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Discovery of Biomarkers for Systemic Lupus Erythematosus Using a Library of Synthetic Autoantigen Surrogates

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Abstract

Antibodies to a wide range of self-antigens, including those directed against nucleic acids or nucleic acid-binding proteins are the essential biomarkers for diseases such as systemic lupus erythematosus (SLE). Highly complex libraries of nonamers consisting of *N*-substituted glycines (peptoids) were screened for compounds that bound IgG from patients with SLE and earlier, incomplete autoimmune syndromes. Peptoids were identified that could identify subjects with SLE and related syndromes with a high sensitivity (70%) and specificity (97.5%). Immobilized peptoids were used to isolate IgG from both healthy subjects and SLE patients that reacted with known RNA-binding proteins. In the case of SLE patients, the peptoid-purified IgG reacted with several autoantigens, suggesting that the peptoids are capable of interacting with multiple, structurally similar molecules. These results show that the measurement of IgG binding to peptoids can identify subjects with high levels of pathogenic autoantibodies.

1. Introduction

The worldwide prevalence of all autoimmune diseases taken together is estimated to be 3-5% (Hayter and Cook, 2012). Some, such as rheumatoid arthritis, are relatively easy to identify due to characteristic physical findings and specific autoantibodies to citrullinated protein antigens (Demoruelle and Deane, 2011). Others, such as systemic lupus erythematosus (SLE) are much more difficult to diagnose. These patients typically have non-specific symptoms and the clinically available autoantibodies lack the sensitivity or specificity to be used as diagnostic tests in the absence of disease-defining clinical features. For example, the presence of anti-nuclear antibodies (ANA) is highly sensitive, identifying

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nearly all SLE patients, but they are not specific and are seen in other autoimmune diseases such as systemic sclerosis, polymyositis, autoimmune hemolytic anemia, rheumatoid arthritis (RA), and multiple sclerosis (Solomon et al., 2002). ANA are also present at low titer in ~25% of the healthy adult population and at a clinically significant level in ~5% (Wandstrat et al., 2006). Other autoantibodies such as anti-double stranded DNA or anti-Smith (Sm) are more specific for SLE but only seen in a fraction of lupus patients on presentation (Kavanaugh et al., 2002; Benito-Garcia et al., 2004).

It is recognized that ANA and other autoantibodies are present for years before the onset of clinical SLE and potentially predict the transition from unaffected individuals to those with disease (Arbuckle et al., 2003; Olsen et al., 2012). Again, a lack of specificity precludes their use as truly predictive biomarkers. Autoantibodies remain attractive prognostic candidates due to their central role in the pathogenesis of autoimmune diseases such as SLE. What is necessary, then, is a platform that will detect disease-defining autoantibodies with increased sensitivity and specificity, and at the same time distinguish early disease states from normal variation.

Several approaches have been tried to find specific antibodies associated with autoimmune or infectious diseases. Some of the approaches used a focused panel of antigens known or presumed to be important for the condition, while others have used a less biased “whole proteome” technology (Robinson et al., 2002; Lueking et al., 2003; Kanter et al., 2006; Gibson et al., 2010; Hecker et al., 2012). We have described the successful use of an unbiased library of synthetic ligands to identify antibody biomarkers of Alzheimer’s disease (Reddy et al., 2011) and neuromyelitis optica (Raveendra et al., 2013). The ligands are peptoids, oligomers of *N*-substituted glycine. These ‘peptoids’ can be synthesized easily with a high degree of structural diversity using a ‘split and pool’ strategy resulting in libraries of millions of compounds representing a vast chemical space (Figliozzi et al., 1996). While the native antigen cannot possibly be present in this collection of abiological molecules, the goal of the experiment is to identify an “antigen surrogate” that will occupy the antigen-binding site of the disease-specific antibodies with sufficient affinity and selectivity to retain it from serum. There is precedent for this approach in that peptoids can have specific protein binding activity. Their interactions with proteins can be optimized by rational chemical design and have been used as functional ligands for molecules such as VEGF receptor 2, an antigen-specific T cell receptor, and PKB/Akt (Udugamasooriya et al., 2008; Gocke et al., 2009; Tal-Gan et al., 2010). We have used un-biased peptoid libraries to identify antibodies that can characterize patients with SLE without regard to the antigenic specificity of the antibody.

2. Materials and methods

2.1 Human subjects

All serum samples for this study came from subjects enrolled in the Dallas Regional Autoimmune Disease Registry. The UT Southwestern Medical Center Institutional Review Board approved this study, and all subjects gave informed consent. Subjects were healthy controls if they had no personal or family history of autoimmune disease, and patients with RA and SLE met the 1987 and 1997 disease classification criteria of the American College of Rheumatology, respectively (Tan et al., 1982; Arnett et al., 1988; Hochberg, 1997). Patients were categorized as “incomplete lupus erythematosus” (ILE), if they had evidence of an antinuclear antibody and one or two other features of SLE as previously described (Olsen et al., 2012).

