Immune Responses to Ro60 and Its Peptides in Mice. I. The Nature of the Immunogen and Endogenous Autoantigen Determine the Specificities of the Induced Autoantibodies

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Summary

Anti-Ro60 autoantibodies are found in a variety of autoimmune disorders including systemic lupus erythematosus (SLE), Sjögren’s syndrome, primary biliary cirrhosis, and active hepatitis. They are the most prevalent autoantibodies in normal individuals and in asymptomatic mothers of infants afflicted with neonatal lupus. In the present study, immune responses to recombinant human Ro60 (rhRo60) and recombinant mouse Ro60 (rmRo60) and selected Ro60 peptides in non–SLE-prone mice were investigated. Multiple T and B cell epitopes were identified in Ro60. Immunizations with either xenogeneic or autologous Ro60 induced autoantibodies to a diverse group of autoantigens. In addition to La and Ro52, proteins in the small nuclear ribonucleoprotein (snRNP) particles such as SmA, SmB, SmD, and 70-kD U1-RNP were unexpectedly identified as targeted antigens. In the studies involving synthetic Ro60 peptides, both human and mouse Ro60 316–335 peptides, which differ in three amino acids, were found to contain dominant cross-reactive T cell determinants. Immunizations with these peptides induced autoantibodies to Ro60, La, SmD, and 70-kD U1-RNP without autoantibodies to Ro52, SmA, or SmB. With human Ro60 316–335 as the immunogen, additional autoantibodies reactive with the Golgi complex were found. In contrast to the immunodominance of both human and mouse Ro60 316–335 peptides, the T cell determinant in human Ro60 316–335 was dominant, whereas that in the mouse peptide was cryptic. Immunization with human Ro60 441–465 induced primarily anti-peptide Abs. Mouse Ro60 441–465 failed to induce an antibody response. These results show that both the nature of the immunogen and the immunogenicity of the related endogenous antigen are important in determining the specificities of the autoantibodies generated. They have significant implications for proposed mechanisms on the generation of complex patterns of autoantibodies to a diverse group of autoantigens in SLE patients.

Key words: systemic lupus erythematosus • determinant spreading • tolerance • autoimmunity • T and B cell epitopes

Systemic lupus erythematosus (SLE) is an autoimmune disorder with antinuclear Abs as a prominent feature (1–3). Among the targeted autoantigens in this disease, Ro60 (or Sjögren’s syndrome A, SS-A) and La (SS-B), which are physically associated with hYRNAs, constitute a major class of SLE-related autoantigens, and Abs to them have been shown to be associated with specific clinical presentations (2, 4). The presence of Abs to these autoantigens in normal individuals and in asymptomatic mothers of infants affected with neonatal lupus suggests that there is a lack of tolerance to these autoantigens or that the tolerance can be readily broken. In this regard, autoantibodies to Ro60 and La have been induced in non–SLE-prone mice by immunizations with recombinant proteins (5–8).

Recently, this antigenic system has been utilized to show the role of intramolecular and intermolecular epitope spreading in the diversification of the autoimmune re-
response to these antigens (5–10). Because of the availability of mouse as well as human recombinant proteins, La has been used extensively as the immunogen in many of these studies. In particular, emphasis has been made by Reynolds et al. (6) that there is hierarchical self-tolerance to T cell determinants (epitopes) within La and that cryptic or non-dominant T cell epitopes are capable of breaking tolerance, leading to a diversified autoantibody response.

Because of the observation that the most prevalent autoantibodies in asymptomatic normal individuals are anti-Ro60 Abs (4), it is of considerable interest to explore the immune response to this autoantigen in normal non–SLE-prone mice. In this investigation, the immune responses to both autologous and rhRo60 in mice were studied. These responses involve multiple T and B epitopes within the immunogens. In addition to the induction of Ab to La and Ro52, these responses unexpectedly generated autoantibodies to multiple proteins in the small nuclear ribonucleoprotein (snRNP) particle. Evidence has been obtained that tolerance to the dominant T cell epitopes in Ro60 can be readily overcome, leading to a diverse autoimmune response.

