Anti-Sm Autoantibodies in Systemic Lupus Target Highly Basic Surface Structures of Complexed Spliceosomal Autoantigens

Micah T. McClain, Paul A. Ramsland, Kenneth M. Kaufman and Judith A. James

*J Immunol* 2002; 168:2054-2062; doi: 10.4049/jimmunol.168.4.2054
http://www.jimmunol.org/content/168/4/2054

**References**
This article cites 40 articles, 8 of which you can access for free at:
http://www.jimmunol.org/content/168/4/2054.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2002 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Anti-Sm Autoantibodies in Systemic Lupus Target Highly Basic Surface Structures of Complexed Spliceosomal Autoantigens

Micah T. McClain,† Paul A. Ramsland,‡ Kenneth M. Kaufman,*§ and Judith A. James2*†

Autoantibodies directed against spliceosomal proteins are a common and specific feature of systemic lupus erythematosus. These autoantibodies target a collection of proteins, including Sm B, B′, D1, D2, and D3. We define the common antigenic targets of Sm D2 and D3 and examine their role in spliceosomal autoimmunity. Our results define nine major common epitopes, five on Sm D2 and four on Sm D3. These epitopes have significantly higher (more basic) isoelectric points than do nonantigenic regions. In fact, this association is of sufficient power to make isoelectric point an excellent predictor of spliceosomal antigenicity. The crystallographic structure of Sm D2 and D3 is now partially described. The anti-Sm D2 and D3 antigenic targets are located on the surface of the respective three-dimensional complexed proteins, thereby suggesting that these epitopes are accessible in the native configuration. All but one of these nine epitopes conspicuously avoid the specific regions involved in intermolecular interactions within the spliceosomal complex. One of the D3 epitopes (RGRGGRGMR) has significant sequence homology with a major antigenic region of Sm D1 (containing a carboxyl-terminal glycine-arginine repeat), and anti-D3 Abs cross-react with this epitope of Sm D1. These results demonstrate that spliceosomal targets of autoimmunity are accessible on native structure surfaces and that cross-reactive epitopes, as well as structural associations of various spliceosomal Ags, may be involved in the induction of autoimmunity in systemic lupus. The Journal of Immunology, 2002, 168: 2054–2062.

S
ystemic lupus erythematosus (SLE)3 is a heterogeneous, idiopathic autoimmune disorder unified by the presence of a host of autoantibodies, many of which recognize Ags from the cell nucleus. Several of the common antigenic targets of these responses include dsDNA, nuclear ribonucleoproteins (nRNPs), Sm, Ro/SS-A, La/SS-B, and ribosomal P. Of these autoantibodies, anti-Sm and anti-dsDNA are sufficiently discriminating to be part of the American College of Rheumatology classification criteria for SLE (1). In SLE patients, as much as 20% of the entire Ig repertoire may bind Sm proteins (2). These anti-Sm autoantibodies individually target a number of components of the spliceosome, namely Sm B (26 kDa), B′ (27 kDa), D1 (16 kDa), D2 (16.5 kDa), D3 (18 kDa), E/F (11 kDa doublet), and G (<10 kDa).

Protein sequences of Sm B, B′, D1, D2, D3, and E have been known for some time. The last of these to be defined were Sm D2 and D3 by Lehmeier et al. (3), mAbs coprecipitate Sm D1 and Sm D2, but not Sm D1 and D3, suggesting a specific interaction between the D1 and D2 molecules. Sm D3 and Sm B′ have also been shown to bind to one another (4). Indeed, Kambach et al. (5) recently described the crystalline structure of the D1/D2 complex, as well as the B′/D3 complex. This study revealed the specific crystallographic structural details of the protein-protein interactions that help make up the spliceosomal complex.

Previous work has detailed the prevalence and possible clinical significance of the autoantibodies binding to Sm B, B′, or D1 (6–10). In addition, the precise sequential antigenic regions of these proteins recognized by human lupus autoantibodies are described (7, 8). Among the most important of these sequential epitopes is the glycine-arginine (GR)-rich carboxyl region of Sm D1, which is recognized by virtually all Sm-precipitin positive lupus patient sera (8, 10–14). This region of Sm D1 also shows striking sequence homology to a portion of the Sm D3 protein, and immunization with this region of D1 results in the production of anti-D3 Abs (13). The (GR)-rich portions of the Sm D1 and D3 proteins thus appear to be significant targets of the anti-Sm response. This raises the question of whether these autoantibodies (as well as anti-D2) arise through cross-reactivity with other previously antigenic proteins or through a mechanism by which their close physical association with these more commonly antigenic proteins promotes the loss of tolerance. This mechanism of physical association has been suggested for Ro/La, the nucleosome, and other autoantigen systems as well (15–17).