2.2 Autoantibody testing

Solid phase measurement of ANA was performed by ELISA (INOVA) as previously described (Wandstrat et al., 2006). Simultaneous measurement of extractable nuclear antibodies (ENA) was performed using a multiplex bead assays as described (Wandstrat et al., 2006). These methods have been shown to correlate with standard clinical indirect immunofluorescence and ELISA measurements of these antibodies (Olsen et al., 2012).

2.3 Peptoid library synthesis

Two different 'one-bead one-compound' combinatorial libraries of peptoids (oligo-*N*-substituted glycines) were synthesized onto 75µm TentaGel beads using a split and pool method (Figliozzi et al., 1996). The first library had the general structure of NH₂-X₈-diaminobutane-methionine-TentaGel, where X is aminoethylamine, allylamine, glycine, 4-(2-aminoethyl)-benzenesulfonamide, cyclohexylamine, 3,4-dimethoxyphenethylamine, isobutylamine, diaminobutane, methylbenzylamine, benzylamine, piperonylamine, N-(3'-aminopropyl)-2-pyrrolidinone, or ethanolamine. The second library was configured as NH₂-X₇-methoxyethylamine-methoxyethylamine-methionine-TentaGel, where X is allylamine, glycine, cyclohexylamine, furfurylamine, isobutylamine, methylbenzylamine, methoxypropylamine, benzylamine, piperonylamine, or ethanolamine. This library was constructed to minimize the dominance of charge effects due to the diaminobutane (a lysine equivalent) and to test more hydrophobic molecules. The methionine linker was coupled in the usual way, while the peptoid residues were coupled using the sub-monomer method (Zuckermann et al., 1992) with microwave irradiation to accelerate reactions (Olivos et al., 2002). Proper library synthesis was confirmed by CNBr cleavage of compounds from a sample of isolated beads and subsequent analysis by tandem mass spectrometry.

2.4 On-bead library hybridization and magnetic screening

A modification of the on-bead library magnetic screening method (Astle et al., 2010) was employed using ~ 375,000 swelled library beads washed with PBST (PBS-0.1% Tween 20), then blocked with blocking buffer (1:1 mixture of PBST-1% BSA and SuperBlock Blocking Buffer (Thermo Scientific, Rockford, IL)) for 1 hr at RT. For the first library, serum aliquots from 46 ANA-negative, healthy individuals were pooled and diluted in 1ml of blocking buffer to have a total IgG concentration at 40 µg/mL and then incubated with the blocked library beads overnight at 4° C. The beads were washed with PBST eight times, re-suspended in blocking buffer, and mixed with 50 µl of goat anti human IgG-Dynabead conjugate prepared by mixing 50 µl of Dynabead M-280 streptavidin (Invitrogen, Grand Island, NY) and 10 µg of biotinylated goat F(ab')₂ anti human IgG (SouthernBiotech, Birmingham, AL) for 2 hr at RT with gentle agitation. Library beads that bound anti-human IgG were removed by placing the tube in a strong magnetic field. The wash/magnetic isolation step was repeated twice and then the remaining library beads (theoretically depleted of non-specific IgG binding peptoids) were incubated overnight with pooled serum from 47 SLE patients. "Hit" peptoid-beads were isolated with anti-IgG coupled to Dynabeads. The peptoids were cleaved from the TentaGel beads and sequenced using a 4800 MALDI TOF/TOF.

The second library was screened in a similar manner, except that it was first depleted using pooled serum from 18 healthy control subjects who had a positive ANA. This was done to remove potential false positive hits representing non-pathological autoimmunity. The library was then screened for peptoids that bound to pooled serum from 18 patients who have ILE.

Re-synthesis of hit peptoids was performed on Polystyrene AM RAM resin (Polymere GmH, Tübingen, Germany) employing a cysteine linker. After all amines were coupled,

peptoids were cleaved off the resin by incubating with a 95% TFA, 2.5% triisopropylsilane, and 2.5% water mixture for 2 h, purified using HPLC and verified by MS.

2.5 Peptoid ELISA

Maleimide activated 96-well plates (Pierce Biotechnology, Rockford, IL) were used to immobilize the sulfhydryl-containing SLE hit peptoids at 0.03 to 0.05mM in 0.1 M sodium phosphate, 0.15 M sodium chloride, 10 mM EDTA; pH 7.2. The peptoid-immobilized plates were blocked with PBST-1% BSA-5% goat serum for 1h at RT. After washing with PBST, the blocked plates were incubated with diluted serum samples for 2hr at RT. Washed plates were then incubated for 30 min at RT with goat anti human IgG-Fc-HRP conjugate (Bethyl Laboraories, Montgomery, TX), diluted at 1:30,000 in blocking buffer, then with TMB substrate for 16min at RT. All samples were run in duplicate. Each ELISA plate contained both the healthy control (HC) serum pool and an SLE serum pool. Results are reported as the absorbance at 450 nm after background subtraction normalized to the HC pool values. Inter-experiment validity was checked by comparing the results for the SLE positive control.