Materials and Methods

Cloning and Expression of Recombinant Antigens. The cDNAs encoding human Ro60, 70 KD U1–RN P (gifts from Jack Keene, Duke University, Durham, NC), SmB and SmD (from Joe Craft, Yale University, New Haven, CT), were cloned into the pQE expression vectors (Qiagen Inc., Chatsworth, CA) to generate recombinant fusion proteins with a 6XHis tag. Mouse Ro52 in pQE expression vector was a gift from James McCluskey (Flinders Medical Center, Bedford Park, South Australia). SmA cDNA from the pET expression vector was a gift from Joe Craft. For cloning of mouse Ro60 cDNA, two Axa10 libraries were used: a mouse liver 5'- stretch plus cDNA library from Clontech (Palo Alto, CA) and 33BTE-67, a mouse λ-6 T cell hybridoma library from Rebecca L. O'Brien (National Jewish Medical and Research Center, Denver, CO). They were screened with a 1.8-kb, EcoRI/NcoI DNA fragment of human Ro60 under nonstringent conditions. Two independent clones, MuT 10.1 (2-kb insert from T-cell library) and Mu1 23.1 (2.3-kb insert from liver cDNA library) were obtained from screening 1.2 × 10^6 colonies. Their DNA sequences were determined and data were analyzed using Eugene (Molecular Biology Information Resource, Baylor Medical College, Houston, TX) and GCG (Wisconsin Package, Version 8; Genetic Computer Group, Madison, WI) software. MuT 10.1 and Mu1 23.1 had an overlap of 1.446 kb. The combined sequence of these two clones was 85% homologous to the human Ro60 sequence. It lacked a 170-bp fragment at the 5' end. 5' RACE (11) was used to amplify the missing 170-bp fragment. The entire coding region of mouse Ro60 was generated by PCR using WEHI 7.1 cDNA and cloned into the KpnI and HindIII sites of the pQE expression vector. Mouse La was similarly cloned from the liver cDNA library screened with full-length human La cDNA. The complete cDNA encoding mouse La was cloned into pQE expression vector. Recombinant proteins were expressed in Escherichia coli. Recombinant antigens expressed in pQE vectors were purified under denaturing conditions following manufacturer's instructions. Purified proteins were dialyzed against distilled water, and stored at -70°C until use. Recombinant Sm was purified as described by Fatenejad et al. (12).

Synthetic Peptides. Overlapping peptides spanning the entire sequence of hrRo60 and mRo60 were synthesized on an automated peptide synthesizer, AMSi 422 (Gilon Inc., Middleton, WI) using Fmoc Chemistry. Peptides were analyzed and purified by reverse phase HPLC and their masses confirmed by mass spectrometry. Peptides used for immunizations were made in the Biomolecular Research Facility, University of Virginia.

Immunization. 6–8-wk-old female SJL/J and A/J (both from National Cancer Institute, Bethesda, MD) and BALB/cBy mice (Jackson Laboratory, Bar Harbor, ME) were maintained in the animal facility at the University of Virginia. For in vitro lymph node cell (LNC) proliferative responses, mice were immunized with either 100 µg of recombinant protein or 50 µg of synthetic peptide emulsified in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI) in one hind footpad and at the base of the tail. For analysis of antibody production, animals were immunized initially as described above. They were subsequently immunized with 50 µg of antigen emulsified in incomplete Freund's adjuvant (Difco Laboratories) intraperitoneally, on days 14 and 28. Controls were immunized with adjuvant alone in a similar way. Tail bleeds from mice were collected at different time points postimmunization, and sera were assayed for specific Abs.

Lymph Node Proliferation Assays. 2 wk after immunization, draining lymph nodes were removed and single-cell suspensions were prepared. LNCs were cultured in 96-well plates at 3 × 10^4 cells/well in DMEM (BioWhittaker Inc., Walkersville, MD) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 2 mM l-glutamine, nonessential amino acids, sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO BRL, Gaithersburg, MD), and 5 × 10^−3 M 2-mercaptoethanol, with or without antigens in triplicates. The cells were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 4 d. Plates were pulsed with [H]thymidine (NEN Research Products, Boston, MA), 0.5 µCi/well, during the last 16 h of culture. The cells were harvested onto glass fiber filters (Wallac Oy, Turku, Finland) using a semiautomated cell harvester (Skatron Instruments Inc., Sterling, VA) and radioactivity measured by liquid scintillation counting using a Betaplate counter (LKB Instruments Inc., Piscataway, NJ). The results are expressed, either as stimulation index (SI), determined by dividing the mean triplicate antigen-specific cpm by mean triplicate cpm from wells without antigen, or as mean triplicate ΔCPM (mean triplicate cpm with antigen minus mean triplicate cpm without antigen).