The ability to compare Ab fine specificity to defined three-dimensional Ag structure is highly significant and could potentially aid in answering questions of autoantibody origin. In the Ro and Sm B/B′ systems in lupus, affinity purification of Abs using native human Ag leads to a consistent increase in binding to most of the antigenic octapeptides (9, 18). Thus, those Abs binding to native Ag seem to comprise the same set of Abs that bind to the octapeptides. This implies that autoantibody binding to octapeptides primarily reflects binding to surface structures and defines minimal to no cryptic epitopes with respect to the Ro and Sm B/B′ autoantigens in human
SLE. Based upon these data, the current hypothesis is that in systemic autoimmune responses many spliceosomal Abs preferentially target regions of an Ag that are localized to the autoantigen surface. The mechanism of determinant spreading in the SLE has also been proposed to involve structures that are positioned near one another on the surface of the three-dimensional structure (20). Amazingly, very few autoantigens have defined crystalline structures, which would allow testing of this hypothesis. Such data could prove invaluable in defining conformational epitopes of these autoantigens.

Although potential surface position on the crystal structure is an important factor in determining antigenicity of peptide sequences, other means are undoubtedly significant. Previous observations have suggested that the isoelectric point (pI) of the octapeptide could be used to predict spliceosomal autoantibody binding, especially with Sm B/B’ and nRNP A (9, 19). Basic octapeptides (pI > 10.4) are vastly over-represented in the antigenic regions of these proteins, whereas more acidic or neutral octapeptides (pI ≤ 7.0) are more commonly nonantigenic. These differences may simply reflect a propensity for more basic structures to localize to the surface upon folding, or other mechanisms might be at play.

We now evaluate two additional spliceosomal peptides for autoantibody fine specificity to test the hypothesis that pI can be used to predict spliceosomal autoantibody binding. Our findings confirm that higher pls in the primary sequence significantly increase the likelihood that spliceosomal structures will become autoimmune epitopes. These findings, coupled with earlier data, also suggest that the relationship between pl and antigenicity is a feature common to nuclear autoantigens in systemic lupus (9, 19). In addition, we have used the overlapping octapeptides of the Sm D2 and Sm D3 proteins on solid phase supports to define structures bound by anti-Sm autoantibodies. The epitopes identified tend to be found on the surface of the proposed crystalline structures of the B’/D3 and D1/D2 complexes.

Materials and Methods

Sera selection

SLE patients were selected from the Oklahoma Clinical Immunology Serum Repository who met the Revised Classification Criteria for lupus set forth by the American College of Rheumatology (1) and whose sera contained precipitating levels of anti-Sm by Ouchterlony immunodiffusion. Patients thus tested variably displayed a wide array of autoantibody profiles, including anti-Sm, anti-dsDNA, anti-Ro, anti-La, and/or anti-nRNP autoantibodies. Of the 12 patients in whom anti-D2 or anti-D3 was detected, all were female, seven were African American, four were Caucasian, and one was a Native American. These patients ranged in age from 18 to 30 years, with an average age of 23. All SLE patients had carried a history of arthritis, and Sjögren’s patients were matched for sex and ethnicity and were age-matched to within 5 years of their case.

Cloning and expression of Sm D2 and Sm D3

The cDNA coding for the Sm D2 and D3 proteins was obtained from the American Type Culture Collection (Manassas, VA; accession numbers AA314384 and AA084844). The PCR was used to amplify D2- or D3-specific cDNA using primers that incorporated a 5’ EcoRI site (gCGGATCTCATAGCTCCTCCATAC for D2 and gGGGATCTGTCGTATGTTGC for D3) and M13 reverse primers (for plasmid; New England Biolabs, Beverly, MA). PCR products were purified and ligated into the pCRII cloning vector (Invitrogen, Carlsbad, CA) and sequenced.

Sm D2 and D3 pCRII clones were then digested with EcoRI and HindIII and ligated into the Escherichia coli pMal-c2 expression vector (New England Biolabs). Transformed cells were incubated with 0.3 mM isopropyl β-D-thiogalactoside to induce expression of Sm D2 or D3 fusion proteins with an N-terminal bacterial maltose binding protein moiety. Fusion protein was isolated from whole bacterial lysate by purification on an amylose column and subsequent elution with 10 mM maltose, 20 mM Tris, 1 mM EDTA, 200 mM NaCl (pH 7.4).

ELISAs

Standard solid-phase assays were used to measure the Ab reactivity in human sera or in murine mAbs. One microgram of Ag (purified Sm D2 or D3 fusion proteins, maltose-binding protein, or whole Sm) was coated per well in polylysine plates. Assays were performed using a protocol previously described in detail (7, 8).

