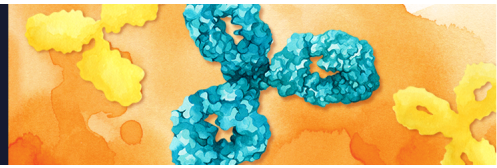


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# A SmD Peptide Induces Better Antibody Responses to Other Proteins within the Small Nuclear Ribonucleoprotein Complex than to SmD Protein via Intermolecular Epitope Spreading<sup>1</sup>

Umesh S. Deshmukh,<sup>2</sup> Harini Bagavant, Davis Sim, Vyankatesh Pidiyar, and Shu Man Fu

Autoantibody response against the small nuclear ribonucleoprotein (snRNP) complex is a characteristic feature of systemic lupus erythematosus. The current investigation was undertaken to determine whether activation of SmD-reactive T cells by synthetic peptides harboring T cell epitopes can initiate a B cell epitope spreading cascade within the snRNP complex. T cell epitopes on SmD were mapped in A/J mice and were localized to three regions on SmD, within aa 26–55, 52–69, and 86–115. Immunization with synthetic peptides SmD<sub>31–45</sub>, SmD<sub>52–66</sub>, and SmD<sub>91–110</sub> induced T and B cell responses to the peptides, with SmD<sub>31–45</sub> inducing the strongest response. However, only SmD<sub>52–66</sub> immunization induced T cells capable of reacting with SmD. Analysis of sera by immunoprecipitation assays showed that intermolecular B cell epitope spreading to U1RNA-associated A ribonucleoprotein and SmB was consistently observed only in the SmD<sub>52–66</sub>-immunized mice. Surprisingly, in these mice, Ab responses to SmD were at low levels and transient. In addition, the sera did not react with other regions on SmD, indicating a lack of intramolecular B cell epitope spreading within SmD. Our study demonstrates that T cell responses to dominant epitope on a protein within a multiantigenic complex are capable of inducing B cell responses to other proteins within the complex. This effect can happen without generating a good Ab response to the protein from which the T epitope was derived. Thus caution must be taken in the identification of Ags responsible for initiating autoimmune responses based solely on serological analysis of patients and animals with systemic autoimmune disorders. *The Journal of Immunology*, 2007, 178: 2565–2571.

Autoantibodies to the small nuclear ribonucleoprotein (snRNP)<sup>3</sup> complex are often found in patients with lupus and related disorders (1). The snRNP particle is composed of several different polypeptides named 70 kDa, A, C, and Sm proteins (B/B', D1, D2, D3, E, F, G) that are associated with different RNA molecules rich in uridine (2). The presence of anti-Sm autoantibodies is considered specific for the diagnosis of systemic lupus erythematosus (SLE) (3). Longitudinal analyses of sera from SLE patients and lupus prone mouse models have demonstrated that autoantibody responses to the snRNP particle evolve over a period of time (4–7). Initially few polypeptides are targeted by the Abs and a progressive recognition of additional polypeptides of the snRNP particle ensues. This phenomenon has been termed “intermolecular epitope spreading,” wherein an immune response to initial target Ags leads the immune responses to Ags targeted later (8). Based on the serological analyses of patient sera

and mouse models, different snRNP proteins have been proposed to be the Ags responsible for initiating the epitope spreading cascade (7, 9). Experimental model systems, using immunization of mice and rabbits with recombinant RNP or synthetic peptides, have provided evidence for this hypothesis (10). Despite these observations, molecular mechanisms for intermolecular epitope spreading have not been clearly defined (11, 12). The recent observations that autoantibodies to RNPs are present several years before the diagnosis of SLE (9, 13) provides a strong impetus for elucidating the mechanisms of epitope spreading, which may be an important factor leading to end organ damage.

We have previously demonstrated that the B cell epitope spreading patterns within the snRNP complex are determined by the immunizing polypeptide (14). In A/J strain of mice immunized with SmD, epitope spreading to U1RNA-associated A ribonucleoprotein (A-RNP) and SmB was observed. However, in mice immunized with A-RNP, epitope spreading occurred only to the 70-kDa protein. In addition, in mice immunized with SmB, epitope spreading occurred to A-RNP with minimal reactivity toward SmD. In our model system, we have clearly demonstrated that the autoantibodies arising out of epitope spreading were of IgG isotype, recognized native Ags, and immunoprecipitated the whole snRNP complex. One of the mechanisms proposed for intermolecular B cell epitope spreading within the snRNP complex is that of intrastuctural help (15). In this process, T cells reactive with one component of a multiantigenic complex provide help to B cells reactive with other components of the same antigenic complex. In experimental model systems using whole proteins such as SmD to study epitope spreading, presence of multiple T and B cell epitopes increases the complexity of cellular interactions involved in epitope spreading. In addition, formation of immune complexes with the immunogen and their possible involvement in the activation of B cells can add further complexity to the process. As a consequence, addressing the mechanisms for epitope spreading has been difficult.

