE patients positive for anti-ribosomal P, anti-RNP-A and anti-Ro52/Ro60/La antibodies respectively. Patients fulfilled at least four of the eleven ACR criteria for SLE [4], including the presence of antibodies to double-stranded DNA by Farr assay (Trinity Biotech, Bray, County Wicklow, Ireland). The study was approved by the Clinical Ethics Committee of the Flinders Medical Centre.

2.2. Preparation and specificity analysis of anti-SmD autoantibodies

Anti-SmD IgGs were purified from ELISA plates (Maxi-Sorp; Nunc, Roskilde, Denmark) using a simple elution method. In brief, plates were coated with 1 μ g/ml of purified bovine native SmD protein (confirmed by mass spectrometric sequencing as SmD1, SmD2, SmD3) diluted in 0.03M carbonate buffer (Arotec Diagnostics, New Zealand), blocked with 1% BSA in phosphate buffered saline (PBS), incubated with serum from each subject diluted 1:50 in 1%BSA/PBS for one hour at 37 °C, washed four times with PBS, and bound Ig fraction eluted with 200 μ l of 0.1M glycine and 0.5M NaCl, pH 2.3. Eluted Ig fractions were neutralized in 1M Tris HCL, pH 8.0 and concentrated to 10 μ g/ml on an Amicon concentrator.

The activity and specificity of ELISA plate-purified Igs for SmD were determined by testing the starting sera (diluted 1:100), unbound fractions (normalized to each starting serum), and bound/eluted Igs (2.5 ug/ml) for reactivity against native SmD, native RNP-A (Arotec Diagnostics), and native Ro60 (Arotec Diagnostics) by ELISAs as described previously [13]. Kappa and lambda distribution of bound anti-SmD Igs was determined by ELISA using specific rabbit anti-human kappa and anti-human lambda antisera (Dako, Denmark) detected by HRP-labelled donkey anti-rabbit antisera (Jackson ImmunoResearch, USA). Purified anti-Sm Igs were also tested for reactivity with native Sm antigen by indirect immunofluorescence of HEp-2 cells (ImmunoConcepts, Sacramento, USA) and by counterimmunoelectrophoresis (CIEP) using rabbit thymus extract and anti-Sm and anti-RNP reference controls.

2.3. Two-dimensional gel electrophoresis (2-DE)

2-DE was performed as previously described [13] with the following modifications; 13-cm, non-linear immobilized pH 3-11 IPG strips (Bio-Rad, USA) were used in the first dimension and 8-15% gradient Criterion stain free TGX gels (Bio-Rad), at 300V using a Criterion electrophoresis system (Bio-Rad) in the second dimension. Imaging was performed using a Gel Doc EZ Imager (Bio-Rad).

2.4. Mass spectrometry (MS)

In-solution digests were performed on plate-purified Igs, while in-gel digests were performed on H- and L-chain spots excised from 2-DE gels using Trypsin Gold (Promega, USA) as described previously [13]. Analysis of peptides was carried out using high-end-Q-TOF mass spectrometer (AB Sciex, USA) coupled to an Eksigent nanoLC 400 HPLC. Samples were applied to a C18 trap (Eksigent, USA) and eluted onto a 15 cm C18 column (Nikkyos Technos, Japan) using a 2-40% acetonitrile gradient over 33 min. The instrument was operated in high sensitivity positive ion mode; charge state of +2 to +5 ions selected; with

one MS scan followed by 20 MS/MS scans. At least two technical and biological replicates were performed for each sample.

2.5. Protein sequence data analysis

MS data was analysed using Peaks Studio v7.0 software (Bioinformatics Solution Inc, Canada) using the following parameters: 2 missed cleavages; precursor m/z tolerance of ≤ 10 ppm; product ion error tolerance of 0.01 Da; precursor charge state of +2 to +4; database search performed against a combined ImMunoGeneTics (IMGT) (<http://www.imgt.org>) and the Uniprot 2010-06 database as described previously [14].

