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Ro60 Peptides Induce Antibodies to Similar Epitopes Shared Among Lupus-Related Autoantigens

Umesh S. Deshmukh,* Janet E. Lewis,*† Felicia Gaskin,‡ Prashant K. Dhakephalkar,* Carol C. Kannapel,* Samuel T. Waters,*† and Shu Man Fu*‡†

The coexistence of autoantibodies to ribonucleoproteins (RNP) in sera of patients with systemic lupus erythematosus has been attributed to intermolecular determinant spreading among physically associated proteins. Recently, we showed that murine Ab responses to rRo60 or Ro60 peptides were diversified unexpectedly to small nuclear RNP. In this investigation, the mechanisms for this autoantibody diversification were examined. Intramolecular determinant spreading was demonstrated in mice immunized with human or mouse Ro60 (128–285). Immune sera depleted of anti-peptide Ab immunoprecipitated Ro60-associated mY1 and mY3 RNA and remained reactive to a determinant on Ro60 (128–285). Absorption with the immunogen depleted the immune sera completely of anti-Golgi complex Ab (inducible only with human Ro60 (316–335)) and anti-La Ab, and reduced substantially Ab to SmD and 70-kDa U1RNP. Mouse rRo60 completely inhibited the immune sera reactivity to La, SmD, and 70-kDa U1RNP. However, La, SmD, and 70-kDa U1RNP preferentially inhibited the antisera reactivities to these Ags, respectively. Affinity-purified anti-La Ab were reactive with Ro60, La, SmD, and 70-kDa U1RNP. These results provide evidence that a population of the induced autoantibodies recognized determinants shared by these autoantigens. Lack of sequence homology between Ro60 (316–335) and La, SmD, or 70-kDa U1RNP suggests that these determinants are conformational. Interestingly, similar cross-reactive autoantibodies were found in NZB/NZW F1 sera. Thus, a single molecular mimic may generate Ab to multiple RNP Ags. Furthermore, cross-reactive determinants shared between antigenic systems that are not associated physically (Ro/La RNP and small nuclear RNP) may be important in the generation of autoantibody diversity in systemic lupus erythematosus. The Journal of Immunology, 2000, 164: 6655–6661.

Systemic lupus erythematosus (SLE) is characterized by the presence of autoantibodies to multiple cellular constituents, including dsDNA, snRNP, and the Ro/SSA and La/SSB RNP complex (1–4). The patterns of these autoantibodies in SLE patients are complex, and some of these Ab have pathogenic potential. It has been hypothesized that this complexity is attained through diversification of Ab responses within the same Ag (intramolecular determinant spreading) or to different Ags (intermolecular determinant spreading). The origin of these autoantibodies has been a topic of intense study. The proteins from snRNP and the Ro/La-RNP complex have been used to study the role of intramolecular and intermolecular determinant spreading in the diversification of autoantibody production to the relevant autoantigens (5–11). The data from these studies are compatible with the interpretation that intramolecular and intermolecular determinant spreading play a major role in the generation of these autoantibodies.

Recently, we have shown that immunization of mice with either recombinant mouse Ro60 (rmRo60) or recombinant human Ro60 (rhRo60) or a 20-mer Ro60 peptide induced autoantibodies of diverse specificity (10). In addition to anti-Ro60, anti-La, and anti-Ro52 Ab, Ab to SmD and 70-kDa U1RNP were also detected. In addition, sera from mice immunized with peptide hrRo60 (316–335) stained the Golgi complex. The induction of these autoantibodies to autoantigens within the snRNP complex was unexpected. These results could not be explained by the particle hypothesis, which states that the immune response in SLE is driven by multimeric complexes such as snRNP, the Ro/La complex, and/or the nucleosomes (12). As there is no evidence for the physical association of the proteins in the snRNP particle with Ro60, our results suggest additional mechanisms are involved in the diversification of autoantibody response initiated through a single Ag. In this study, we have investigated the mechanisms involved in the diversification of Ab responses following immunization with Ro60 peptide (316–335). Evidence is presented to show that Ab diversification occurs through intramolecular epitope spreading and recognition of B cell epitopes shared among different RNP Ags.