2.6 Affinity purification of peptoid-binding IgG

The SLE15 peptoid was coupled to MicroLink™ iodoacetyl gel columns (Pierce Biotechnology, Rockford, IL). IgG from individual subjects was isolated from serum using Melon™ Gel IgG Spin purification kit (Pierce Biotechnology, Rockford, IL). Purified IgG was then incubated overnight at 4° with the SLE15 peptoid column. The flow through was collected by centrifugation ("SLE15-depleted"). The column was washed 3x with 0.5 M NaCl-0.05% Tween 20 then 3x with PBS. Then 100 µl of elution buffer, pH2.8, was added to each column to remove peptoid-binding IgG. Immediately 5 µl of 1 M Tris, pH 9.0 was added to each eluted aliquot.

A human IgG ELISA Quantitation Kit (E80-104, Bethyl Laboraories, Montgomery, TX) was used to measure the IgG concentration of the sample before affinity purification (before column incubation), in the depleted IgG (after column incubation) fraction and in the eluted fraction.

2.7 Autoantigen microarray analysis of peptoid-recognized antibodies

The three IgG samples (before, after and eluted) from the SLE15 peptoid column were analyzed with autoantigen microarrays as described previously (Li et al., 2007; Li et al., 2011). Autoantibodies were detected with Cy3-labeled anti-human IgG and Cy-5 labeled anti-human IgM on parallel arrays. Imaging and calculation of mean fluorescence intensity were carried out as described previously (Li et al., 2011). Values were expressed as normalized mean fluorescent intensities (MFI) using standard control antigens on each slide. Heat maps were generated using Cluster and Treeview software.

2.8 Western blot analysis

Based on the result of autoantigen microarray analysis, eight autoantigens with higher signal in the SLE-peptoid purified samples were selected for Western Blot analysis. 1 µg of each autoantigen (La/SS-B, Ro/SS-A (52), Scl-70, PM/Scl-100, U1-snRNP-68kD, U1-snRNP-A, U1-snRNP-B/B', U1-snRNP-C (DIARECT AG, Freiburg, Germany), as well as the negative control desmoglein 4 (DSG4, Abnova, Taiwan) were loaded in a Criterion TGX 4-20% Precast Gel (Bio-Rad Laboratories, Hercules, CA). In each experiment, the same amount of each antigen was duplicated in four gels. Following electrophoresis, gels were transferred onto Immobilon-P membranes (Millipore, Bellerica, MA). Membranes were blocked with blocking buffer (2.5% BSA and 2.5% non-fat milk in PBST) overnight at 4°, then incubated with 63 ng/mL of purified IgG from different subjects for 2hr at RT, washed and then

incubated for 30 min at RT with goat anti human IgG-Fc-HRP conjugate prior to visualization (SuperSignal West Dura Substrate, Thermo Scientific, Rockford, IL and Amersham Hyperfilm ECL high performance chemiluminescence film, GE healthcare Limited, Buckinghamshire, UK). The protein bands identified in this manner corresponded to the M_r of the proteins as given by the supplier. All membranes were exposed for identical times to minimize inter-experiment variation.

2.9 Statistics

All statistical analyses were done using GraphPad Prism 6.0. The mean values of untransformed ELISA binding data were compared using Mann-Whitney tests. The Kruskal-Wallis test was used to compare multiple groups with Dunn's post-test for the multiple comparisons. Fisher's exact test was used to compare the dichotomous classification in the Table.

3. Results

3.1 Identification of "hit" peptides

The first goal of this study was to determine whether synthetic peptidomimetics could be identified that would bind immunoglobulin from patients with SLE. Two separate strategies were employed for peptide library synthesis and screening. The demographics of the control, ILE, and SLE subjects are shown in Table 1. Subjects were chosen from the Dallas Regional Autoimmune Disease Registry biobank based on diagnosis and ANA titer (by ELISA) such that a wide range of ANA values was obtained. There was no attempt to select samples based on disease features (e.g., thrombocytopenia or anti-Sm) or disease activity. "Test" sets of subjects were used to select the peptides for study while "Validation" sets were used to determine the test characteristics (sensitivity, specificity, likelihood ratios). The first library consisted of 10-mers synthesized from 13 different amines. An invariant diaminobutane-methionine unit was included at the carboxyl terminus and diaminobutane was included in the monomer list to create more hydrophilic structures. This library was first depleted of beads that display peptides that bound to immunoglobulin from a pool of 46 ANA-negative healthy subjects. Then, a serum pool from 47 patients with SLE was incubated with the remainder of the beads (Test Set 1). For the second library, diaminobutane was omitted and the peptides were terminated by methoxyethylamine-methionine. This eliminated the potential charge interactions. This library was first depleted of peptides that bound to a pool of 18 serum samples from healthy control individuals who were found to have elevated levels of ANA. This was done to remove ligands that would react with these autoantibodies, but would not identify diseased individuals. Then peptides that bound to a pool of serum from 19 patients with incomplete lupus erythematosus ILE (Test Set 2) were isolated. ILE subjects were used to try to find ligands that would identify the first changes in serological autoimmunity associated with disease.