Western blots

Affinity-purified Sm D2 and D3 Ags or HeLa cell extracts were subjected to electrophoresis in 12.5% High-N,N,N,N-tetramethylethylendiamine SDS-PAGE gels containing 1% SDS (in 0.15 M Tris-HCl, pH 8.8) using a previously described protocol (8). Proteins were transferred to nitrocellulose and human sera at 1/100 dilutions were added. A secondary Ab consisting of alkaline phosphatase-conjugated anti-human IgG was then added followed by nitrotetrazolium blue/5-bromo-4-chloro-indolyl phosphate as a substrate for the conjugated enzyme.

Other autoantibody assays

Precipitating levels of Sm and/or nRNP autoantibodies were detected using double immunodiffusion. Human sera were tested for antinuclear Abs by a standard antinuclear Ab test against Hep-2 cells (INOVA Diagnostics, San Diego, CA) and for autoantibodies binding to native DNA by a Crithidia assay (Protrac Industries, Kerrville, TX) using previously published protocols (21).

Solid-phase peptide synthesis and autoantibody assays

The 111 possible overlapping octapeptides of the Sm D2 protein and the 44 overlapping octapeptides of Sm D3 were prepared on the ends of polyethylene pins using solid-phase peptide chemistry as described previously (7, 8). Anti-peptide assays were conducted at a 1/100 dilution of patient or control serum using a modified ELISA technique, which we have previously described in detail (7, 8).

Antibody affinity purifications and depletion

Recombinant Sm D2 and D3 were prepared as described above. These individual proteins were separately, covalently bound to cyanogen activated Sepharose 4B by the method recommended by the manufacturer (22–23). Each serum sample tested was circulated through this column for a total of four to six passes and the absorbed serum was then concentrated back to the original volume. More than 90% of the anti-protein reactivity was removed as measured by standard ELISA. Anti-Sm D2 or D3 Abs were eluted from the column with 3 M sodium thiocyanate. Total protein concentration was used to adjust dilutional effects caused by the column absorptions, as previously described (24).

Inhibition of anti-Sm D2 and anti-Sm D3 autoantibodies

Inhibition experiments were performed as has been described (24). Briefly, linear peptides comprising the individual epitopes of Sm D2 and Sm D3 defined by the octapeptide assays were obtained (Molecular Biology Core Facility, Oklahoma City, OK). Standard solid phase assays were then performed as described above, with an additional 2 h inhibition step before addition of human sera. In this step, diluted serum samples were incubated with varying levels of the linear peptides, fusion proteins, or no peptide at all, with peptide concentrations of 1–50 µg/ml. Both uninhibited and inhibited samples were then tested for binding to the D2 or D3 fusion proteins using a standard ELISA (7, 8). Additionally, similar inhibitions were performed before analyzing these same serum samples against both fusion proteins and HeLa cell-derived extract in Western blots, under both denaturing and non-denaturing conditions, as described above and previously (8, 19).

Structural analysis of Sm D2 and Sm D3 sequential epitopes

The Protein Data Bank (25) was accessed to extract coordinates for the crystal structures of two complexes of Sm proteins, D1-D2 (Protein Data Bank code 1B34) and D3-B’ (Protein Data Bank code 1D3B) (4). Using these coordinates, secondary structures were calculated and displayed for the Sm D2 and Sm D3 proteins using the MOLMOL program (25). Solvent-accessible surface areas were calculated with the WHAT IF program using a solvent probe radius of 1.4 Å and the following van der Waals radii: 1.8 Å for carbons, 1.4 Å for oxygens, 1.7 Å for nitrogens, and 2.0 Å for sulfurs. Molecular surface areas calculated by the WHAT IF program have been shown to be within 5% of Connolly’s method (27, 28).
Results

Characterization of anti-Sm D2 and anti-Sm D3 lupus patient sera
Forty-one anti-Sm precipitin-positive SLE patients were selected from the Oklahoma Clinical Immunology Serum Repository. These sera were also tested for reactivity with Sm and nRNP by standard ELISA (Table I). All patients positive for Sm by Ouchterlony immunodiffusion also had positive results by standard ELISA. Conversely, all normal control and Sm-negative patient sera used in this study were shown to be negative for Sm by ELISA and Ouchterlony.

Western blots with HeLa cell extract revealed that seven of 41 (17%) anti-Sm-positive SLE patients were positive for Sm D2 and nine of 41 (22%) were positive for Sm D3 Abs. These patients exhibited binding to bands at 16.5 kDa (Sm D2) and 18 kDa (Sm D3). Four of these patients bound to both D2 and D3 (Table I). All patients who had reactivity with Sm D2 or Sm D3 also demonstrated binding to Sm B/B' on the HeLa cell immunoblots. In addition, all anti-D2-positive patient sera bound Sm D1. No further commonalities were discovered with respect to binding to other self-proteins.