Materials and Methods
Recombinant Ags and synthetic peptides
Recombinant Ags and synthetic peptides were made as reported previously (10). The cDNAs encoding six overlapping fragments of mRo60, B (1–285), C (255–539), D (1–158), E (128–285), F (255–412), and G (382–539) were generated by PCR and cloned into the pQEs30 vector (Qiagen, Chatsworth, CA) to generate recombinant fusion proteins with a 6× His tag. The rmRo60 fragments were purified by Ni-NTA affinity chromatography under denaturing conditions, as per the manufacturer’s instructions. Purified recombinant Dermatophagoides pteronyssinus protein (Der p2)

Received for publication November 3, 1999. Accepted for publication March 28, 2000.

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2 This study was supported by National Institutes of Health Grants R01 AR-42027, R01 AR-42465, RO1 AI-45199, RO1 AI-43248, K11 AR-01906, P30 CA-44579, and P50 AR-45222. U.S.D. is supported partly by a postdoctoral fellowship from the Arthritis Foundation, USA.
3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; hrRo60, human Ro60; mRo60, mouse Ro60; mLs, mouse La; RNP, ribonucleoprotein; Sm, smith; snRNP, small nuclear RNP.

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0022-1767/00/$02.00
with 6X His tag was kindly provided by Dr. S.-S. J. Sung (University of Virginia, Charlottesville, VA).

Synthetic peptides hRo60\textsubscript{316-355} (KARIHPFHILIALETYKTGH), mRo60\textsubscript{316-335}, (KARIHPFHILIALEYTGRH), and hRo60\textsubscript{344-360} (PAG-GTDCLSPLMIWAQKTNTPADVFI) were synthesized and purified by reverse-phase HPLC. Their purity was confirmed to be >95% by mass-spectrometric analysis.

**Immunization**

Six- to 8-week-old female SJL/J and A/J mice from the National Cancer Institute (Bethesda, MD) were maintained in the animal facility at the University of Virginia. Mice were immunized with either purified recombinant proteins or peptides, as described previously (10). The NZB/NZW F\textsubscript{1} and SNF (SWR x NZB F\textsubscript{1}) mice were from The Jackson Laboratory (Bar Harbor, ME). Mice were bled through the tail vein at different time points.

Ab absorption and competitive inhibition

Absorption of sera with synthetic peptides was done as described previously (10). For absorption of sera with recombinant Ags, murine La (mLa) was coupled with cyanoegen bromide-activated Sepharose 4B beads (Pharmacia Biotech, Piscataway, NJ) following manufacturer’s instructions. Ag-coupled beads at increasing concentrations were mixed with variable amounts of Sepharose CL-6B (Pharmacia Biotech) to make the total absorbent volume to 100 ml and incubated overnight with PBS containing 3% BSA. After removal of excessive supernatants from the beads, 500 ml of 1/50 diluted pooled sera was added to the beads. After 1 h incubation at room temperature, the supernatant was used in slot blot analysis. For immunoprecipitation, 5 ml of the pooled immune sera was diluted to 500 ml and mixed with 400 ml of Ro60\textsubscript{316-335} coupled to Sepharose. After incubation for 1 h, 100 and 400 ml of the supernatant were used, representing 1 and 4 ml of the undiluted absorbed sera, respectively. For immunofluorescence, 1 ml of the immune serum was diluted to 200 ml and mixed with 200 ml of beads. The supernatant was used for staining. For affinity purification, Ab bound to the beads were eluted with 0.1 M glycine-HCl buffer, pH 2.7. Eluates were immediately neutralized with 1 M Tris and used in slot blots. For competitive inhibition experiments, pooled sera were diluted in PBS containing 0.1% Tween-20 (PBST) and 3% BSA and mixed with various amounts of purified recombinant Ags in a final volume of 500 ml to achieve the desired final concentrations of the recombinant proteins. The sera from mice immunized with synthetic peptides were used at a dilution of 1/500. Sera from NZB/NZW F\textsubscript{1} mice were used at a final dilution of 1/250. After a 45-min incubation at room temperature, samples were centrifuged in a microfuge for 5 min and the supernatants were used in slot blots and Western blots.