3. Results

3.1. Purification of anti-SmD IgGs from anti-Sm-positive sera

To purify anti-Sm autoantibodies for analysis of clonality by 2-DE and mass spectrometric sequencing, we eluted antibodies bound to native SmD-coated ELISA plates from the sera of six SLE subjects with mixed anti-Sm/RNP/Ro60 specificities. Monospecificity of eluted IgGs for anti-SmD was confirmed by testing starting sera, unbound and eluted fractions on individual SmD/RNP-A/Ro60 ELISAs (Fig. 1A). In addition, eluted IgGs gave a speckled nuclear immunofluorescence pattern with nucleolar and chromosomal sparing on HEp-2 cells (Fig. 1B), and formed a precipitin line of identity to an anti-Sm reference serum and partial identity to an anti-RNP reference serum on CIEP, confirming reactivity with native Sm antigen (Fig. 1C). Assessment of clonality of plate-purified anti-SmD IgGs by reduced 2-DE revealed two H-chain clusters of spots ranging from pI 6.4-7.6 (identified as IGHV3-7 on gel plug digests) and pI 8-9.2 (IGHV1-69) together with two distinct clusters of kappa (K) L-chain spots ranging from pI 6.6-7.6 (IGKV2-28) and pI 8.2-9.2 (IGKV3-20) (Fig. 2). Similar charge variants have been reported for clonotypic anti-Ro60

and anti-La autoantibodies and are presumed to arise from post-translational modifications [13,15].

3.2. SmD-reactive clonotypic IgGs are public and share common amino acid replacement mutations.

Mass spectrometric analysis was performed on solution trypsin digests of plate-purified anti-Sm IgGs from all six SLE patients. The lower limit of detection of H- and L-chain constant and V-region peptides by this method was determined as 50 pg/ml using serial tryptic digests of a mouse monoclonal antibody of known concentration as a surrogate IgG. This revealed the common expression of two IgG1 H-chain species, IGHV3-7 and IGHV1-69, and two kappa L-chains encoded by IGKV2-28 and IGKV3-20 respectively, confirming the plug digest data. No private clonotypes were expressed by individual patients. No lambda L-chain peptides were identified in plug or solution digests of plate-purified anti-SmD, showing kappa L-chain restriction at the level of L-chain protein sequencing. Kappa restriction was verified immunochemically by SmD ELISA (n=6 anti-SmD-positive SLE sera) probed with anti-kappa and anti-lambda antisera [mean (SD) anti-SmD kappa 0.60 (0.04) OD units; anti-SmD lambda 0.05 (0.01) OD units]. Sequencing of joining (J)-regions also revealed common usage of IGJH4 and IGJH6 while the two L-chain clonotypes were restricted to IGJK2 and IGJK4. As discussed previously, proteomic methodology cannot generally obtain full sequence through the H-chain diversity (D) region [15]. Detailed anti-Sm V-region tryptic peptide maps are shown in Supplementary Fig. 1 and reveal multiple clonotypic peptides with public (shared among patients) and private amino acid replacement mutations consistent with selection of intraclonal variants by self-antigen. The public mutations are summarised by a proteomic heat map and are present in both framework regions (FR) and complementary determining regions (CDRs) of the H- and L-chains (Fig. 3). The most common substitutions were a threonine to serine at position 14 of IGKV2-28 and a glutamine to lysine at position 6

of IGKV3-20, both present in 5/6 anti-Sm-positive SLE patients. In control experiments, no IgG tryptic peptides were detected by mass spectrometry of elutes from SmD-coated ELISA plates treated with normal human sera (n = 4) and SmD antibody-negative lupus sera with specificities for ribosomal P, RNP-A and Ro52/Ro60/La antibodies respectively (data not shown).