Six peptides were isolated from the first library and were termed SLE10-SLE15. Seven peptides were isolated from the second screen and were termed ILE1-ILE7. These peptides were cleaved from the beads using CNBr and their structures determined by tandem MS/MS. They were then re-synthesized in bulk and coupled to maleimide-activated ELISA plates via their sulfhydryl residue. The binding characteristics of two of the selected peptides, SLE15 and ILE2 to the discovery pools (Test Sets) used to select the peptides are shown in Figures 1A and 2A, respectively. SLE15, ILE2, and ILE7 were selected for further characterization. They had the greatest reactivity to the pooled SLE or ILE serum, and the lowest signal from control serum pools. Their chemical structures are shown in Figure 3. In each case, there is very low binding to serum from healthy controls and significantly higher binding to pooled serum from SLE patients. This validates the original peptide selection

(Figure 1A). Two pools of serum from lupus patients were tested. Both of them contained antibodies to chromatin but differed in the presence of anti-Ro autoantibodies. These are the most common specific autoantibodies in patients with SLE. For both peptoids, there is some background binding to control serum, particularly at higher concentrations, but significantly greater binding to serum from lupus patients. In the case of the ILE peptoids, there was no significant binding to healthy control subjects who had high titers of ANA either as a pooled sample, or individually (see below).

Since patients with autoimmunity may have higher levels of IgG in their serum, all analyses were done on dilutions of serum corrected for measured total IgG levels. In some experiments, purified IgG from either pooled or individual serum samples was used. No difference in peptoid binding was seen between serum and purified IgG (not shown).

3.2 Validation of SLE15 and ILE2

A comparison of the binding of separate validation sets of healthy and lupus individuals is shown in Figures 1B and 2B for SLE15 and ILE2, respectively. In each case, the binding of IgG from the lupus patients was significantly greater than the binding of IgG from the control individuals. The receiver-operating characteristic curves for SLE15 and ILE2 are shown in Figures 1C and 2C. In both cases, IgG binding to the peptoids accurately classifies subjects with SLE. However, ILE2 binding is much more robust, with an area under the curve of 0.90 versus 0.72 for SLE15. For SLE15, the maximum positive likelihood ratio of 7.45 was obtained at a sensitivity of 19.6% (9.8-33.1%) and a specificity of 97.4% (86.2-99.9%, $p = 0.0003$). For ILE2, a maximum positive likelihood ratio of 28.0 was obtained at a sensitivity of 70.0% (53.5-83.4%) and a specificity of 97.5% (86.8-99.9%, $p < 0.0001$).

Panels 1D and 2D show the peptoid binding of IgG from individuals with other conditions: healthy controls, RA, immune alteration (i.e., ANA⁺ healthy individuals, IA); and ILE. While a subset of subjects with ILE have much higher binding to SLE15 than healthy control subjects, the binding in these two groups overall is not significantly different. For ILE2, there was an apparent gradient of peptoid binding to the serum of RA patients without ANA, RA patients with positive ANA, and ILE patients such that each group is significantly different from healthy controls.

3.3 Comparison of SLE15 and ILE2 binding

The distribution of control serum binding to the peptoids as determined by ELISA was not normal (Figures 1B and 2B). However, a log transformation of the ELISA result for ILE2 did fit a Gaussian distribution (Figure 4A, $r^2 = 0.88$). Similar results were seen for SLE15 and a second ILE peptoid, ILE7 (not shown). Simultaneous ELISA binding assays for the three peptoids were performed on a panel of control subjects. A two dimensional plot of the log transformed ELISA data for two structurally related peptoids, ILE2 and ILE7 (Figure 4B) shows that they are highly correlated (Pearson $r = 0.90$, $p < 0.0001$) suggesting that these two peptoids are measuring the same immunoglobulin ligands. However, a similar analysis for SLE15 and ILE2 measurements (Figure 4C) has a much lower degree of correlation ($r = 0.44$, $p = 0.004$). While the antigens recognized by ILE2/7-binding IgG molecules are not yet known, this suggests that they are not identical to those seen by SLE15, and that the methodology of using libraries composed of different amine monomers is capable of identifying different subsets of serum immunoglobulin. ANA levels among all subjects (healthy and lupus) generally fell into two groups. Both groups displayed a wide range of overlapping values for IgG binding to ILE2 (Figure 4D) or SLE15 (not shown). This suggests that the antibodies bound to the peptoids are distinct from those picked up by ANA in traditional immunofluorescence testing. Finally, the levels of peptoid-binding IgG

in SLE patients were compared to the presence of clinically relevant antibodies to extractable nuclear antigens. There was no significant difference in either SLE15 or ILE2 binding in subjects who did or did not have elevated levels of antibodies to chromatin (anti-DNA), Sm, U1-RNP, 52 kDa Ro, 60 kDa Ro or La antigens (not shown).

The binding of IgG to SLE15 and ILE7 was simultaneously determined on serum samples from controls or individuals with RA, ILE, and SLE (Figure 5). Using the fitted normal distribution of the log transformed data from healthy control subjects, cut off values corresponding to 2 standard deviations above the mean were determined. Nearly all the control individuals fell into the quadrant that was bounded by these values (Figure 5A). Approximately half of the RA patients had ILE7 binding but only one had SLE15 binding above the conservative upper limit of normal. Both ILE and SLE patients had similar characteristics. Approximately half of the subjects had levels of peptoid binding that overlapped the control subjects and half had both ILE7 and SLE15 binding. These findings further support the concept that the two classes of peptoids, ILE2/7 and SLE15 are identifying different classes of immunoglobulin biomarkers that can distinguish separate autoimmune conditions, e.g., early versus established SLE.