Slot blot analysis

Slot blot analysis was conducted as described previously (10). Bound Ab were detected with HRP-labeled goat anti-mouse IgG, and blots were developed using enhanced chemiluminescence (Pierce, Rockford, IL).

Western blotting

WEHI 7.1 cell extracts were run on a 10% SDS-PAGE. Proteins were transferred overnight to nitrocellulose paper. The nitrocellulose paper was cut into 3-mm strips and used for blotting, as described previously (10). Each strip represents a cell extract from 1.5 x 10\textsuperscript{6} cells.

**Immunoprecipitation**

Imune sera either untreated or absorbed with synthetic peptides were used to immunoprecipitate the \textsuperscript{32}P-labeled mYRNA associated with mRo60, as described previously (10). Human antisera reactive with Ro60, La, and RNP were obtained from Center for Disease Control (CDC). Human antisera reactive with Sm was from one of the lupus patients seen in the lupus clinic, University of Virginia. These sera were used as standards. The precipitated RNA were electrophoresed and processed for autoradiography.

**Immunofluorescence**

Reactivity of sera with Golgi was studied by indirect immunofluorescence, as described previously (10). For disruption of Golgi, HeLa cells were preincubated with medium containing brefeldin A at a concentration of 5 \mu g/ml, for 30 min. Cells were then fixed in methanol for 7 min at -20°C and used as substrate.

**Results**

**Immunization with xenogeneic and autologous Ro60\textsubscript{316-355} peptides induced Ab to Ro60, which cannot be absorbed by the immunizing peptides**

We recently showed that immunization of SJL mice with either xenogeneic or autologous Ro60\textsubscript{316-355} peptides induced Ab reactive with epitopes outside the area of the molecule covered by the immunizing peptide (10). Some of these Ab were cross-reactive with the immunizing peptide. Other Ab were still reactive with Ro60 after the immune sera were depleted of Ab reactive with the immunizing peptide. In addition, Ab capable of precipitating Ro60-associated RNA were detectable by day 24 postimmunization. In control mice immunized with adjuvants, these Ab were not detected. To further document that there was intramolecular determinant spreading, the immune sera depleted of Ab to peptide hRo60\textsubscript{316-335} were tested for their ability to precipitate \textsuperscript{32}P-labeled mYRNAs associated with mRo60. The results are shown in Fig. 1. The panel on the left shows immunoprecipitation patterns obtained with the CDC standard and other reference sera. It is of interest to note that the reference anti-La serum precipitated three dominant RNA species, which correspond to Y3RNA, Y4RNA, and Y5RNA. It has been suggested that the Y4 and Y5 RNA species are absent in rodents (13). In view of this finding, these data need to be reinvestigated. In lane 1, 1 ml of the pooled immune sera from SJL mice immunized with the human peptide precipitated labeled Y1RNA and Y3RNA (Fig. 1, lane 1). The pooled serum sample, depleted of >99.9% of anti-human Ro60\textsubscript{316-335} Ab, also precipitated the labeled mYRNAs, although the intensities of the bands were appreciably reduced (Fig. 1, lane 2). With 4 ml of the pooled immune sera, there was little difference between the unabsorbed and absorbed sera (Fig. 1, lanes 3 and 4), suggesting that the absorbed sera had an excess of Ab. Similar results were obtained with the pooled serum sample from SJL mice immunized with the autologous peptide absorbed with mRo60\textsubscript{316-335} (Fig. 1, lanes 5 and 6).

Further evidence for intramolecular determinant spreading was obtained. Six fragments were made covering the entire span of
mRo60. As shown in Fig. 2, pooled sera from mice immunized either with hRo60 316–335 or mRo60 316–335 after absorption with the immunizing peptides to deplete anti-peptidic Ab react with the whole Ro60 or its fragments Ro60 1–285 and Ro60 128–285. These results indicate that Ab to a determinant(s) in Ro60 128–285 were induced by immunization with either hRo60 316–335 or mRo60 316–335.