4. Discussion

This study reveals that secreted anti-SmD autoantibody proteomes in unrelated patients with SLE are dominated by IgG1 kappa-restricted clonotypes specified by two distinct H- chains encoded by IGHV3-7 and IGHV1-69 gene segments and two L-chains derived from IGKV3-20 and IGKV2-28. While the finding of two common H- and L-chains is consistent with a bclonal anti-SmD autoantibody repertoire in lupus, the precise chain pairings are yet to be determined. Importantly, common usage of L- and H-chain V and J gene segments can now be extended from linked sets of anti-Ro60/Ro52/La responses to the SmD protein component of the Sm/RNP autoantibody cluster [13-15]. Taken together with the shared mutational signatures (Fig. 3), these findings imply similar if not identical molecular pathways of anti-Sm autoantibody production in individual to individual. Although shared IGV usage has been reported for both infectious agents and autoantigens, this is generally H-chain restricted and has not been studied at the level of the secreted antibody proteome [17-22]. While the relative importance of recombinatorial bias versus antigen-driven clonal selection events in shaping serum anti-SmD autoantibody proteomes remains unclear, the extensive V-region somatic hypermutation and intraclonal diversification observed at the level of SmD autoantibody proteomes are consistent with multiple rounds of Sm antigen-driven B-cell affinity maturation in germinal centres of secondary lymphoid organs.

The proteomic workflow developed herein utilised a simple ELISA plate-based method to purify anti-SmD autoantibodies from as little as one millilitre of complex SLE sera, followed by combined electrophoretic analysis and de novo and database-driven mass spectrometric sequencing. The methodology requires as little as 20 micrograms of native or recombinant antigen for antibody isolation and sequencing of V-region clonotypic peptides; can be completed within a few days; and be used to determine V-region signatures for any serum autoantibody or humoral anti-viral response for which purified antigen or epitope is available. While these “front end” improvements have reduced autoantibody purification and sequencing times by days, sample size remains limited by the complexity of immunoglobulin bioinformatics analysis. The high-end- Q-TOF mass spectrometry used herein performs higher mass accuracy protein sequencing and better protein coverage than Orbitrap mass spectrometry used in previous studies, making it more suited to de novo peptide sequencing [23]. High-mass accuracy mass spectrometric L-chain constant region sequencing enables direct analysis of kappa/lambda restriction at the protein level, as opposed to standard immunochemical methods that may vary with antigen purity and specificity of anti-L-chain antibodies. Our finding of an absolute kappa restriction of purified anti-SmD IgGs as indicated by a unique kappa peptide profile by L-chain sequencing and kappa-specific anti-SmD solid phase immunoassay, differs from a 1980s study that used a different, antigen source and anti-L-chain [24].

We anticipate that the discovery of shared anti-Sm autoantibody clonotypes will open new pathways of research for this prototypic systemic autoantigen, with unique V-region clonotypic peptides detected by mass spectrometric sequencing being potential novel biomarkers of anti-Sm autoantibody production. For example, mutated Sm-specific V-region clonotypic peptides might be used in targeted mass spectrometric platforms such as multiple reaction monitoring to quantitate specific V-region peptides in complex serum mixture, or to

monitor expression of anti-Sm clonal turnover in patients undergoing treatment [14,25]. A priority will be to sequence linked RNP/Sm autoantibody populations to test whether intermolecular sharing of autoreactive clonotypes holds true for the Sm/RNP autoantigen cluster. A long-term goal will be to correlate secreted autoantibody proteomes derived by mass spectrometry with Sm-specific B cell and plasma cell receptor repertoires in blood and tissues.

In conclusion, we use a novel proteomic workflow to determine the molecular signatures of anti-SmD autoantibodies from unrelated patients with SLE. Remarkably, the response is restricted to the same H- and L-chain immunoglobulin families and shares V-region amino acid replacement mutations, a finding of pathognomonic and diagnostic significance for anti-Sm immunity in lupus.

AUTHOR CONTRIBUTIONS

All authors provided substantial contribution to acquisition of data, revising it critically for important intellectual content, and all authors approved the final version to be published. Dr Gordon has full access to all of the data in the study and takes responsibility for the integrity of data and the accuracy of the data analysis.