ELISA. Microtiter plates, Immulon 2 and Immulon 4 (Dynatech Inc., Chantilly, VA) were coated with rhRo60 and synthetic peptides, respectively, at a concentration of 2 µg/well, in carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. The wells were then blocked with PBS containing 3% BSA (200 µl/well). Sera diluted in PBS containing 0.1% Tween 20 (PBST) and 3% BSA were added to the washed plates. Bound antibodies were detected with peroxidase-conjugated goat anti–mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL), using the substrate o-phenylenediamine (0.05%) (Sigma Chemical Co., St. Louis, MO), 0.06% hydrogen peroxide in citrate-phosphate buffer, pH 5.0. Reaction was stopped after 15 min by the addition of 50 µl/well of 2.5 N sulfuric acid. Absorbance was read at 490 nm. Volumes of the diluted sera, Abs, and substrates were 100 µl/well, and incubations were for 2 h at room temperature. Plates were washed five times with PBST between steps.

Immunofluorescence. HeLa cells or NIH/3T3 cells were grown on round coverslips (1 cm diameter). The cells were fixed in methanol for 7 min at −20°C. Sera diluted in PBS containing 1% BSA were added. After 2 h, bound antibodies were detected with
rhodamine-coupled goat anti–mouse IgG. In between steps, coverslips were washed three times with PBS.

Antibody Absorption. Peptides hr60,316–335 and hr60,441–465 were coupled to CNBr activated Sepharose 4B beads (Pharmacia Biotech Inc., Piscataway, NJ) following the manufacturer’s instructions J5TA, a peptide corresponding to amino acids 330–342 of ZP3, which is a protein in the mouse zona pellucida, was also coupled to beads and used as a control in absorption experiments. The beads were incubated overnight with PBS containing 3% BSA at 4°C. Sera were diluted in PBS containing 3% BSA and incubated with the beads for 2 h at room temperature. The absorbed sera were assayed for anti-peptide activity in ELISA and other antibody activity.

Western Blotting. WEHI 7.1 cell extract was run on a 7.5% SDSPAGE and transferred overnight onto nitrocellulose paper. After a 5% milk protein blocking step, sera diluted in PBS containing 5% milk protein were incubated with the nitrocellulose paper. Bound antibodies were detected with peroxidase-labeled goat anti–mouse IgG, and blots were developed using enhanced chemiluminescence (Pierce Chemical Co, Rockford, IL). All incubations were for 2 h at room temperature, and blots were washed three times in between steps.

Slot Blot. The slot blot apparatus from Millipore Inc. (Bedford, MA) was used. Each slot had a length of 8-mm. Purified recombinant antigens were loaded at a concentration of 5 μg/slot in 8 M urea. The 8 mm strips were cut into three equal parts. After a blocking step with PBS containing 5% milk protein overnight at 4°C, the strips were incubated with diluted sera and the bound Abs were detected in a manner similar to that described in the preceding paragraph.

Immunoprecipitation of mYRNAs Associated with Ro60. The mYRNAs associated mR60 were immunoprecipitated as described by Craft and Hardin (13). Briefly, WEHI 7.1 cells were suspended at 2.5 × 10⁶ cells/ml in phosphate-free RPMI 1640 supplemented with 5% dialyzed FCS. The cells were grown for 14 h in the presence of 10 μCi/ml of ³²P (NEN Research Products). The ³²P-labeled RNA associated with Ro60 were immunoprecipitated with immune and control sera. The precipitated RNA were electrophoresed and revealed by autoradiography.