Immunological testing of anti-Sm D2 and anti-Sm D3 lupus patients' sera
All sera were also tested for the presence of other common lupus autoantibodies by immunofluorescence and Ouchterlony. All Sm D2- and Sm D3-positive patients had positive anti-nuclear Abs. Anti-dsDNA Abs were detected in 67% (six of nine) of the anti-D2-positive patients and 57% (four of seven) of anti-D2-positive patients. Anti-nRNP autoantibodies were found in 57% (four of seven) of Sm D2-positive patients and 67% (six of nine) of the Sm D3-positive patients (Table I). The patient controls used in the study exhibited a wide range of autoantibody profiles.

Fine specificity mapping of Sm D2
Sera from the seven patients positive for Sm D2 Abs were tested for binding to the overlapping octapeptides of the D2 protein by our modified solid-phase ELISA. These serum samples exhibited significant reactivity with various portions of the Sm D2 protein, including both N- and C-terminal regions. Patient sera contained Abs that on average recognized five major regions of Sm D2. These major epitopes are defined as being bound by >3 SDs above the normal mean and as being antigenic in at least 50% of the patients tested (Fig. 1). These antigenic regions include: epitope 1, octapeptides 1–6 (aa 1–13); epitope 2, octapeptides 30–34 (aa 30–41); epitope 3, octapeptides 74–77 (aa 74–84); epitope 4, octapeptides 83–98 (aa 83–105); and epitope 5, octapeptide 111, the last full octapeptide of the protein (aa 111–118; see Table II). Epitope 4 showed the most significant binding of any of the antigenic regions, with average anti-D2 binding of six SDs above the normal mean, with maximum reactivity on octapeptide 87 (aa 87–94).

Individual patients showed a moderate amount of variation between specific epitopes bound (Table III). All seven D2-positive patients tested significantly bound (an average of ≥3 SDs above the normal mean) fifteen of the 111 octapeptides. Two of these common antigenic regions contain octapeptides bound at least six SDs above the normal mean. All tested sera recognize epitope 4, whereas epitopes 1 and 2 are antigenic in nearly all (six of seven) tested specimens. Some aspects of the binding profile of this autoantibody response are conserved between patients with some modest variability from patient to patient.

One of the SLE patient sera with anti-Sm reactivity but no detectable anti-D2 binding by Western blot showed minimal binding to epitope 3; however, no other autoantibody binding was noted to epitopes 1, 2, or 4 in this patient. None of the other SLE patient controls without anti-Sm showed any evidence of binding to the major D2 epitopes. In addition, none of the normal control sera tested bound octapeptides in epitopes 1, 2, 3, or 4. Some reactivity with epitope 5, however, was noted in ~20% of control sera. In viewing these results, we realized that all of these normal (nonaffected) sera binding epitope 5 were obtained from laboratory personnel. The level of binding in these sera was significantly below that of the patient binding in these regions, and none of these sera bound Sm D2 or D3 by ELISA or Western blot. When using sera

Table I. Autoantibody binding profiles of study patients and representative controls

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>B/B'</th>
<th>Sm</th>
<th>nRNP</th>
<th>ANA</th>
<th>dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:360</td>
<td>1:30</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:360</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:3240</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:1620</td>
<td>1:810</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:9720</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:3240</td>
<td>1:2430</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:3240</td>
<td>1:30</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:1080</td>
<td>1:90</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:3240</td>
<td>1:90</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:1620</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:360</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:9720</td>
<td>1:30</td>
</tr>
<tr>
<td>Patient controls (negative for Sm D2 and D3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:360</td>
<td>1:30</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1:30</td>
<td>1:30</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1:90</td>
<td>1:90</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1:3240</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1:1080</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1:360</td>
<td>1:30</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1:360</td>
<td>1:120</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1:3240</td>
<td>1:90</td>
</tr>
</tbody>
</table>

*Reactivity to Sm D1, D2, D3, and B/B' are detected by Western blotting against HeLa extract and purified Ags. Binding to Sm and nRNP are measured by standard ELISA with positives being 3 SD above the normal mean. Antinuclear (ANA) and anti-dsDNA Abs are tested by immunofluorescence. Antinuclear Ab titers >1:40 and dsDNA titers >1:10 are considered positive. +, Binding detected; —, no detectable levels of binding above background.
collected from nonaffected individuals without previous exposure to laboratory work, no reactivity with any of the Sm D2 octapeptides was noted. No other significant binding to the overlapping octapeptides was detected in lupus patient sera negative for Sm D2 by ELISA and Western blot.