Immunization of SJL mice with hRo60 316–335 generated cross-reactive Ab to the Golgi complex

Ab reactive with the Golgi complex were detected in a titer greater than 1:1000 in the immune sera from SJL mice immunized with hRo60 316–335. The staining pattern is shown in Fig. 3A. The staining was not apparent when HeLa cells were treated with brefeldin A, an agent known to disrupt the Golgi complex (Fig. 3B). The reactive Ab were removed by incubation of the immune sera with the immunizing peptide coupled to Sepharose beads (Fig. 3C), but not with the control peptide JS7A, a peptide corresponding to aa 330–342 of mouse ZP3, a protein in the zona pellucida (Fig. 3D).

In another experiment using soluble peptides as the inhibitor, hRo60 316–335 at 0.1 μM inhibited the staining reaction, while 10 μM of mRo60 316–335, which did not induce Ab reactive with the Golgi complex, did not abolish the staining. The three amino acid differences between hRo60 316–335 and mRo60 316–335 provide an explanation for the inability of the autologous peptide to induce and to absorb the cross-reactive Ab. Similar anti-Golgi complex Ab were detected in A/J and BALB/c mice immunized with the human peptide. It is of interest to note that anti-Golgi complex Ab were not detected in mice immunized with the rhRo60 and that the recombinant protein failed to absorb the Ab to the Golgi complex. These results indicate that the relevant epitope is not accessible on the surface of rhRo60.

Intermolecular determinant spreading to RNP in response to immunization with human and mouse Ro60 316–335 peptides was due to the emergence of Ab to the epitopes shared by these autoantigens

Immunization with peptide hRo60 316–335 induced Ab to La, SmD, and 70-kDa U1RNP in eight of eight SJL/J mice (10), whereas none of the mice (six of six) immunized with control peptide from zona pellucida generated these Ab. Because there was little individual variation of responses among the immunized mice, pooled sera from these mice were employed in all subsequent studies. Absorption experiments with either the immunizing peptide or the recombinant proteins were conducted to determine the mechanism for this intermolecular determinant spreading. Fig. 4 shows a representative experiment with the pooled sera from SJL mice immunized with hRo60 316–335. The unabsorbed sera reacted with Ro60, La, SmD, and 70-kDa U1RNP (Fig. 4, lane 1). No reaction to Ro52 was detected. Diluted, pooled sera were incubated with Sepharose beads coupled to different peptides. The immunizing peptide, hRo60 316–335, abolished the reaction of the pooled immune serum sample to La and reduced its reactivity to 70-kDa U1RNP markedly, and to SmD and Ro60 to lesser degrees (Fig. 4, lane 2). No reaction to Ro52 was detected. Diluted, pooled sera were incubated with Sepharose beads coupled to different peptides. The immunizing peptide, hRo60 316–335, abolished the reaction of the pooled immune serum sample to La and reduced its reactivity to 70-kDa U1RNP markedly, and to SmD and Ro60 to lesser degrees (Fig. 4, lane 2). The control peptides, JS7A and hRo60 441–465, had no appreciable effect on the reactivity of the pooled sera (Fig. 4, lanes 3 and 4). Under these experimental conditions, hRo60 316–335 Sepharose beads had no effect on the reaction of a immune serum from a SJL mouse immunized with hRo60 441–465 (Fig. 4, lane 7).
In contrast, hRo60\textsuperscript{441–465}-Sepharose beads effectively removed all the Ab to Ro60 and Ro52 in the immune serum (Fig. 4, lane 6). Absorption experiments to determine whether all the reactivity to SmD and 70-kDa U1RNP from the pooled anti-human Ro60\textsuperscript{316–335} immune sera could be removed by increasing the amounts of Sepharose beads did not yield interpretable results because the control absorbents at the increased volumes also reduced the immune serum reactivity toward these proteins. Similar results were also obtained with the immune sera from mice immunized with mRo60\textsuperscript{316–335}.