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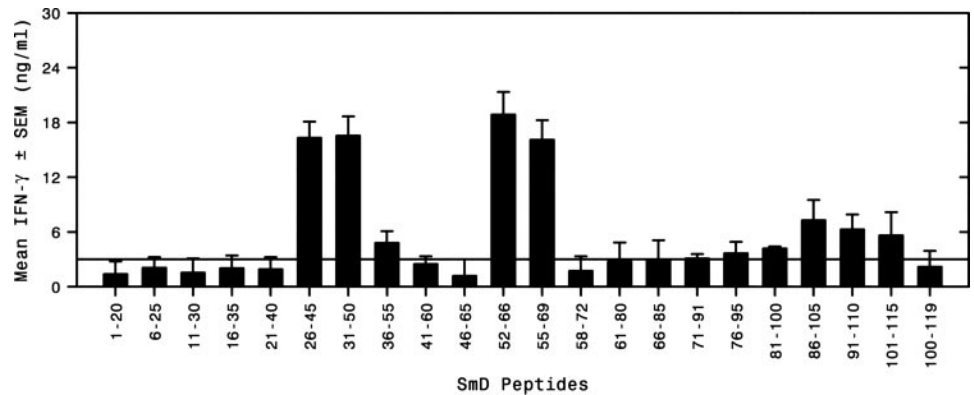
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<sup>3</sup> Abbreviations used in this paper: snRNP, small nuclear ribonucleoprotein; A-RNP, U1RNA-associated A ribonucleoprotein; SLE, systemic lupus erythematosus; LNC, lymph node cell.

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**FIGURE 1.** T cell epitopes on SmD1 are present within aa 26–50, 52–69, and 86–115. Draining LNCs from A/J mice immunized with recombinant SmD were cultured in presence of synthetic peptides for 44 h, and 100  $\mu$ l of culture supernatants were used in duplicates to estimate IFN- $\gamma$  by ELISA. Data are represented as the mean IFN- $\gamma$   $\pm$  SEM expressed in nanograms per milliliter from two independent experiments. The mean background IFN- $\gamma$  was 2.88 ng/ml and is represented by the solid horizontal line in graph.



Recent studies have suggested that molecular mimicry at single B cell epitope level may play an important role in the initiation of autoantibody responses in SLE (13). However, the role of T cell epitope mimicry in initiating an epitope spreading cascade in SLE has not been described. To address the mechanisms for epitope spreading within the snRNP particle, we decided to determine whether immunization with synthetic peptides containing T cell epitopes of SmD can induce epitope spreading. This study shows that immunization of mice with an immunodominant T cell epitope on SmD induces intermolecular B cell epitope spreading. Interestingly, despite the initiation of a T cell response against SmD, dominant Ab responses were not generated against SmD but were against A-RNP and other cellular proteins.

## Materials and Methods

### Synthetic peptides

Panel of overlapping synthetic peptides spanning the entire sequence of mouse SmD1 (aa 1–119) was obtained from Dr. C. David of the Mayo Clinic (Rochester, MN). The peptides were 20 aa long with an overlap of 15 aa. Due to synthesis and purification difficulties, three peptides were 15 aa long with an overlap of 13 aa. Peptides SmD<sub>31–45</sub> GVDVSMNTH LKAVKM, SmD<sub>52–66</sub> PVQLETLSIRGNNIR, and SmD<sub>91–110</sub> KREAV AGRGRGRGRGRGR were obtained from the Biomolecular Research Facility at the University of Virginia (Charlottesville, VA). All peptides were HPLC purified and were at >90% purity. Peptides were solubilized in water, aliquoted, and stored at  $-80^{\circ}\text{C}$  until use. Preparation of recombinant SmD1 has been previously described (14).

### Mice and immunization

All mouse experiments were approved by the Animal Care and Use Committee at the University of Virginia. Female A/J mice were purchased from The Jackson Laboratory and kept in specific pathogen-free conditions in the University of Virginia vivarium. For lymph node cell (LNC) proliferation experiments, mice were immunized in one footpad and the base of the tail with 100  $\mu$ g of recombinant SmD emulsified in IFA (Difco) or with peptides emulsified in CFA. For Ab experiments, mice (four to five per group) were immunized by s.c. route with peptides emulsified in CFA. Controls were immunized with only CFA. Mice received additional i.p. injections of peptides emulsified in IFA on days 14 and 28. Controls were injected with only IFA. At each injection a dose 50  $\mu$ g of peptide was used. Mice were bled through the tail vein at different time points, and serum was separated and stored at  $-20^{\circ}\text{C}$  until use. In all, three independent cohorts of mice were used for peptide immunizations and Ab analysis.