3.4 Using peptoid binding to classify subjects

Individuals can be classified as peptoid-binding positive and peptoid-binding negative based on an upper limit of normal defined as the mean + 2SD from the control population. This dichotomous assessment gives a more stringent assessment of peptoid binding than the continuous measures in Figure 1B and 2B. All evaluable subjects for this study were then categorized according to ILE2 binding by this method. As shown in Table 2, nearly all control subjects lack ILE2 binding, while 61% of SLE patients are ILE2-positive. The RA and ILE patients are intermediate at 40% and 39% respectively. Peptoid binding conferred an increased relative risk for disease over control in each of the three conditions, with values of 2.3, 4.0, and 2.5 for RA, ILE, and SLE, respectively. These were all statistically significant. ILE2 binding also conferred a significant relative risk of 1.6 for SLE versus RA. Using these more conservative data, the sensitivity for classifying SLE versus control based solely on ILE2 binding was 61% (47.9-72.9%) with a specificity of 97.5% (86.8-99.9%) and a positive likelihood ratio of 24.4.

3.5 Purification of IgG using immobilized peptoid

The immunoglobulins bound by SLE15 were characterized by autoantigen microarray and by Western blot. First, IgG was purified from individual subjects with high levels of SLE15 binding by ELISA. Next, the IgG was passed over a column of SLE15 peptoid covalently bound to iodoacetyl-activated resin. Non-binding IgG was collected as a “depleted” pool, and SLE15 binding IgG was eluted with pH 2.8 buffer. The binding of the different fractions from eight individuals is shown in Figure 6. All subjects had low, but measureable levels of SLE15 binding IgG prior to column purification. The post-column flow through had little or no peptoid binding, even though identical amounts of total IgG were tested. The IgG that bound to and was eluted from the column had up to ten times the specific SLE15 binding activity of the flow-through fraction. The specificity of the column depletion was tested by comparing the purified and peptoid depleted fraction to bind common antigens including rubella and varicella zoster virus. While binding to SLE15 was diminished from 50-99% (Figure 6), binding to viral antigens was only diminished 0-28% (not shown).

The three fractions of IgG from four individuals were then tested for their ability to bind to 80 native autoantigens in a microarray format (Li et al., 2007). The binding of IgG to the array is detected by immunofluorescence and normalized to the total IgG signal. For ease in analysis, the antigen binding data can be clustered and visualized as a traditional heat map

(Figure 7). Surprisingly, the purification of IgG on the SLE15 column did not result in a single antigen-reactive species. Instead, the SLE15 purified IgG was poly-reactive with a number of previously described autoantigens, particularly those associated with SLE, including snRNPs, Ro, La, several tRNA synthetases (Jo-1, PL-7, PL-12) as well as thyroid antigens such as thyroglobulin and thyroid peroxidase. To prove that the SLE15-purified IgG had reactivity to these antigens, Western blots with purified proteins were performed. As shown in Figure 8, the IgG purified by the peptoid columns had reactivity to a number of nuclear antigens, although each subject had a unique pattern. Most subjects, including the ANA+ healthy controls had antibodies to 52 kDa Ro, while the patients with incomplete lupus and SLE typically had antibodies to Ro as well as to several protein components of small nuclear RNPs, including U1-A, U1-B/B', and U1-68kD. Together, these data support the conclusion that the SLE15 peptoid binds to a family of autoantibodies rather than acts as a specific antigen surrogate.

4. Discussion

The goal of this study was to assess the utility of a novel biomarker platform in SLE. A highly diverse library of *N*-substituted glycine oligomers was probed for compounds that identified patients with SLE as well as related phenotypes using stored serum samples. Several peptoids were identified that could characterize patients with a high degree of accuracy. Moreover, several of these compounds appear to be able to discriminate between healthy individuals with a positive ANA, and those with evidence of clinically significant autoimmunity suggesting that the two methods can be used as complementary tests. It should be noted, though, that ANA positivity in this study was performed using a solid-phase assay that is typically less sensitive than indirect immunofluorescence (Meroni and Schur, 2010) although the level of ANA by ELISA has shown to correlate significantly with the reactivity of ANA on Hep-2 cells in previous studies (Olsen et al., 2012).

Seropositivity to the peptoids SLE15 and ILE2 has a positive likelihood ratio (LR) of roughly 7.5 and 28, respectively, when used to classify the SLE patients compared to the healthy controls. This compares to a +LR of 4.2 for the traditional ANA test (Solomon et al., 2002). If a more stringent cut-off for seropositivity is used, and the data from healthy individuals and patients with RA are combined (Table 2), the +LR is 3.4 compared to the value of 2.2 for the ANA when used to differentiate lupus from disease controls and healthy individuals overall. In the subjects studied here, the +LR for ANA comparing lupus to healthy controls is 2.0 due to the fact that the control subjects were selected to have an ANA prevalence of ~50%. By comparison, two other serological tests that are commonly used in the diagnosis of lupus are antibodies to double stranded DNA, which has an estimated +LR of 16.4 (Kavanaugh et al., 2002) and is most typically performed by indirect immunofluorescence, and antibodies to the Sm proteins of the U1 ribonucleoprotein complex, which are determined by ELISA on purified protein and has a mean +LR of 12 (Benito-Garcia et al., 2004). In the subjects studied here, the +LR for antibodies to chromatin (the ELISA version of anti-DNA) was 8.0.