The results of competitive inhibition experiments with purified recombinant proteins are shown in Fig. 5. In the upper panel, the pooled immune sera from SJL mice immunized with hRo60\textsuperscript{316–335} were shown to react with Ro60, La, SmD, and 70-kDa U1RNP. A total of 20 μg/ml of Ro60 abolished the reactivity of the pooled sera to La and reduced the reactivity substantially to Ro60, SmD, and 70-kDa U1RNP. At 100 μg/ml, Ro60 inhibited the Ab reactivity to all Ags. La, SmD, and 70-kDa U1RNP at differing concentrations inhibited the reactivity of the pooled immune sera to La, SmD, and 70-kDa U1RNP without an appreciable reduction of reactivity to Ro60. The possibility that lack of inhibition of Ro60 reactivity by other recombinant proteins was due to presence of excess amount of anti-Ro60 Ab was considered. Pooled immune sera were used at a dilution of 1/2000. At this dilution, a limited amount of anti-Ro60 Ab was present in the diluted sera because mRo60 at a concentration of 20 μg/ml completely inhibited the reactivity to Ro60, La, SmD, 70-kDa U1RNP, GST, and Der p2 had no effect on this reactivity even at a concentration of 100 μg/ml (data not shown). These results suggest that the cross-reactive Ab represent a fraction of Ab population induced by the peptide immunization. Similar results were obtained with sera from mice immunized with mrRo60 as shown in the middle panel.

The presence of cross-reactive Ab in the immune sera was further confirmed by performing competitive inhibition experiments, employing extracts from WEHI 7.1 cell line as substrate in Western blot analysis. The results are shown in the lower panel of Fig. 5. Lanes 1–3 show the reactivity of human antisera to La, Sm, and RNP. Lane 4 is reactivity of pooled sera from mice immunized with mrRo60. The pooled sera from mice immunized with the peptide (lanes 5 and 11) reacted with proteins at molecular mass of 70 kDa, 46 kDa (La), and 16 kDa (SmD) and multiple proteins between 21 kDa and 30 kDa. These reactivities were inhibited either completely or significantly by recombinant proteins mRo60 (lane 6), mLa (lane 7), SmD (lane 8), and 70-kDa U1RNP (lane 9). The control protein GST (lane 10) had no effect on these reactivities. The reactivity to Ro60 and 70-kDa U1RNP in slot blots and Western blots does not correlate. It is interesting to note that only sera from mice immunized with mrRo60 react with a 60-kDa protein, whereas those from mice immunized with the peptide are not reactive. It is possible that only limited epitopes on Ro60 are recognized by sera from mice immunized with the peptide, which are maintained on the recombinant protein, but are destroyed on the protein from cell extracts. Despite these discrepancies, the experiments employing cell extracts do confirm our finding of generation of cross-reactive Ab.
Additional experiments were done with recombinant mLa coupled to Sepharose beads. As shown in Fig. 6, increasing amounts of La Sepharose beads from 1 to 25 μl were incubated with the pooled immune sera. Anti-La reactivity was completely removed with 4 μl of La beads. Additional immunoabsorbent was required to remove all the reactivity to SmD. Ab eluted from 10 μl of La beads, which were incubated with the immune sera, showed strong reactivity to La, SmD, and 70-kDa U1RNP and weak reactivity to mRo60. Ab eluted from 25 μl La beads reacted readily to mRo60.

The cross-reactive Ab were detected at the earliest time point of 14 days after the initial immunization. These Ab, which are cross-reactive with the RNP of interest, disappeared from the immunized SJL mice 60–120 days after the initial immunization with either human or mouse peptide. In contrast, Ab to the peptide and to mRo60 persisted 270 days after the initial immunization, when the mice were sacrificed. Repeated attempts to show that there was T cell determinant spreading by demonstrating T cell proliferative responses to mLa, SmD, or 70-kDa U1RNP have not been successful (data not shown).