### LNC proliferation and T cell epitope mapping

LNC proliferation studies were performed in two independent cohorts of mice. The [ $^3\text{H}$ ]thymidine incorporation assays were performed as previously described (16). The stimulation index was calculated using (mean triplicate cpm with Ag)/(mean triplicate cpm without Ag). A stimulation index  $>2$  was considered as positive. For epitope mapping studies, mice were immunized with recombinant SmD in the footpad and base of the tail, and 12 days later draining LNCs were harvested. Cells were plated in 24-well plates at a concentration of  $4 \times 10^6$  cells per well in 1 ml of DMEM supplemented with 10% FBS, 2 mM L-glutamine, nonessential

amino acids, sodium pyruvate, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and  $5 \times 10^{-5}$  M 2-ME. SmD peptides at a final concentration of 50  $\mu$ g/ml were added to each well, and cells were incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 40–44 h. Culture supernatants (100  $\mu$ l) were used to determine IFN- $\gamma$  by ELISA (BD Pharmingen), following the manufacturer's instructions.

### T-T hybridomas

T cells obtained from mice immunized with synthetic peptides were fused with T cell fusion partner BW5147 and plated in 96-well plates (17). Hybrid cells were HAT (hypoxanthine, aminopterin, thymidine medium) selected for 10 days and expanded in 24-well plates. Reactivity with synthetic peptides and SmD was tested by ability of syngenic spleen cells fed with either peptides or SmD to activate IL-2 production in the T-T hybrids. Generally,  $10^5$  T cells were incubated with  $2.5 \times 10^5$  spleen cells in presence or absence of Ags for 14 h. IL-2 in culture supernatants was estimated by ELISA (BD Pharmingen) following manufacturer's instructions. Selected hybrids were cloned by limiting dilution method.

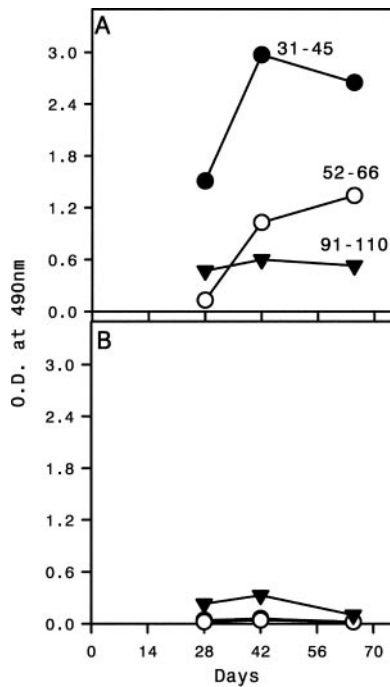
### Ab analysis

Anti-peptide Abs were assayed by ELISA as previously described (16). Reactivity to native polypeptides was determined by immunoprecipitation using in vitro translated,  $^{35}\text{S}$ -labeled A-RNP, SmB, and SmD as previously described (14). Reactivity to whole snRNP particle was determined by immunoprecipitation using cell extracts from metabolically labeled WEHI 7.1 cells with [ $^{35}\text{S}$ ]methionine (14). To determine cross-reactivity between A-RNP and immunizing peptides, absorption and elution studies were performed as previously described (16, 18). Briefly, 5  $\mu$ l of pooled sera were absorbed with 50  $\mu$ l of A-RNP coupled-Sepharose beads. The beads were washed extensively with PBS containing 0.1% Tween 20 until the OD<sub>280</sub> of wash buffer was between 0.01 and 0.05. Bound Abs were eluted in 1 ml of glycine-HCl buffer (pH 2.7) containing 0.5% BSA, neutralized immediately with 1 M Tris, and used in ELISA.

## Results

### Mapping of T cell epitopes on SmD

A/J mice were immunized with purified recombinant SmD protein emulsified in IFA. After 10 days, the draining LNCs were stimulated with overlapping peptides of SmD for 40–44 h and IFN- $\gamma$  produced was determined by ELISA. The results are shown in Fig. 1. The data are represented as mean IFN- $\gamma$   $\pm$  SEM from two independent experiments. Multiple peptides on SmD, within aa 26–55, 52–69, and 86–115 were able to induce IFN- $\gamma$  production. Although peptides SmD<sub>26–45</sub>, SmD<sub>31–50</sub>, SmD<sub>52–66</sub>, and SmD<sub>55–69</sub> were dominant in this assay, peptides within aa 86–115 gave much lower levels of IFN- $\gamma$  production. LNC proliferation measured by [ $^3\text{H}$ ]thymidine incorporation assay was also used to map T cell epitopes. Peptides within the same areas were stimulatory with the same hierarchy (data not shown), and LNCs obtained from control mice immunized with only IFA did not proliferate in response to peptide stimulation. Thus, at least two immunodominant T cell epitopes are present on SmD within aa 26–50 and aa 52–69. Because both peptides SmD<sub>26–45</sub> and SmD<sub>31–50</sub> gave very similar stimulation, we deduce that SmD<sub>31–45</sub>



**FIGURE 2.** Immunization of A/J mice with synthetic peptides from SmD1 induces anti-peptide Ab responses, with peptide 31–45 inducing the highest response followed by peptides 52–66 and 91–110. Pooled sera obtained at different time points were used at multiple dilutions for the detection of anti-peptide Abs by ELISA. Representative data from a group of mice are shown at 1/200 serum dilution and are expressed as mean duplicate of OD at 490 nm. *A*, Reactivity to peptide 31–45 (●) in peptide 31–45 immunized mice; to peptide 52–66 (○) in peptide 52–66 immunized mice; and to peptide 91–110 (▼) in peptide 91–110 immunized mice. *B*, Minimal reactivity to peptides SmD<sub>31–45</sub> (●), SmD<sub>52–66</sub> (○), and SmD<sub>91–110</sub> (▼) in sera of mice immunized with adjuvants only.