Results

The immune R responses to rhRo60 W are directed to M uliplet T and B determinants. T and B cell responses to rhRo60 were studied in SJL/J (H-2d), BALB/c (H-2b), and A/J (H-2a). All three strains mounted a strong T cell proliferative response to rhRo60 (Fig. 1 A). Strong antibody responses were also induced. Abs to the immunogen were readily detected 14 d after the initial immunization. By day 30, high antibody titers were generated and reactivity to rhRo60 was detectable at a serum dilution of 1:100 (Fig. 1 B). Control mice injected with CFA alone did not give specific T and B cell responses. Two other experiments gave similar results.

A panel of overlapping peptides spanning the entire sequence of hr60 was made and used to map T and B epitopes recognized by mice immunized with rhRo60. In preliminary experiments, peptides at a concentration of 20 μM were found to be optimal in recalling T cell responses in vitro. A peptide was considered to contain a T epitope(s) if it gave an SI > 2.0 in at least three independent experiments. Fig. 2 A shows representative results for SJL/J mice. M ultiplet peptides recalled the proliferative response in varying magnitudes. Peptides hr60,411–415 and hr60,316–335 were the most dominant peptides, followed by peptide hr60,316–335, hr60,441–465, hr60,401–425, and hr60,481–505. M ultiplet T cell determinants were also mapped in A/J and BALB/c mice, and the results are summarized in Fig. 2 B.
The B cell epitopes on Ro60 were mapped in ELISA, using microtiter plates coated with synthetic peptides. Fig. 3 shows results for sera obtained on days 14 (closed bars) and 30 (open bars) after immunization. Three clusters of reactivities were detected, with most determinants located on the C O O H - terminal region of the molecule (hR o60 amino acids 401–538). B cell epitopes were also mapped in the middle portion of the molecule, hR o60 amino acids 240–315. The sera recognized only two peptides from the NH2-terminal region. While peptide hR o601–25 was recognized by all strains, peptide hR o6061–85 was recognized only in SJL/J and A/J strains. Similarly, peptides hR o60251–285, hR o60456–475, and hR o60466–485 were recognized in SJL/J and A/J but not in BALB/c. Peptide hR o60201–225 appears to be specific to BALB/c and peptide hR o6016–335 for SJL/J.

Both T and B cell responses to rmR o60 were inducible. Because of a sequence difference between human and mouse R o60, the immune response generated by hR o60 immunization could be strongly mediated by these differences between the xenogeneic and autologous forms of this autoantigen. To determine whether responses to autologous R o60 could be demonstrated, the cDNA encoding mouse R o60 was cloned and expressed. Our sequence was in agreement with that reported by Wang et al. (14). There is 90% homology at the amino acid level between the mouse and human R o60, and the amino acid differences scatter throughout the whole sequence. Immunization with purified rmR o60 induced T cell proliferative responses in all strains of mice (Fig. 4). The response in SJL/J mice was much higher than that in BALB/c and A/J. Proliferative responses were not seen in the animals immunized with only CFA, indicating specificity of the response.

A set of peptides (20 mers with an overlap of 10) spanning the entire sequence of mR o60 was synthesized in order to undertake T cell epitope mapping. Only 3 peptides, mR o60121–140, mR o60281–300, and mR o60441–460, were able to recall the proliferative responses in SJL/J after immunization with rmR o60 (Fig. 5). The most dominant peptide was mR o6011–30. Interestingly, this peptide overlaps the dominant T cell determinant mapped in hR o60, peptide hR o60466–485. These results indicate a lack of tolerance to this region on the R o60 antigen. The other dominant epitope mapped on hR o60 was in peptide hR o60441–460. However, the mR o60 peptides mR o60441–460 and mR o60461–470 that span this region could not recall the proliferative responses.
The panel of peptides was also used to map B cell determinants on mR060. As shown in Fig. 5 B, multiple peptides reacted with the pooled sera at day 67 postimmunization. These B cell determinants are present throughout the whole span of mR060. Immune sera at later time points gave similar reactivity patterns.

Both Xenogeneic rhR060 and Autologous rmR060 Induced Intermolecular Determinant Spreading. Sera from SJL mice immunized with rmR060 and rhR060 were positive for antinuclear antibodies (ANA) (Fig. 6, A and B, respectively). Although results are shown for pooled sera, all mice had ANA in their sera. Such antibodies were not detected in mice immunized with only CFA (Fig. 6 C).