In two of the four cohorts, the proportions of subjects who have SLE were more likely to be Hispanic or African American. It is possible, but unlikely, that a specific autoantibody exists that is restricted to these ethnic groups. It is possible that higher titers of certain autoantibodies are more common in these groups. While this was not seen in the other two cohorts in this study it merits further investigation.

We, and others, have used whole antigen (protein or lipid) microarrays to characterize the sera of subjects with autoimmune diseases (Robinson et al., 2002; Kanter et al., 2006; Li et al., 2007; Gibson et al., 2010; Li et al., 2011). The advantages of these arrays are that they

reveal qualitative patterns of autoimmunity specific to disease states. These patterns can be unexpected, such as the finding of increased reactivity to skin antigens seen in healthy ANA-positive subjects (Olsen et al., 2012). These multiplexed autoantibody assays can also be quantitative, as we have shown the increase in certain IgG autoantibodies and the decrease in IgM autoantibodies as subjects progress from less definite to more definite forms of SLE. However, these arrays are limited in that they are inherently biased to known autoantigens. The use of peptoids allows the unbiased assessment of protein binding to classify subjects.

While the overall goal of unbiased proteomics screens such as the ones described here is typically to develop better methods of providing diagnostic or prognostic information, there is no *a priori* assumption about how that will occur. It is possible to develop assays for unknown biomarkers as has been done for Alzheimer's Disease (AD) (Reddy et al., 2011) and lung cancer (Hooks et al., 2011). A library of 8600 peptoids was screened with serum from Alzheimer's disease (AD) patients and three peptoids were isolated that were able to correctly classify the presence of AD in a subsequent validation study of 16 patients. The target(s) of the antibodies that react with these peptoids are unknown. Similarly, specific peptoid-based ligands have been developed that specifically bind to lung cancer cells although the cell surface protein target of the peptoids is unknown. These studies illustrate the utility of peptoid/protein interactions as useful biomarkers, even without the identities of the natural ligands.

It is also possible to develop improved ways of detecting known biomarkers. A library of over 100,000 peptoids was screened with serum from patients with neuromyelitis optica (NMO), a demyelinating condition characterized by cytotoxic antibodies to aquaporin 4. Several peptoids were isolated that bound specifically to anti-aquaporin 4 and could correctly identify NMO patients in 13 of 15 cases (Raveendra et al., 2013). Finally, the peptoid screens can identify novel biomarkers whose identity can be learned through secondary analysis. Previously unknown autoantibody specificities in autoimmune pancreatitis were detected using pooled IgG to screen against a random peptide library (Frulloni et al., 2009). Antibodies to a peptide derived from the *H. pylori* plasminogen binding protein were detected in 33 of 35 patients with autoimmune pancreatitis, but only 5 of 110 with pancreatic cancer. The specificity of this reactivity was confirmed by Western blot. These findings are consistent with a possible role for *H. pylori* infection in the pathogenesis of autoimmune pancreatitis.

When this study of SLE was begun, there were no assumptions as to which of these answers would be obtained. For SLE15, the conclusion is an unexpected finding that this one ligand is capable of binding multiple different autoantibodies. The fact that the immobilized SLE15 depleted the SLE15 binding IgG from the pool of purified IgG argues that the peptoid is not just non-specifically binding all immunoglobulin. There are several possible explanations. Each of the peptoids can exist in many different conformations due to rotation about the secondary amino nitrogen atoms. This may result in not only lowered affinity for each peptoid as only a fraction of the molecules are in the correct orientation, but also in lowered specificity of binding to related molecules. By adding a methyl group to the alpha carbon, peptoid tertiary amines with more rigidity and higher affinity for specific ligands can be obtained (Gao and Kodadek, 2013).

The peptoids may be interacting with conserved protein binding regions of the autoantibodies, such as C1q or FcR binding domains. To date, inhibition studies with purified antigens or C1q have not been done. Second, the autoantibodies may, in fact, cross-react with one another. For example, immunization of mice with a peptide surrogate for anti-DNA antibody ligands leads to the development of antibodies not only to the immunizing