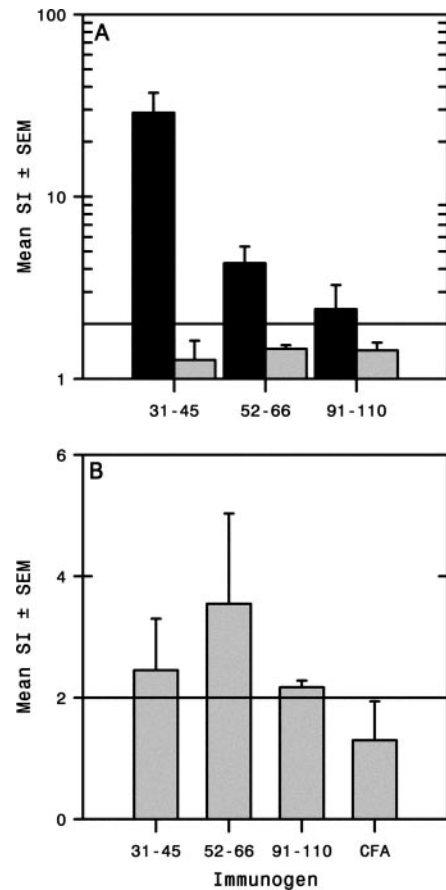
is the core T cell epitope in this region. Similarly, SmD<sub>52–66</sub> is a dominant T epitope. In the regions of aa 86–115, multiple minor T cell epitopes may be present.

#### *SmD<sub>31–45</sub>, SmD<sub>52–66</sub>, and SmD<sub>91–110</sub> induce anti-peptide Abs in immunized mice*

Three peptides, SmD<sub>31–45</sub>, SmD<sub>52–66</sub>, and SmD<sub>91–110</sub> from the three T cell antigenic regions were chosen for analysis to determine whether they were capable of inducing anti-peptide Abs. Sera from A/J mice immunized with these three SmD peptides were analyzed for reactivity with respective peptide immunogens in ELISA. Data from a representative experiment are shown in Fig. 2. Sera obtained at different time points were pooled and 5-fold dilutions (from 1/200 to 1/625,000) of sera were tested in ELISA. The data in Fig. 2*A* show reactivity to the immunizing peptides at a 1/200 dilution. SmD<sub>31–45</sub> induced the highest anti-peptide Ab response followed by SmD<sub>52–66</sub> and SmD<sub>91–110</sub>. In comparison, control mice immunized with adjuvants (Fig. 2*B*) did not show any reactivity to the peptides, except with peptide 91–110. Analysis of individual mouse sera showed similar results. In addition, similar results were also obtained in an additional cohort of mice.

#### *Immunization with peptide SmD<sub>52–66</sub> activates T cells that recognize peptides processed from whole SmD*

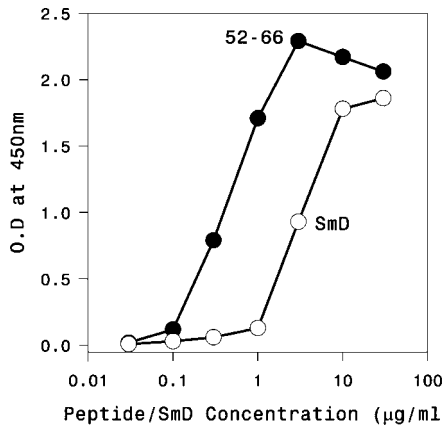
To determine whether T cells from A/J mice immunized with peptides SmD<sub>31–45</sub>, SmD<sub>52–66</sub>, and SmD<sub>91–110</sub> would proliferate in response to immunizing peptides and SmD, LNC proliferation assays were performed. The results are shown in Fig. 3 and are



**FIGURE 3.** LNC proliferative responses to peptide immunogens and SmD in peptide- and adjuvant-immunized mice. T cell responses to SmD peptides were studied by LNC proliferation and [<sup>3</sup>H]thymidine incorporation assays. Proliferative responses to peptides are shown at 30 µM concentration and to recombinant SmD at 10 µg/ml concentration. Data are represented as mean stimulation index (SI) ± SEM from two independent experiments. The stimulation index for each experiment was calculated as (mean triplicate cpm with peptide)/(mean triplicate cpm without peptide). A stimulation index >2.0 was considered positive and is indicated by a horizontal line in each panel. *A*, Activation (■) of LNCs from peptide 31–45 immunized mice by peptide 31–45; from peptide 52–66 immunized mice by peptide 52–66; and from peptide 91–110 immunized mice by peptide 91–110. Peptide 31–45 induced highest proliferative responses followed by peptides 52–66 and 91–110. The peptides do not induce proliferative responses in LNCs obtained from adjuvant-immunized mice (□). *B*, Recombinant SmD (10 µg/ml) induced highest proliferative responses in LNC obtained from mice immunized with peptide 52–66.