All mice immunized with either rmR060 or rhR060 produced high titers of Abs to the immunogens. In both groups of mice, immunoprecipitating Abs to native mR060 were induced (data not shown). In addition to expected reactivity to La and R o52 (5–9), Abs to SmA, SmB, SmD, and U1 RNP associated 70 kD protein were also observed (Fig. 7). In the control mice immunized with CFA, only weak reactivity to La, SmD, and U1 RNP associated 70 kD protein was observed in two of seven mice studied. Surprisingly, recombinant dihydrofolate reductase (DHFR)-6XHis protein included as a control protein was reactive with some of the immune sera. As presented later, this reactivity was also found in some of the sera from mice immunized with either human or mouse R060316–335, which do not have the 6XHis tag. Thus it is concluded that this reactivity was not directed against the 6XHis tag. Similar results were obtained in an additional experiment. These results suggest that intermolecular epitope spreading had occurred in mice immunized with either rhR060 or rmR060.

Intermolecular and Intramolecular Antibody Diversification Is Induced by Peptide hR060316–335 but Not by Peptide hR060441–465. The presence of multiple T and B epitopes on both rhR060 and rmR060 makes it difficult to address the phenomenon of spreading and the mechanisms involved due to the complexity of the immune response elicited. For this purpose, we used synthetic peptides representing the dominant T cell epitopes on hR060 as immunogens.

Sera from mice immunized with peptide hR060316–335 had very high titers of antipeptide Abs. The titers peaked between days 60 and 90. In addition, Abs to nonhomologous peptide hR060401–425 were detected as early as 24 d after immunization. The titer against hR060401–425 increased with time up to 60 d postimmunization. With time, the immune sera had increasing amounts of Abs with more diverse specificities. These sera were capable of immunoprecipitating native R060 as shown in Fig. 8. Two cytoplasmic R N A moieties, namely mY1 and mY3 associated with mR060 (15), have been shown to be precipitated by R-specific antisera. The appearance of Abs to the nonhomologous peptide and to the native R060 determinant(s) represents intramolecular determinant spreading in response to the immunization with the peptide hR060316–335.

Antibody diversification to multiple intracellular determinants was also evident in that the pooled immune sera recognized multiple cellular proteins in W EHI 7.1 cell extract in Western blot analysis (Fig. 9). Of particular interest was the protein recognized at 48 kD (Fig. 9, lanes 2–4), which was also recognized by the CDC anti-La reference serum (Fig. 9, lane 1), suggesting that intermolecular epitope spreading to La might have occurred. This reactiv-

![Figure 6](image1)  
**Figure 6.** Induction of ANA as following immunization with R060 antigens. Sera obtained on day 67, postimmunization, were pooled and used at a dilution of 1:200 to stain methanol-fixed HEK cells (detected by indirect immunofluorescence). a, b, and c represent sera from mice immunized with rmR060, rhR060, and CFA, respectively.

![Figure 7](image2)  
**Figure 7.** Intermolecular determinant spreading of antibody responses in SJL/J mice immunized with R060 antigens. Reactivities of sera with different ribonucleoproteins are shown in dot blots. Each lane represents a serum sample at a dilution of 1:250.

![Figure 8](image3)  
**Figure 8.** Immunoprecipitation of mYRNAs associated with mR060. W EHI 7.1 cells were labeled with 32P, and RNA species were immunoprecipitated and visualized by autoradiography. Lanes 1–4 represent pooled sera on days 24, 37, 60, and 90, respectively, from SJL/J mice immunized with hR060316–335. Lanes 5–7, represent pooled sera on days 37, 60, and 90, respectively, from mice immunized with CFA. Lane 8 is control, without any serum, and lane 9 is a human anti-R060 reference serum from the Center for Disease Control.
ity was not observed in adjuvant immunized mice (Fig. 9, lanes 5–7). These immune sera also reacted with additional protein bands at both high and low molecular weight ranges. Based on this result, the identity of the proteins recognized by these sera can only be speculated on. To identify other autoantigens, the sera were checked for reactivity to purified recombinant ribonucleoproteins in slot blots (Fig. 10). All sera reacted strongly with rmR o60. Interestingly, reactivity to La, SmD, and U1 RNP associated 70 kD protein was also detected. In a few mice, reactivity to DHFR was observed. However, no reactivity to R o52 and SmB, which have a 6X H is tag, was observed. Immunization of mice with a control peptide JS7A did not generate antibodies reactive with these proteins, although very weak reactivity to R o60 was observed in two of six control mice (Fig. 10).