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Table 1. Serological characteristics of patients with systemic lupus erythematosus (SLE) *

Patient	Age/Sex	ANA (Pattern/ Titer)	Anti- dsDNA (IU/ml)	ENA	CIEP
SLE1	59/female	Speckled/2560	>100	Sm, RNPA, Ro60, Ribo P	Sm, RNP, Ro60
SLE2	30/female	Speckled/2560	>100	Sm, RNPA, RNP 70, Ro60,	Sm, RNP, Ro60
SLE3	46/female	Speckled/2560	>100	Sm, RNPA, RNP 70	Sm, RNP
SLE4	45/female	Speckled/2560	80	Sm, RNP A	Sm, RNP
SLE5	49/female	Speckled/2560	70	Sm, RNPA, Ro-60	Sm, RNP, Ro60
SLE6	66/female	Speckled/2560	90	Sm	Negative

Antinuclear antibodies (ANA) were detected using HEp2 slides; anti-double stranded DNA (dsDNA) measured using Farr assay with a value ≥ 10 IU/ml considered positive; and extractable nuclear antigen (ENA) autoantibodies detected by line blot assay (Euroimmun, Germany) and counterimmunoelectrophoresis (CIEP) using rabbit thymus extract.

FIGURE LEGENDS

Fig. 1. Specificity of anti-Sm IgGs purified from SmD-coated enzyme-linked immunosorbent assay (ELISA) plates from sera of 6 patients with systemic lupus erythematosus (SLE) containing mixed anti-Sm/RNP-A/Ro60 specificities. (A) Purified anti-Sm IgGs tested by ELISA using native SmD, RNP-A and Ro60 proteins. Starting sera, bound and unbound fractions are compared. Bars show the mean \pm SEM of duplicate optical density values. (B) Indirect immunofluorescence of HEp-2 probed with anti-Sm IgG (2.5 ug/ml) from a representative patient with SLE. (C) Counterimmunoelectrophoresis of purified anti-Sm IgGs (0.05ug/well) tested against rabbit thymus extract (RTE) with anti-Sm and anti-RNP reference controls.

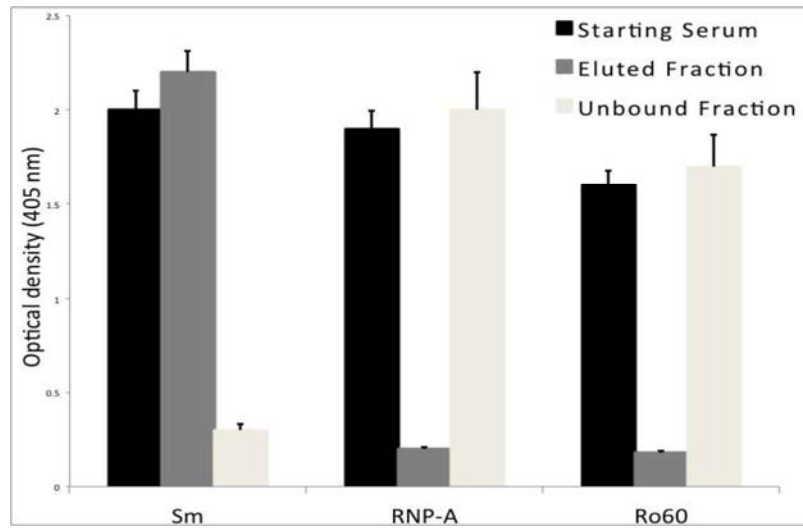
Fig. 2. Clonal restriction of anti-SmD IgGs purified from the sera of patients SLE1 and SLE6. (A) Reduced 2-dimensional gel electrophoresis of SLE1 anti-Sm reveals 2 heavy chain (H-chain) clusters of spots ranging from pI 6.4-7.6 (gel plug 1, identified as IGHV3-7) and pI 8-9.2 (gel plug 2, identified as IGHV1-69) together with 2 clusters of light chain (L-chain) spots ranging from pI 6.6-7.6 (gel plug 3, identified as IGKV2-28) and pI 8.2-9.2 (gel plug 4, identified as IGKV3-20). (B) The same pattern of H-and L-chain clusters with identical IGHV and IGKV gene families is observed for SLE6. H-chain spots have been overexposed in order to visualize L-chain spots.