Cross-reactive autoantibodies were also generated in A/J mice

Similar experiments were conducted in A/J mice to show that cross-reactive Ab to the RNP are also generated in response to immunization with hRo600316–335. As shown in Fig. 7, immunization with the human peptide induced Ab reactive with Ro60, La, Ro52, SmD, and 70-kDa U1RNP (Fig. 7, lane 1). Ro60 removed all the reactivity to these autoantigens (Fig. 7, lanes 2–5). La, SmD, and 70-kDa U1RNP inhibited the reactivity of the immune sera to La, Ro52, SmD, and 70-kDa U1RNP without appreciably reducing its reactivity to Ro60. With the immunizing peptide as the inhibitor, similar results were obtained. The striking difference from the immune sera obtained in SJL mice is that anti-mouse Ro60 reactivity was completely inhibited by the immunizing peptide.

Detection of autoantibodies cross-reactive with various autoantigens in NZB/NZW F1 mice

The possibility that cross-reactive Ab are generated due to the unique conformations presented by the Ro60 peptides was considered. In addition, we were interested in finding out whether Ab recognizing such cross-reactive determinants were present in sera of lupus-prone mice. The NZB/NZW F1 mouse has been considered a murine model for human SLE (14). Thus, sera from 5-month-old NZB/NZW F1 mice were pooled and used in immunoblot analysis. As shown in Fig. 8, the sera were still reactive with SmD and 70-kDa U1RNP at 1/800 dilution. At 1/100 dilution, reactivities were detected against all the autoantigens tested. No reactivity was seen against the control protein, Der p2. The reactivities of the sera from male or female mice were similar. The incidence of these reactivities was determined in sera from individual mice (data not shown). Reactivity to Ro60 was observed in 7 of 14 mice; reactivity to La, SmD, and 70-kDa U1RNP was obtained in 12 of 14 mice. The pattern of reactivity obtained in NZB/W F1 mice was distinct from that obtained with pooled sera from age-matched SNF1 mice, which represent another murine lupus model. The sera were strongly reactive with La, whereas reactivity to SmD and 70-kDa U1RNP was only detectable at a serum dilution of 1/100. Both sera had similar quantities of total IgG: 3.5 mg/ml in NZB/W F1 and 3.8 mg/ml in SNF1.

The reactivities of the pooled sera against Ro60, La, and Ro52 were unexpected and allowed us to determine whether cross-reactive determinants shared by these Ags play a role in the detection of these autoantibodies. As shown in Fig. 9, at 1/250 dilution, pooled sera from NZB/NZW F1 female mice reacted with mLa, SmD, and 70-kDa U1RNP. A total of 10 μg/ml of SmD inhibited reactivity to mLa and 70-kDa protein. With 50 μg/ml of SmD,
concentrations of 2–50 μg/ml in inhibition experiments. Recombinant SmD and mLa were used at 1/250 dilution. Ab are able to immunoprecipitate native Ro60. One of the targeted determinants spreading to regions outside the immunogen. These break tolerance to endogenous Ro60 and induce intramolecular by the data that both mouse and human Ro60 peptides 316–335 Ro60, which can be targeted for autoimmunity. This is exemplified (15). Thus, this peptide represents a potentially important region of sera from patients with primary and secondary Sjogren’s syndrome almost all the reactivities to these three autoantigens were abolished. Similar results were obtained with mLa as the inhibiting protein.

Discussion
The evolution of a highly heterogeneous population of autoantibodies reactive with multiple autoantigens in SLE patients has been attributed to the phenomenon of intermolecular determinant spreading. We are investigating this phenomenon using Ro60 as a model Ag. In a recent publication (10), evidence has been presented to show that immunization of mice with either autologous or xenogenic Ro60 or its peptides induces a diverse autoantibody response. In addition to Ab against the immunogen, Ab to La, Ro52, and proteins in the snRNP particles were detected in the immunized mice. According to the current view, after initiation of immune response to a single Ag, diversification of Ab response occurs to other Ags that are physically associated. Thus, in our case, the detection of Ab responses to proteins within the snRNP complex was surprising, as there is no evidence for physical association between proteins of the Ro/La-RNP and snRNP. The mechanisms for this phenomenon have been investigated in this study. Employing multiple techniques, we have obtained extensive data that suggest that substantial amounts of Ab to other autoantigens were generated by immunization with peptide Ro60316–335 and that these Ab are reactive to similar epitopes shared by Ro60 and the relevant reactive autoantigens. The lack of amino acid sequence homology between peptide Ro60316–335 and La, SmD, and the 70-kDa U1RNP suggests that the cross-reactive Ab recognize shared conformational determinants. Similar data have been obtained in several studies where the presence of multiple B and T cell epitopes on Ro60 was not possible to assign a specific region on the molecule, which generates cross-reactive Ab.