peptide, but also autoantibodies to double-stranded DNA, ribonucleoprotein, cardiolipin and laminin (Putterman and Diamond, 1998). Some of these polyreactive, germline encoded antibodies have both nephrotoxic and neurotoxic potential (Zhang et al., 2009). Their pathogenicity may be related to the class switching from IgM to IgG, as has been suggested for the evolution of individuals with serological autoimmunity to more complete expression of the lupus phenotype (Li et al., 2007). It is possible that the peptoid ligands represent a surrogate for a common structural motif in many autoantigens recognized by lupus sera. For example, a peptide ligand has been identified that binds to and inhibits IgG that recognizes both DNA and the NMDA receptor in patients with lupus (Bloom et al., 2011). Molecular analysis of monoclonal autoantibodies derived from lupus-prone mice demonstrated conserved binding motifs, as well as cross-reactivity explained, in part, by the binding of nucleic acid to the immunoglobulin (Liang et al., 2004; Chang et al., 2009). The binding of purified immunoglobulin to SLE15 after treatment with RNase and/or DNase eliminates the unlikely possibility that the peptoid was actually binding attached nucleic acid. Immunoglobulin G subclass quantification (not shown) showed no difference between the IgG pools depleted of SLE15 binding IgG or purified by immobilized SLE15. A final possible explanation for the binding of both SLE15 and ILE2/7 to a subset of IgG molecules from subjects with SLE is that these generally hydrophobic peptoid molecules are recognizing soluble immune complexes. Immune complexes containing DNA, RNA, and nucleic acid binding proteins have been recognized for decades as central to SLE, not only as diagnostic and prognostic biomarkers, but also as important mediators of disease pathogenesis (Reviewed in Elkon and Wiedeman, 2012; Rekvig et al., 2012; Tan, 2012). Immune complexes from lupus serum stimulate peripheral blood mononuclear cells, particularly plasmacytoid dendritic cells to produce type I interferon, a hallmark of the disease (Lovgren et al., 2006; Garcia-Romo et al., 2011; Lande et al., 2011).

Soluble immune complexes in lupus sera have been analyzed by precipitation in polyethylene glycol (PEG) or by their binding to C1q (Skinner and Maddison, 1990; Ahlin et al., 2012). The two methods are not completely identical, as lupus patients also produce autoantibodies to the collagen like domain of C1q that bind independent of immune complexes (Uwatoko and Mannik, 1988; Mannik and Wener, 1997). Detailed analysis of PEG precipitated immune complexes from lupus patients suggests that the circulating complexes are enriched in antibodies binding RNA but not anti-double stranded DNA (Ahlin et al., 2012). This is consistent with the frequent finding of antibodies to Ro52 and RNP in the SLE15 purified IgG from both lupus and control sera. In contrast to anti-DNA, neither antibodies to RNA-associated antigens or RNA-containing immune complexes seem to be associated with SLE disease activity. While preliminary studies have suggested that PEG precipitates contain SLE15 and ILE2 reactivity (not shown), current work is directed at a quantitative analysis as well as longitudinal studies of lupus patients at different levels of disease activity.

5. Conclusions

Highly diverse libraries of *N*-substituted glycine polymers (peptoids) were probed for their ability to react specifically with the IgG fraction of serum from patients with systemic lupus erythematosus. Several peptoids were identified that could be used to classify individuals with lupus compared to controls with high sensitivity and specificity, and with test characteristics comparable to or better than standard serological tests. Importantly, this assay could differentiate healthy subjects who had evidence of autoimmunity as measured by the standard anti-nuclear antibody test from subjects with true pathological autoimmunity. Certain peptoids were used to purify polyclonal autoantibodies from lupus patient serum, suggesting that these abiological molecules detect classes of IgG found in disease, rather than single antigen-specific immunoglobulin.

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Highlights

- Peptoid libraries yield compounds that react with disease-specific IgG.
- Ligands were discovered that classify patients with systemic lupus erythematosus.
- A peptoid binding ELISA had moderate sensitivity and high specificity for lupus.
- Peptoid-binding IgG reacts with several disease-associated autoantigens.

FIGURE 1.

Binding of serum IgG to peptoid SLE15. A. Titration of IgG binding to peptoid SLE15 using serum pools from healthy control (HC) individuals who lacked detectable ANA (closed circle), SLE patients who had high levels of anti-chromatin but lacked anti-Ro antibodies (closed triangle), and SLE patients who had high anti-chromatin and anti-Ro antibodies (closed square). B. Binding of serum IgG from a validation set of control individuals and SLE patients to peptoid SLE15. Results are expressed as the ratio of binding compared to a reference serum pool from control subjects. C. Receiver operating characteristic curve for the association of SLE15 binding with the diagnosis of SLE compared to control subjects. The sensitivity and specificity are 19.6% (9.8-33.1%) and 97.4% (86.2-99.9%), respectively with a positive likelihood ratio of 7.45. $p=0.0003$. D. Comparison of peptoid SLE15 binding in different conditions, HC – healthy control, RA – rheumatoid arthritis, IA – “immune alteration”, i.e., ANA-positive healthy controls, ILE – incomplete lupus erythematosus.

FIGURE 2.

Binding of serum IgG to peptoid ILE2. A. Titration of IgG binding to peptoid ILE2 using serum pools from ANA-negative HC (closed circle), ANA-positive HC (closed square), ILE patients (closed triangle), anti-Ro-negative SLE patients (inverted triangle), and anti-Ro-positive SLE patients (diamond). B. Binding of serum IgG from a validation set of control subjects and SLE patients to peptoid ILE2. C. Receiver operating characteristic curve for the association of ILE2 binding with the diagnosis of SLE compared to control subjects. The sensitivity and specificity are 70.0% (53.5-83.4%) and 97.5% (86.8-99.9%), respectively, with a positive likelihood ratio of 28.0. $p < 0.0001$. D. Comparison of peptoid ILE2 binding in different conditions.