Fig. 3. Variable (V)-region peptide heat map of compiled de novo sequencing data from 6 patients with systemic lupus erythematosus (SLE) showing public (shared among patients) mutations. (A) Heavy chain (H-chain) V-region sequences align with germline IGHV1-69 and IGHV3-7. (B) Light chain (L-chain) V-region sequences align with germline IGKV2-28 and IGKV-3-20. (C) H-chain joining (J)-region with IGHJ4 and IGHJ6 germline sequence and L-

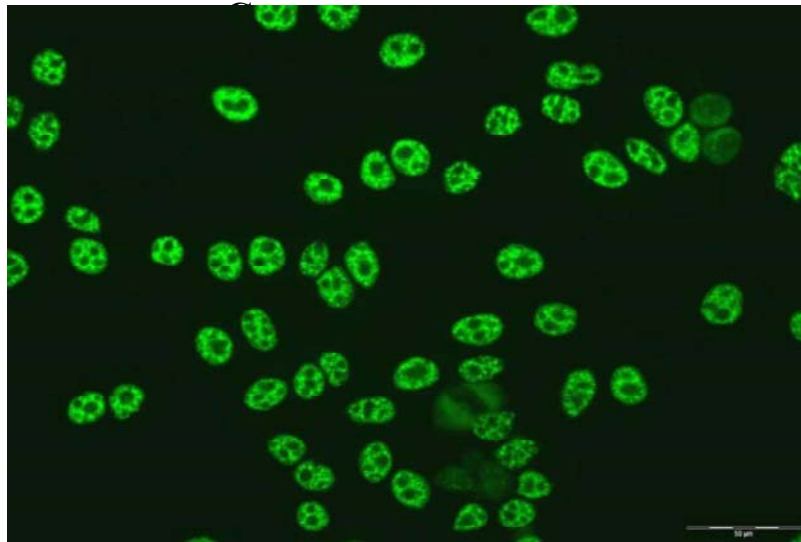
chain J-regions aligned with IGKJ2 and IGKJ4. Common amino acid replacement mutations that diverge from the germline sequence are depicted in the text and color-coded according to the frequency of the mutation detected in the SLE patient cohort analyzed. Dots indicate amino acid matching to the germline sequence derived from the ImMunoGeneTics database. Germline complementary determining regions (CDR) are underline.

Fig. 1

A



B



C

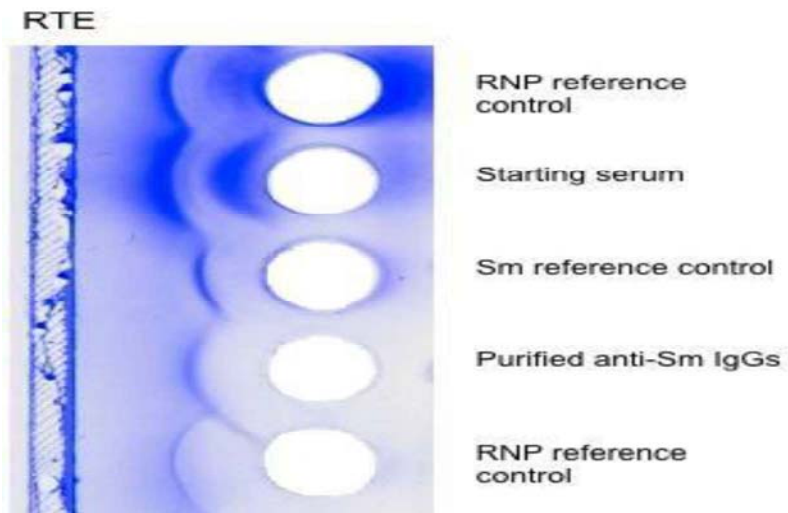


Fig. 2