The synthetic peptide Ro60316–335 contains a dominant, non-cryptic T cell determinant (10). Moreover, the N-terminal domain of this peptide represents a dominant B cell epitope recognized by sera from patients with primary and secondary Sjogren’s syndrome (15). Thus, this peptide represents a potentially important region of Ro60, which can be targeted for autoimmunity. This is exemplified by the data that both mouse and human Ro60 peptides 316–335 break tolerance to endogenous Ro60 and induce intramolecular determinant spreading to regions outside the immunogen. These Ab are able to immunoprecipitate native Ro60. One of the targeted epitopes has been mapped to the region on Ro60 spanning aa 128–285. Absorption experiments indicate that reactive epitopes are not cross-reactive with the immunizing peptides. Thus, true intramolecular epitope spreading occurs in our experimental system.

We and others have relied on the use of recombinant proteins for studying Ab diversification in experimental model systems (6–10). Although sera from immunized mice could readily immunoprecipitate native Ro60, they reacted weakly with 35S-labeled SmD (data not shown). Thus, the majority of cross-reactive Ab appear to be of the nonprecipitating type. This may be due to unique epitope specificity of Ab or due to their affinity. Ab that lack the ability to immunoprecipitate lupus-associated Ags have been demonstrated in the sera of patients with lupus (16, 17). Another possibility is that the synthetic peptide used for immunization has distinctive conformations that are also present on the recombinant proteins employed for immunoassays. To rule out this possibility, we used sera from unimmunized NZB/NZW F1 mice. The NZB/NZW F1 mouse is considered one of the best models of lupus. Autoantibodies to Ro60, La, and peptides within the snRNP complex were readily detected in the pooled serum. Pooled sera from SNF1 mice were employed for comparison. In contrast to the NZB/NZW F1 mice, only Ab reactive with La were dominant. In a limited study employing sera from normal mice, such Ab were detected at lower serum dilutions (data not shown). At the present, it is not clear whether this represents background reactivity in immunoassays or true Ag-Ab interaction. Thus, it is possible that these Ab in normal mice may represent a pool of polyreactive autoantibodies present at low titers. The ability of both mLa and SmD to competitively inhibit the NZB/NZW F1 serum reactivities to La, SmD, and 70-kDa U1RNP provides strong evidence that autoantibodies cross-reactive with a broad group of autoantigens are generated in the absence of specific immunization. Recently, we have affinity-purified anti-SmD Ab from the sera of two patients with lupus. By immunodiffusion, both patients had anti-Ro60 Ab. Anti-Sm Ab were not detected. However, by ELISA, one of them had predominantly IgM anti-Sm Ab, while the other had IgG anti-Sm Ab. The affinity-purified anti-Sm Ab reacted with hRo60 (data not shown). These results provide strong evidence that cross-reactive autoantibodies to a variety of RNP are readily detectable in sera of subjects with spontaneous SLE.