FIGURE 3.
Structures of peptoids SLE15, ILE2, and ILE7.

FIGURE 4.

Comparison of ILE2/7, SLE15, and ANA binding. A. Plot of log-transformed serum IgG binding from healthy control subjects to ILE2 and the superimposed Gaussian curve fit to the data. B. Comparison of the log-transformed values for the binding of IgG from healthy control subjects and SLE patients to peptoid ILE2 and ILE7. C. Comparison of the log-transformed serum IgG binding to peptoids ILE2 and SLE15. D. Comparison of the log-transformed serum IgG binding to ILE2 to log-transformed ANA values

FIGURE 5.

Comparison of SLE15 and ILE7 binding in different subjects. The binding of IgG to peptoids SLE15 and ILE7 was determined simultaneously on serum samples from control subjects (A), patients with RA (B), patients with ILE (C), and patients with SLE (D). The binding values were log-transformed and plotted on x- and y-axes, respectively. The dotted lines represent the mean + 2SD obtained from the fitted Gaussian distribution of control subjects (e.g., see. Figure 4A).

FIGURE 6.

Purification of SLE15-binding IgG. IgG purified from serum of different individuals was passed over a column of immobilized SLE15 peptoid. The comparative binding of the input IgG (open columns), the depleted IgG fraction (black columns) and the eluted IgG fraction (shaded column) in a subsequent SLE15 ELISA is shown. All samples were tested at identical IgG concentrations as determined by quantitative ELISA.

FIGURE 7. Autoantigen array of IgG bound to peptoid SLE15. IgG binding to a 76 feature microarray of whole autoantigens was tested using 1) the whole purified IgG fraction from 4 individuals, 2) the “depleted” IgG fraction from the SLE15 column, and the eluted IgG from the SLE15 column. The array was analyzed using Cluster software to produce a heat map of relative binding to each antigen. Analysis of the entire array is shown in panel A and an enlargement of the section with higher than median binding is shown in panel B. Individual subjects are assigned a code indicating the pre-column IgG fraction. The suffix -D indicates the fraction depleted of SLE15 binding IgG and the -E1 suffice the eluted IgG fraction.

FIGURE 8.

Western blot using SLE15 purified IgG. Purified IgG eluted from seven individuals was used to probe Western blots of eight autoantigens identified as potential targets of SLE15-binding IgG from the microarray. A. Healthy control subject; ANA 65 ELISA units. B. Healthy control subject, ANA 43 ELISA units. C. ILE subject; ANA 201 ELISA units. D. SLE subject; ANA 185 ELISA units. E. SLE subject; ANA 118 ELISA units. F. SLE subject; ANA 169 ELISA units.

TABLE 1

Demographics of Subjects Used to Select Peptoids

Population	N	Median Age (Range)	Female (%)	Hispanic (%)	African American (%)
Test Set 1 ^a					
Control	46	27 (22-59)	63	9	15
SLE	47	38 (18-80) ^c	96	15 ^e	49 ^d
Validation Set 1					
Control ^d	38	29 (22-81)	71	16	13
SLE	50	42 (18-68) ^e	96	32 ^e	36 ^e
Test Set 2					
Control (ANA pos)	18	29 (22-81)	78	17	28
ILE	19	41 (25-66) ^e	100	11 ^e	67 ^e
Validation Set 2					
Control	40	27 (22-79)	63	3	28
SLE	40	38 (19-67) ^e	83	40 ^d	43 ^e

^aPools of serum from individuals in the Test Sets 1 and 2 were used to screen peptoid Libraries 1 and 2, respectively. Individual samples from Validation Sets 1 and 2 were used to determine the sensitivity and specificity of SLE15 and ILE2 respectively.

^b50% of the Control subjects in each Validation Set had antinuclear antibodies as detected by solid phase assay.

^cp<0.05, Student's t-test

^dp<0.005, Fisher's exact test

^eNot significant

TABLE 2

Use of ILE2 to classify subjects^a

	Condition			
	Control	RA	ILE	SLE
ILE2 positive	1	12	7	39
ILE2 negative	39	28	11	25
P values^b				
	Control	RA	ILE	SLE
RR (CI)				
Control		0.0008	0.0007	<0.0001
RA	2.3 (1.7-3.2)		0.7633	0.0074
ILE	4.0 (2.2-7.1)	1.2 (0.57-2.7)		0.1132
SLE	2.5 (1.8-3.4)	1.6 (1.1-2.2)	1.2 (0.95-1.6)	

^a Subjects with different conditions were characterized as ILE2-positive or ILE2-negative based on the analysis done for Figure 5.

^b Fisher's exact test was applied to each combination of conditions. The p value for association of ILE2 positivity is shown to the right of the diagonal and the relative risk (95% CI) is shown to the left of the diagonal.