Fine specificity mapping of Sm D3

Sera from the Sm D3-positive patients (as determined by immunoblot) were also tested for reactivity with the respective overlapping octapeptides. Numerous regions of Sm D3 were recognized as antigenic; however, the greatest binding was seen with the carboxyl-terminal portion of the protein (Fig. 2). Sm D3-positive patients produced Abs against four major regions of the D3 polypeptide. These common antigenic regions were defined as having average binding greater than three SDs above the normal mean for each octapeptide.

These common antigenic regions include: epitope 1, octapeptides 24–30 (aa 24–37); epitope 2, octapeptides 60–64 (aa 60–68); epitope 3, octapeptides 76–99 (aa 76–106); and epitope 4, octapeptides 104–119 (aa 104–126; see Table II). The most significant anti-Sm D3 binding in patient sera was with octapeptide 81 of epitope 3 (PMLKSMKN), which was bound on average at nearly 11 SDs above normal average binding. Epitope 2 also has significant binding with multiple octapeptides from this epitope, having average reactivity greater than six SDs above the normal mean. Substantial reactivity was seen from octapeptides 76–99, in that every octapeptide in that range was recognized. All anti-D3 patient sera tested demonstrate reactivity with epitopes 3 and 4 (Table III). None of these octapeptides were significantly bound by normal control sera. In addition, the region described as the (GR)-repeat is contained within epitope 4 (primarily octapeptide 110). This sequence bears close homology to an antigenic sequence from the Sm D1 protein (10, 11). This epitope is significantly bound by every D3-positive patient tested and, not surprisingly, most of these patients also exhibited anti-D1 reactivity. None of the patient controls or normals, however, exhibited detectable reactivity with the overlapping octapeptides of Sm D3.

In addition, the monoclonal Ab Y12, which has been shown previously to bind to Sm D1, D3, and B/B’s, was tested for reactivity with the overlapping octapeptides of Sm D2 and D3 (Ref. 8; data not shown). The Y12 monoclonal showed little significant reactivity with the Sm D2 octapeptides. This monoclonal, however, did exhibit impressive binding to the C-terminal octapeptides of D3, specifically amino acids 109–120 (epitope 4). These data were not unexpected because the Y12 Ab has also been shown to react with the C-terminal portion of D1, which encompasses a (GR)-repeat portion of that protein as well (8, 29).

**Affinity-purified anti-Sm D2 and D3**

Lupus patient Abs specific for Sm D2 or Sm D3 were purified by affinity column chromatography using recombinant Sm D2 or D3. This resulted in removal of >95% of the anti-Sm D2 or anti-Sm D3 reactivity from these samples, as detected by standard ELISA. In addition, the Abs eluted from the Sm D2 column showed the same specificity for D2 by our octapeptide assay as did the original serum samples from which they were taken (Fig. 3). Similar results were detected after purification of anti-D3 Abs. Anti-D3 Abs do not cross-react with the octapeptides of Sm D2. The eluted Abs

---

**Table II. Amino acid sequences of antigenic regions of Sm D2 and Sm D3**

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major antigenic regions of Sm D2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Major antigenic regions of Sm D3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

* Anti-D2- or Anti-D3-positive patient sera bind all epitopes depicted at >3 SD above the normal mean.
from the D3 column do show reactivity to the overlapping octapeptides from the C-terminal end of Sm D1, specifically limited to those encompassing the (GR)-rich portion of this protein. The affinity-purified anti-D2 Abs, however, do not bind to either D1 or D3 by our octapeptide assay or by Western blot. Also, neither purified anti-D2 nor purified anti-D3 Abs showed any reactivity with the overlapping octapeptides of Sm B/B’ or with any other common lupus autoantigen (data not shown).

Inhibition of anti-D2 and anti-D3 autoantibodies

The addition of Sm D2 and D3 fusion proteins decreased binding of the appropriate SLE sera to Sm D2 or D3, respectively, from HeLa cell extract on Western blots under both denaturing and non-denaturing conditions (data not shown). Additionally, prior inhibition of anti-Sm D2 or D3 sera with Sm D2 or D3 peptides caused notable decreases in binding to D2 and D3 fusion proteins in standard ELISAs. Peptides comprising epitopes 1 and 4 of D2 variably inhibited anti-D2 binding, from 6 to 40% for epitope 1 and from 17 to 42% for epitope 4. When combined, these two peptides demonstrated an additive inhibitory effect, blocking up to 60% of anti-D2 Abs. Similar results were seen upon inhibition of anti-D3 Abs with D3 peptides. Peptides comprising D3 epitope 3 were capable of inhibiting up to 61% of anti-D3 binding, whereas epitope 4 of D3 inhibited up to 38%. Combined, these two D3 peptides inhibited up to 90% of some patients’ anti-D3 reactivity by solid-phase ELISA. D2 peptides caused no inhibition of anti-D3 binding, nor did D3 peptides inhibit anti-D2 reactivity.