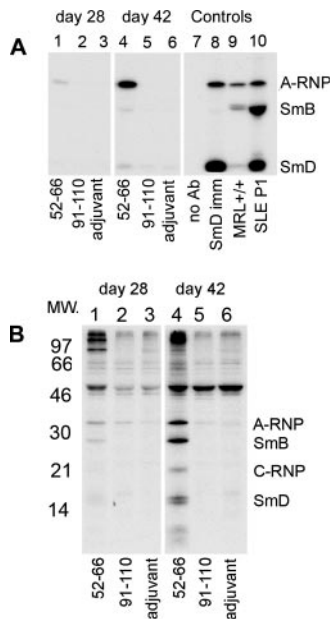
represented as the mean stimulation index ± SEM from two experiments. All three peptides were able to induce LNC proliferative responses, with SmD<sub>31–45</sub> giving the highest response, followed by peptides SmD<sub>52–66</sub> and SmD<sub>91–110</sub> (Fig. 3*A*). LNCs obtained from mice immunized with adjuvant alone did not show any significant proliferative response. To determine whether the T cells from peptide-immunized mice were reactive with peptides generated through the processing and presentation of whole SmD, syngenic APCs were fed with recombinant SmD and used in the LNC proliferation assay (Fig. 3*B*). T cells from mice immunized with SmD<sub>52–66</sub> gave significantly higher mean stimulation index, in comparison with mice immunized with SmD<sub>31–45</sub> and SmD<sub>91–110</sub>. T cells from CFA-immunized mice did not proliferate in response to SmD.

To further confirm that SmD<sub>52–66</sub> activates T cells that recognize peptides generated through the processing and presentation of

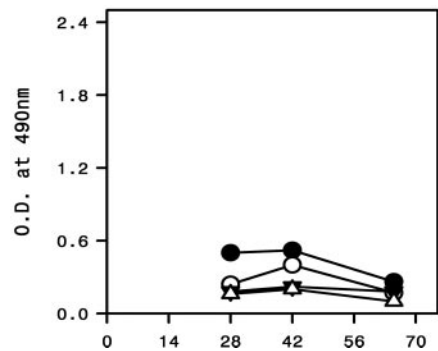
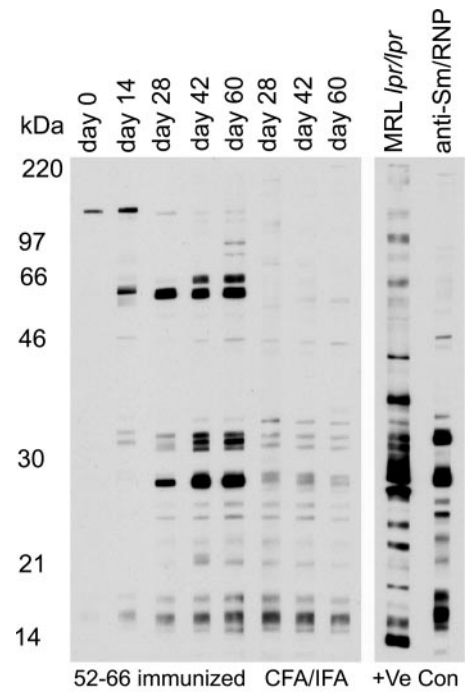


**FIGURE 4.** Activation of T cell hybridoma 13.6 by SmD<sub>52-66</sub> and SmD. T-T hybridoma 13.6 cells ( $10^5$ ) were mixed with syngenic spleen cells ( $2.5 \times 10^5$ ) and different concentrations of either peptide SmD<sub>52-66</sub> or recombinant SmD protein in duplicates in 96-well plate in a final volume of 0.2 ml. After 14 h of incubation, 50  $\mu$ l of culture supernatant was used to estimate IL-2 production by ELISA. Data are from a representative of three experiments and are expressed as mean duplicate OD at 405 nm.

whole SmD, T-T hybridomas reactive with SmD<sub>52-66</sub> were generated. Activation of a representative hybridoma, 13.6, by synthetic peptide and SmD is shown in Fig. 4. Both peptide SmD<sub>52-66</sub>



**FIGURE 5.** Analysis of autoantibody reactivity by immunoprecipitation shows intermolecular B cell epitope spreading in SmD<sub>52-66</sub>-immunized mice. *A*, In vitro translated and <sup>35</sup>S-labeled A-RNP, SmB, and SmD were immunoprecipitated using pooled sera (five mice/group) obtained on day 28 (lanes 1–3) and day 42 (lanes 4–6) postimmunization. Ten microliters of serum was used. Ags used for immunizing different groups of mice are indicated at bottom. Controls (lanes 7–10) are: No Ab, pooled sera from SmD-immunized mice (SmD imm), pooled sera from MRL<sup>+/+</sup> mice (MRL<sup>+/+</sup>), and serum sample from a SLE patient (SLE P1). Data are from one representative of three experiments. The photograph shown is from the same gel with identical exposure. *B*, Only sera from SmD<sub>52-66</sub>-immunized mice are capable of immunoprecipitating the entire snRNP particle. Cell extracts from metabolically labeled WEHI 7.1 cells were used to immunoprecipitate the snRNP particle. Data are from a representative of two immunoprecipitation experiments.