The presence of similar epitopes on SLE-associated autoantigens has been previously reported using human and mouse polyclonal Ab and mAb. The cross-reactivity between the Sm proteins has been localized to the carboxyl amino acid motifs of SmB and SmD proteins (18, 19). This motif has also been shown to react with Ab recognizing ribosomal protein S10 (20). It has also been shown that the proline-rich regions on these proteins are responsible for the cross-reactivity (21). Ab recognizing proteins not sharing homologous sequences or domains, such as those against ribosomal P proteins and Sm (22, 23), DNA and Sm (24), and DNA and ribosomal protein S1 (25) indicate the presence of shared conformational determinants within these Ags. Although these studies demonstrate cross-reactive Ab, the inciting Ags remain unknown. Our study clearly demonstrates that Ab recognizing multiple RNP can be generated experimentally in mice. Moreover, immune responses to the Ro/La-RNP and snRNP antigenic systems can be linked through shared conformational determinants. The lack of sequence homology between the hrRo60316–335 peptide and other relevant autoantigens suggests that these Ab recognize shared structural determinants among various autoantigens important in SLE. This conformation is not shared by another domain on Ro60 represented by peptide 441–465. Immunization of mice with this peptide did not generate Ab reactive with La, SmD, and the 70-kDa U1RNP (10). However, cross-reactive Ab to Ro52.
were generated (Fig. 4, lane 5). Thus, it is possible that different regions on Ro60 will mimic different antigenic domains presented on other autoantigens. Reynolds et al. (26) have demonstrated generation of Ab reactive with Ro52, in mice immunized with a peptide from La. Similarly, Scofield et al. (27) have shown generation of Ab reactive with La in animals immunized with Ro60 peptides. However, in these studies it is not clear whether these Ab are cross-reactive. Relevant to this discussion is the finding of Puttermann and Diamond (28). In their studies, immunization of mice with a synthetic peptide representing a B cell epitope for anti-dsDNA induced Ab cross-reactive with dsDNA.

It has been stressed that intermolecular epitope spreading within autoantigens physically associated with each other, such as nucleosomes (DNA and histones), snRNP (Sm peptides and U1RNP associated with small RNA), and Ro/La-RNP (Ro60 and La associated with YRNA), is responsible for the generation of Ab heterogeneity (11). Craft and colleagues have provided strong evidence for this phenomenon using the Ags involved in the snRNP complex (reviewed in Refs. 11 and 29). Our results demonstrate that additional mechanisms exist for the generation of Ab diversity. The generation of Ab reactive with multiple autoantigens as a result of an immune response to a single peptide has significant implications in the pathogenesis of systemic autoimmune disorders such as SLE. The hRo60 316–335 peptide has three amino acids that are different from the autologous peptide. Its ability to induce a diversified autoantibody response indicates that foreign Ags with molecular mimics to autoantigenic determinants should be considered seriously as the initiating Ags. Induction of intramolecular spreading by peptide Ro60 316–335 indicates that endogenous Ag is processed and presented. Thus, our findings suggest that a single mimic may be sufficient to generate an apparently diversified Ab response against Ags that may or may not be physically associated.

Our model of epitope spreading is different from that of James and Harlcy (30), employing peptide from SmB/B′ protein. In their model, Ab to other components in the snRNP particle were generated and increased in complexity over a period of time. This may be possible because the induced Ab react with proteins that are physically associated. Thus, a T cell response to endogenous SmB′/B′ induced through peptide immunization would be able to sustain Ab response to other physically associated proteins through intermolecular help (5). These investigators have not studied T cell spreading in their model. In our model, disappearance of cross-reactive Ab to La, SmD, and 70-kDa U1RNP indicates that these Ab depend on the presence of the antigenic mimic. This observation suggests that in human SLE, continued or repeated immunization may be required. Our current thinking is that for sustaining Ab response to these Ags, the mimic has to be able to break tolerance at the T cell level. This is exemplified by our earlier finding that a robust Ab response to mRo60 is generated in mice immunized with peptide hRo60 316–335, whereas peptide hRo60 241–465 fails to do so, because only peptide Ro60 316–335 breaks tolerance to endogenous Ro60 (10). In our model, we have not detected spreading of T cell responses to other proteins. This, along with the observation that little if any specific autoantibodies are generated to other autoantigens, indicates that T cell response to other autoantigens may be required to generate specific autoantibodies. The marked variation of autoantibody specificity in SLE patients suggests that different foreign mimics may be the initiating Ags in these patients. It is thus reasonable to postulate that in addition to intermolecular determinant spreading, multiple antigenic challenges leading to T cell activation to multiple autoantigens are required to induce autoantibodies with the complex specificities observed in SLE patients.