Characteristics of Sm D2 and Sm D3 antigenic regions

The major antigenic regions of the Sm D2 protein have an average pI = 10.44 compared with pI = 6.67 for nonantigenic areas. The octapeptides comprising the major epitopes of Sm D3 also have a basic average pI = 10.80 compared with regions not commonly bound by patient sera (pI = 6.55). Both of these differences reached statistical significance with p < 0.0001 (Student’s t test).

In addition, those less common epitopes bound significantly in individual patient sera (≥3 SDs) but which failed to reach significance when reactivity was averaged to form the composite major epitopes also have a basic average pI = 9.3. pI alone, however, is not completely specific in identification of putative epitopes without additional factors (such as known surface probability from crystal structure, etc.) being considered. Using a pI threshold alone (9.5 for D2 and 10.0 for D3), five nonantigenic D2 octapeptides and four nonantigenic D3 peptides would have been mistakenly designated as antigenic. In addition, pI alone would have mistakenly labeled nine octapeptides of D2 and six octapeptides of D3 as nonantigenic. Thus, for D2 the pI threshold yields an 81% sensitivity and 85% specificity, whereas for the D3 protein, pI has a 90% sensitivity and 92% specificity for determining antigenicity (Fig. 4).
Crystallographic structures of Sm D2 and Sm D3 sequential epitopes

The crystal structures of complexes of Sm D2 (complexed with Sm D1) and Sm D3 (complexed with B/H11032) have recently been published by Kambach et al. (5). Sequential epitopes were mapped on the three-dimensional structures of the Sm D2 and Sm D3 proteins (Fig. 5). Segments of polypeptide that are disordered in the crystal structure of Sm D2 include residues 1–25 and 74–92. These regions can be considered as highly flexible or mobile and correspond to epitopes 1 (aa 1–13), 3 (aa 74–84), and part of epitope 4 (aa 83–105). Coordinates of Sm D3 were available only for residues 3–75, which covered epitopes 1 (aa 24–37) and 2 (aa 60–68), but not epitopes 3 and 4 (see Table II). In this case, the two N-terminal residues are disordered, whereas the C-terminal portion of Sm D3 had been deleted to form stable D3-B/H11032 complexes for crystallization (5).

The Sm domains of D2 and D3 share a common fold (see Fig. 5), which consists of an N-terminal helix and a highly bent or twisted five-stranded antiparallel -pleated sheet (5). In Sm D2, epitope 2 (aa 24–37) represents the helix, the following turn, and first part of a strand. The outermost strand and the C-terminal segment correspond to Sm D2 epitopes 4 (aa 83–105) and 5 (aa 111–118). In Sm D3, epitope 1 (aa 24–37) covered the central strand. Similar to Sm D2 epitope 4, the outermost strand of Sm D3 was covered by epitope 2 (aa 60–68). Thus, two of the major antigenic sites on Sm D2 and Sm D3 are located in very similar three-dimensional positions on the crystal structures (see Fig. 5).

Solvent accessible surface areas of the linear epitopes for Sm D2 and Sm D3 crystal structures are presented in Table IV. Surface areas were calculated for the isolated Sm D2 and Sm D3 portions as well as for their dimers with Sm D1 and Sm B/H11032. A decrease in the accessible surface area of an epitope indicates that a portion is buried upon formation of the dimers. All except one strand of the epitopes (54% epitope 4 of Sm D2 was buried in the D1-D2 complex) presented similar accessible surface areas as monomers and dimers, indicating that these linear epitopes could easily be bound by autoantibodies. Note that residues 83–92 of Sm D2 epitope 4 were within a disordered and probably highly solvent-exposed loop (see Fig. 5).

In summary, the major antigenic regions of Sm D2 and Sm D3 appear to be located mainly on accessible strands and turns or loops between these strands. Two linear epitopes appear on structurally homologous regions of the two spliceosomal proteins. No antigenic regions of Sm D3 are buried by its interaction with Sm B’. Instead, the epitopes of D3 conspicuously avoid the major regions (aa 21–23, 39, and 69–75) thought to be directly involved
secondary structure displayed as directional arrows for respectively (5). Only the Sm D2 and Sm D3 proteins are shown with available in the Protein Data Bank under entry codes 1B34 and 1D3B, tallographic location of antigenic regions of nRNP A (19).