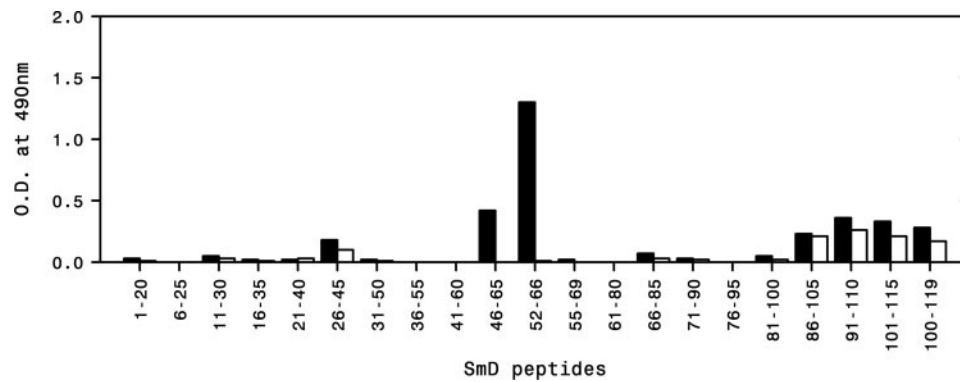


**FIGURE 6.** Analysis of intermolecular epitope spreading to other cellular proteins in SmD<sub>52-66</sub>-immunized mice by Western blotting. Pooled sera (upper panel) obtained at different time points from mice (four per group) immunized either with SmD<sub>52-66</sub> or only adjuvants (five mice per group) were used in Western blots at 1/100 dilution. Pooled sera from MRL-Mp-*lpr/lpr* mice and A/J mice immunized with A-RNP and SmB were used as positive controls. Bound Abs were detected by peroxidase-coupled goat anti-mouse IgG and ECL. Data are from a representative of two experiments. Similar results were obtained in additional groups of mice. Reactivity with recombinant SmD in ELISA is shown (lower panel). Pooled sera at different time points were analyzed for reactivity with SmD. Sera were used at 1/100 dilution from peptide SmD<sub>31-45</sub> (●), SmD<sub>52-66</sub> (○), and SmD<sub>91-110</sub> (▼) immunized and adjuvant (△) immunized mice.

and SmD were able to induce IL-2 production in a dose-dependent manner.

*Intermolecular epitope spreading in SmD<sub>52-66</sub>-immunized mice*

Sera obtained at different times from peptide-immunized mice were analyzed for reactivity to A-RNP, SmB, and SmD by immunoprecipitation using in vitro transcribed, translated, and <sup>35</sup>S-labeled proteins. The Ab analysis was performed in three independent cohorts of mice. Immunization with SmD<sub>52-66</sub> consistently generated Abs capable of immunoprecipitating A-RNP (3 of 3 groups) and SmB (2 of 3 groups). Data from a representative experiment are shown in Fig. 5A. At 28 days after the initial immunization, while Abs to A-RNP were detected in mice immunized



**FIGURE 7.** Sera from SmD<sub>52-66</sub>-immunized mice do not react with SmD peptides except peptides 46–65 and 52–66 indicative of lack of intramolecular B cell epitope spreading within SmD protein. Reactivity of pooled sera obtained 2 mo postimmunization at 1/100 dilutions with different SmD peptides was analyzed in ELISA. Bound Abs were detected with peroxidase-coupled goat anti-mouse IgG. Data are represented as mean duplicate OD at 490 nm and are from a representative experiment. Reactivity of sera from SmD<sub>52-66</sub>-immunized mice (■) and from adjuvant-immunized mice (□) is shown.

with SmD<sub>52-66</sub>, Abs reactive with SmD and SmB were not detected. By day 42, A-RNP reactivity had increased in magnitude and a much weaker reactivity to both SmD and SmB was detected. In contrast, sera obtained from whole SmD-immunized mice show strong reactivity with SmD (Fig. 5A, lane 8). The magnitude of reactivity to A-RNP appears to be very similar between the peptide-immunized and SmD-immunized mice. Analysis of individual serum samples tested at 2 mo postimmunization showed reactivity to A-RNP in four of five mice immunized with SmD<sub>52-66</sub>. Anti-SmD Abs were not detected in any mice. None of these immunoprecipitating Abs were detected in any of the mouse groups immunized with SmD<sub>91-110</sub> or with adjuvants alone (Fig. 5A). Considerable variability was observed in mice immunized with SmD<sub>31-45</sub>, with only one of the three cohorts showing immunoprecipitating anti-A-RNP Abs (data not shown). Again, none of the immune sera reacted with SmD. Thus, further studies were focused on mice immunized with SmD<sub>52-66</sub>.

To determine whether Abs generated in peptide-immunized mice were reactive with intact snRNP particle, WEHI 7.1 cells were metabolically labeled with [<sup>35</sup>S]methionine. Pooled sera at 28 and 42 days postimmunization were used to immunoprecipitate the labeled snRNP particle (Fig. 5B). Only sera from mice immunized with SmD<sub>52-66</sub> were capable of immunoprecipitating the snRNP particle. Reactivity was apparent by 28 days postimmunization, which became much stronger by day 42 and coincides with the appearance of anti-A-RNP Abs in the immunized mice. Sera from groups of mice immunized with peptide SmD<sub>91-110</sub> and adjuvant did not immunoprecipitate the snRNP particle. The sera from SmD<sub>52-66</sub>-immunized mice also immunoprecipitate some additional proteins between molecular mass 97 and 200 kDa by day 28 postimmunization. The identity of these proteins remains to be determined.

Affinity purified anti-A-RNP Abs reacted strongly with A-RNP. These Abs did not react with peptide SmD<sub>52-66</sub>, indicating that Abs reactive with A-RNP are not cross-reactive with the peptide immunogen (data not shown).

#### *SmD<sub>52-66</sub> immunization induces Abs reactive with different cellular proteins*

Sera from SmD<sub>52-66</sub>-immunized mice obtained at different time points were analyzed for reactivity to different cellular proteins by Western blotting using WEHI 7.1 cell extracts. Fig. 6, upper panel, shows reactivity of pooled sera from four mice at 1/100 dilution from a representative experiment. Sera from MRL/Mp-*lpr/lpr* mice and A/J mice immunized with A-RNP and SmB were used as

positive controls. Sera from peptide-immunized mice showed much higher reactivity to different cellular proteins than the adjuvant-immunized mice. The increasing complexity in reactivity with time is indicative of Ab diversification. Of note are proteins around 66, 31, and 29 kDa. This reactivity pattern resembles that observed with anti-Sm/RNP Ab response. Analysis of individual serum samples on day 42 showed similar patterns in three of four mice. The SmD1 protein runs just over the 14-kDa marker in a 12% SDS-PAGE (Fig. 6, second band from the bottom in the MRL/Mp-*lpr/lpr* lane). Weak reactivity to proteins running at this position was observed in sera from peptide-immunized mice. However, this reactivity was similar to that observed in the adjuvant control.

To confirm that reactivity to SmD in peptide-immunized mice was weak, sera were analyzed in ELISA using recombinant SmD as substrate (Fig. 6, lower panel). Sera from all groups of mice reacted weakly with the recombinant protein at low serum dilutions. The hierarchy of reactivity was similar to the peptide reactivity shown in Fig. 2B. On day 60, no difference in reactivity to SmD was seen between the peptide-immunized and adjuvant controls.

#### *Lack of intramolecular epitope spreading in mice immunized with SmD<sub>52-66</sub>*

It was suspected that weak reactivity to SmD1 protein seen in different immunoassays in sera of mice immunized with the SmD peptide was related to a lack of intramolecular B cell epitope spreading within the SmD protein. This relationship was indeed the case as the reactivity to different SmD peptides was analyzed in ELISA. Pooled sera at different time points were screened and reactivity at 2 mo is shown in Fig. 7. None of the sera from peptide-immunized mice reacted significantly with any of the SmD peptides except peptides SmD<sub>46-65</sub> and SmD<sub>52-66</sub>. Both of these peptides contain amino acid sequences from the immunizing peptide. Lack of reactivity to adjacent peptides SmD<sub>41-60</sub> and SmD<sub>55-69</sub> suggests that amino acids on the N-terminal and C-terminal of SmD<sub>52-66</sub> may be critical for reactivity of anti-peptide Abs. The weak reactivity with the peptides in the C-terminal region and peptide 26–45 was seen in both peptide and adjuvant-immunized mice. Overall these data suggest a lack of intramolecular B cell epitope spreading within the SmD protein.

## Discussion

In this study, we mapped the T cell epitopes on SmD in the A/J strain of mouse and demonstrated that immunization with one of

these epitopes SmD<sub>52-66</sub> was capable of inducing Ab responses to other polypeptides within the snRNP complex. A major finding from this study is that this intermolecular B cell epitope spreading occurred without intramolecular B cell epitope spreading within the SmD protein. Our experimental model system uses the A/J strain of mouse. We have previously noted that among the different strains of mice (A/J, B10.BR, BALB/c, BALB.K, C57BL/6J, and C57BL/10J) immunized with SmD, only A/J mice demonstrated intermolecular B cell epitope spreading to A-RNP (19). These data suggest that the T and B cell responses to SmD are strain dependent and some autoantibodies might not be induced in normal mice (20). The lack of epitope spreading in BALB/c mice immunized with SmD in our model system is supported by recent findings of Langnickel et al. (21). BALB/c mice immunized with peptide SmD<sub>183-119</sub> did not generate anti-SmD or anti-dsDNA autoantibodies.