FIGURE 5. Location of sequential epitopes on the Sm D2 and Sm D3 crystal structures. Coordinates for Sm D1-D2 and D3-B’ complexes were available in the Protein Data Bank under entry codes 1B34 and 1D3B, respectively (5). Only the Sm D2 and Sm D3 proteins are shown with secondary structure displayed as directional arrows for B strands, open spirals for A helices, and smooth cylinders for turns and loops. Locations of sequential epitopes are shown in black with other regions of polypeptides shaded gray.

in dimer interactions with B’. Interestingly, one of the areas of Sm D2 (aa 90–102) that interacts to form the dimer with Sm D1 is highly antigenic in D2-positive lupus patients. Furthermore, the location of the Sm D2 and Sm D3 antigenic regions on the exposed surfaces of the spliceosomal subcomplexes is similar to the crystallographic location of antigenic regions of nRNP A (19).

Discussion

High titers of autoantibodies are a hallmark of SLE. Despite their proven importance as disease-specific markers and pathogenic factors, very little is known about the details of autoantibody interaction with specific configurations on the three-dimensional structure of their respective autoantigens. The Sm autoantigen is a key target of lupus autoantibodies, as evidenced by its inclusion on the short list of Abs considered as classification criteria for SLE (1). This study identifies the specific sequential epitopes of Sm D2 and D3 shared by human lupus sera and maps those epitopes on the crystallographic structure of the proteins. These binding patterns are shown to be specific for SLE patients relative to controls and are conserved among individual patient sera.

The octapeptide epitopes of these autoantigens provide several interesting insights into autoimmunity when compared with the crystal structures of the Sm complexes. The area of the Sm D2 protein shown to be involved in binding to Sm D1 (5) consists partly of amino acids 90–102, found in epitope 4 of Sm D2. Interestingly, this is one of the highest areas of reactivity seen in the D2-positive sera. These data initially seem to allow the possibility that the anti-D2 autoimmune response is formed against native D2 Ag, which is not complexed with other spliceosomal proteins. However, only a small segment of a single D2 epitope corresponds to an area of intermolecular interaction. Thus, although the exact mechanisms by which this lupus autoantigen is exposed to immune regulation are as yet undefined, it appears likely that it is seen (at least for the purposes of most autoantibody production) in the context of a whole complex rather than as an individual protein.

The region of Sm D3 that is involved in binding to Sm B’ in vivo consists primarily of aa 21–23, 39, and 69–75 of D3 (5). Interestingly, D3-positive patients make high levels of autoantibodies against aa 60–68 and 76–106, surrounding but conspicuously avoiding one of the major regions (aa 69–75) involved in direct intermolecular bonding to Sm B’ in vivo. In fact, all of the amino acids thought to be involved in these protein-protein interactions lie outside of major D3 epitopes. The comparison of our octapeptide epitopes with known crystalline data also reveals that the patterns created by these epitopes on the surface are similar across the three autoantigens for which this analysis has been performed (Sm D2, Sm D3, and nRNP A), being directed primarily against B’ sheets and the loops connecting B’ sheets. Of course, this could simply be due to the obvious structural similarities between the three-dimensional orientations of these spliceosomal proteins. In addition, when the published human epitopes of Sm B/B’ (7) are compared with the portion of B/B’ complexed with Sm D3, the same pattern surfaces. The region of Sm B/B’ (primarily aa 65–76) involved in close interactions with D3 lies directly in between two of the major Sm B/B’ epitopes. This reinforces our observation that antigenic regions in this system tend to be those that are not involved in intermolecular, intracomplex bonds.

This study, as well as that from Hoch et al. (30), reveals that all lupus patients who bind Sm D3 also bind its complexed partner B/B’ and that all who bind D2 also bind its complexed partner D1. These data fall in line with an increasingly common theme in autoimmunity in which autoantibody specificity pairing seems to be partially explained by physical associations in vivo (15–17, 31–

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Isolated D2</th>
<th>D1-D2 dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (30–41)</td>
<td>194</td>
<td>195</td>
</tr>
<tr>
<td>4a (93–105)</td>
<td>352</td>
<td>164</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Isolated D3</th>
<th>D3-B’ dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (24–37)</td>
<td>233</td>
<td>232</td>
</tr>
<tr>
<td>2 (60–68)</td>
<td>180</td>
<td>126</td>
</tr>
</tbody>
</table>

a Solvent-accessible surface areas are shown in square angstroms and were calculated from the known crystal structures of Sm complexes, D1-D2 (Protein Data Bank code 1B34) and D3-B’ (Protein Data Bank code 1D3B) (5), using the WHAT IF program (26). Sequential epitopes that are not shown above, but appear in Table II, are disordered or missing in the available crystal structures.

b Only residues 93–105 of epitope 4 (83–105) were represented in the crystal structure of the D1-D2 complex.
32). We have demonstrated herein that anti-Sm autoantibodies in lupus are directed primarily against structures that would be located on the surface of native autoantigens in their complexed configurations. Current knowledge of determinant spreading and autoantibody profiles also points to progression from protein to protein known to complex together in vivo as a mechanism in many diseases. Therefore, the importance of the immune recognition and reaction to autoantigens in their native complexed arrangement seems clear.

The question of how these autoantibodies to Sm D2 and Sm D3 arise has not yet been answered. However, it could be that they derive from cross-reactivity between similar sequences from other spliceosomal autoantigens. However, D2 patients bind weakly or not at all to areas of Sm D2 that show similarity to other spliceosomal proteins (D2 aa 57–67 have 65% homology with B/B’ aa 32–42). When coupled with the peptide column data (showing no cross-reactivity of anti-D2 with other Sm proteins), this seems to indicate that spliceosomal cross-reactivity at the level of Ig is not likely to be operating for this specificity. Conversely, one antigenic region of Sm D3, epitope 4, shows significant homology with the (GR)-repeat portion of Sm D1. This carboxy-terminal region of Sm D1 has been determined by several investigators to be the major target of the human and murine lupus anti-Sm D1 response (10–14, 29–30, 33). Immunization with this C-terminal D1 peptide causes lupus-like disease in rabbits as well as the production of anti-D1 and anti-D3 autoantibodies (33) and accelerates disease in lupus-prone animals (New Zealand Black/White mice) (34). The data from the (GR)-repeat portion of the D3 polypeptide, its similarity to D1, and the cross-reactivity of affinity-purified anti-D3 Abs with this region of D1 seem to support the theory of cross-reactivity explaining the appearance of some autoantibodies. An additional group has shown that this same region of Sm D1 is antigenic when the alternating arginines are dimethylated (35). Interestingly, this posttranslational modification may not be absolutely required for antigenicity of these sequences, as has been shown by this and other works (8, 33–34). Additional experiments are underway to understand the overall importance of symmetrical dimethylation of these sequences with regard to lupus autoimmunity.

The other major possibility is that these proteins are seen by the immune system in complex with other spliceosomal proteins already targeted by the immune system and are thus presented by professional phagocytic cells along with previously autoantigenic proteins. This has been shown to be the most likely cause in the case of the Ro (SS-A) and La (SS-B) system in lupus and Sjögren’s syndrome (16, 18). In the Ro/La system, it has been demonstrated that murine immunization with all or portions of these sequences with regard to lupus autoimmunity.

Regardless of the mechanism of development of these autoantibodies, for both Sm D2 and Sm D3 the response is fairly well conserved from patient to patient. This is in direct contrast to the much greater variation seen among patients with the nRNP 70K and nRNP C systems (37, 38). These data are consistent with the autoimmune responses of these patients arising in a similar defined manner rather than from random polyclonal events. Consequently, current data support the anti-Sm D2 and anti-Sm D3 autoantibodies arising through an underlying mechanism that is Ag driven and well conserved between individuals.

The data also reveal that pl acts as an accurate predictor of antigenicity in this system. In the previously studied nRNP A, Sm B’, and Sm D1 proteins, the average pl of antigenic regions was 10.4 compared with the average pl of 6.0 of nonantigenic regions. This observation also holds true with Sm D2 and Sm D3, in that antigenicity clearly increases with pl > 9.0. The combination of localization of epitopes to the surface and antigenicity of these basic epitopes suggests that perhaps high pls simply increase the probability that octapeptides will be situated near the protein surface. Regardless of the reason, the compilation of previous data (9, 19) and our results seem to indicate that high pl is an aspect common to nuclear autoantigens in SLE.

The role of Sm D2 and D3 in the SLE anti-Sm autoimmune response is only beginning to emerge. These experiments describe nine newly appreciated sequential antigenic regions of the spliceosomal complex and show that these antigenic sites also have a high pl, as do other spliceosomal autoantigenic regions. These antigenic regions of Sm D2 and Sm D3 are located on the surface of the crystallographic structure, and concomitant production of Sm B’/D1 and D2/D3 autoantibodies reflects physical associations. Some work has been done detailing the crystal structures of Ags while complexed with specific Abs (e.g., rheumatoid factor (39)). A relatively new approach, presented herein, has been to identify the fine specificity of polyclonal Abs and then to apply those data to the crystal structure of the autoantigen. In fact, we know of only two such cases where a similar study has even been attempted (19, 40). In all likelihood, this is due to the relative paucity of autoantigens that have had their crystal structures solved. This approach allows for efficient analysis of a wide spectrum of Abs specificities. These findings further explain the role of Sm D2 and Sm D3 in the human SLE autoimmune response and will lead to further investigation into potential mechanisms for the induction and perpetuation of these responses.

Acknowledgments

We thank Janeen Arbuckle, Amber Davis, Timothy Gross, and Monica Kirby for their technical assistance; Xana Kim for aid in statistical analysis; and Melissa Arbuckle, and John B. Harley for their invaluable insight and suggestions.

References