Other investigators have used synthetic peptides within the core SmD region used in this study to induce autoantibody responses to the snRNP particle with varying results. Bockenstedt et al. (22) showed that immunization of B10.BR mice with SmD peptide 56-70 did not induce SmD reactive T cells or snRNP reactive Ab. They concluded that T cell epitopes in this region of SmD are cryptic. Our studies in A/J mice show that SmD<sub>52-66</sub> is not a cryptic T cell epitope. Different genetic backgrounds and synthetic peptide design may explain these differences. Similar to our study, Winska-Wiloch (23) reported that immunization of MRL/Mp-*lpr/lpr* mice with SmD peptide 44-67 induced Ab responses to two synthetic peptides of A-RNP without SmD reactive Ab by Western blotting. However, MRL/Mp-*lpr/lpr* mice spontaneously develop anti-A-RNP Abs (5). Thus, determining the role of SmD peptide immunization in epitope spreading in their model is difficult. The result is significant because anti-A-RNP peptide Abs were not induced in normal mouse strains (BALB/c, B10/brown, and C57BL/6) immunized with the SmD peptide.

Although the precise mechanisms for intermolecular B cell epitope spreading are not known, intrastructural T cell help, intermolecular T cell epitope spreading, and activation of B cells through TLRs are thought to be involved. In the SmD<sub>52-66</sub> immunization model, the mechanisms that might be operational are intrastructural T cell help (15, 24, 25). By this pathway, interaction between SmD<sub>52-66</sub> reactive T cells with A-RNP reactive B cells results in the production of anti-A-RNP Abs. This pathway has been invoked in other studies of epitope spreading using synthetic peptides from SmB, La, and Ro60 for immunization (reviewed in Ref. 10). In all these studies, immunization with the peptide epitope induced autoantibodies to the protein from which the peptide was derived, some of it through intramolecular epitope spreading. The novelty of our finding is the lack of intramolecular B cell epitope spreading within SmD, which was evident in weak and transient anti-SmD Ab responses. Whether T cell epitope spreading plays a role in B cell epitope spreading in the peptide induced model was explored by studying T cell proliferative responses against a panel of A-RNP synthetic peptides at different time points. There was no significant difference in T cell responses to A-RNP peptides between the peptide-immunized and adjuvant-immunized mice (data not shown). However, these data do not rule out the role of intermolecular T cell epitope spreading to other polypeptides within the snRNP particle or other regions on the SmD molecule. This possibility will be formally investigated in future. Recent studies have demonstrated the role of TLR3 and TLR7 in immune responses to the Sm-RNP complexes (26-30). In our model system, the TLR7 pathway mediated autoantibody generation may become operational once anti-A-RNP Abs capable of recognizing the whole snRNP particle are formed. The immune

complex-mediated activation through the TLR7 pathway might further amplify the Ab responses within the snRNP particle.

A major implication of our data is on the interpretation of serological studies assigning Ags responsible for initiating autoantibody responses in lupus or other systemic autoimmune diseases. Longitudinal analysis of patients sera showed single autoantibody specificities such as those against Ro60 or SmB/B' or 70-kDa protein at an earliest time point (6, 7, 9). These data were followed by evolution of other Ab specificities over time. These analyses have led to the conclusion that Ro60, 70-kDa protein, and SmB/B' proteins are the Ags initiating autoimmune responses. Lack of detectable Ab responses to SmD1 or other polypeptides within the snRNP complex (A, C, D2, D3, E, F, G) or the Ro-RNP complex (La, Ro52) was the basis for ruling out these as initiating Ags. Our study implies that serological analysis alone might be misleading in identifying Ags initiating autoimmunity in SLE or other systemic autoimmune disorders. Thus, analysis of sera from SmD peptide-immunized mice showing good Ab responses against A-RNP rather than SmD would lead to an erroneous interpretation that A-RNP is the initiating Ag in A/J mice.

Identifying T cell Ags capable of initiating and sustaining autoantibody responses to multiantigenic complexes in systemic autoimmune disorders is important for understanding mechanisms responsible for breaking tolerance to self Ags. One of the mechanisms is molecular mimicry between foreign proteins from infectious organisms or environmental agents and self proteins (31). The promiscuity of TCR to recognize multiple peptides makes molecular mimicry an interesting proposition for initiating autoimmunity. Due to relative simplicity, most of the focus for identifying molecular mimics for lupus-associated Ags has been on B cell epitopes (13, 32). Our study suggests that mimicry at T cell level should also be considered as an important mechanism to activate self-reactive T cells. The T epitope mapped in our study is also targeted by human T cell (33). Using human T cell clones, Talken et al. (33) mapped a dominant T cell epitope within amino acids SmD<sub>53-67</sub>. Thus, molecular mimics of this epitope have the potential to activate SmD reactive T cells in some patients and initiate an epitope-spreading cascade. Identifying the organisms from which these mimics come will be valuable toward determining the environmental risk factors for SLE.

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## Disclosures

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