The diversification of the autoreactivity in response to the immunization with hRo60 316–335 was further documented in our indirect immunofluorescence study. Anticytoplasmic and antinuclear Abs appeared shortly after immunization. More remarkable was the appearance of a population of Abs that stained the Golgi complex (Fig. 11); the anti-Golgi staining was not seen in HeLa cells treated with brefeldin A, which disrupts the architecture of Golgi apparatus (16). Similar results were obtained when a mouse cell line, NIH/3T3, was used as the substrate. Sera from control mice did not give similar staining.

Antibodies reactive to mR o60 epitopes not related to peptide hRo60 316–335 were generated in mice immunized with the peptide (Fig. 12). Sera were absorbed with the immunogen to deplete them of antipeptide antibodies. These sera still recognized rmR o60 in ELISA, indicative of intramolecular epitope spreading (Fig. 12 A). Absorption of sera with a control peptide JS7A had little effect on the reactivity of sera, either to the peptide or to rmR o60 (Fig. 12 A). Results presented in Figs. 8–12 were representative of three experiments.

The immune response against hRo60 441–465, which contains a dominant T cell epitope of hRo60, was markedly different than that against peptide hRo60 316–335. Immunization with peptide hRo60 441–465 generated very high titers

![Figure 9. Reactivity of pooled immune sera with WEHI 7.1 cell extracts in Western blot. Each lane was loaded with protein equivalent to 2.5 × 10⁶ cells. Pooled sera were used at a dilution of 1:100. Lane 1, human anti-La reference serum from CDC; lanes 2–4, day 37, 60, and 90 sera from mice immunized with peptide hRo60 316–335; lanes 5–7, days 37, 60, and 90 sera from mice immunized with CFA.](image)

![Figure 10. Intermolecular determinant spreading of antibody responses in SJL/J mice immunized with hRo60 peptides. Reactivity of sera from mice immunized with peptides hRo60 316–335, hRo60 441–465, and ZP3 peptide JS7A, with different ribonucleoproteins was tested in slot blots. Each lane represents an individual serum, at a dilution of 1:250.](image)

![Figure 11. Anti-Golgi staining patterns of antibodies generated by hRo60 316–335 immunization. HeLa cells grown on coverslips and fixed in methanol were used as substrate. Results are shown for pooled sera (day 60) used at a dilution of 1:200.](image)

![Figure 12. Intramolecular diversification of antibody responses against mR o60 following immunization with synthetic peptides. Pooled sera from mice immunized either with hRo60 316–335 peptide (A), or hRo60 441–465 peptide (B) were absorbed with their respective immunogens and peptide JS7A. Reactivity of unabsorbed and absorbed sera with peptides and mR o60 was determined in ELISA. Results are expressed as mean duplicate O D 490 nm. Open bars denote unabsorbed pooled sera. Cross bars denote pooled sera absorbed with peptide JS7A. The hatched bars denote pooled sera absorbed with hRo60 316–335 in (A) and with hRo60 441–465 in (B).](image)
(10^2–10^6) of antibodies reactive with the peptide in ELISA. Although these antibodies reacted with rmR o60 in ELISA (Fig. 12 B), they did not immunoprecipitate native mR o60 (data not shown). Analysis of antibody reactivities in slot blots (Fig. 10) showed reactivity with rmR o60 in all animals and reactivity to rmR o52 and SmA in only one out of five animals. No reactivity was observed to La, SmB, SmD, U1RNP associated 70 kD protein, and DHFR. Absorption of pooled sera with the immunogen abolished reactivity to the peptide and all reactivity to rmR o60 as shown in Fig. 12 B. Similar results were obtained in two additional experiments involving 10 mice.

Peptide mR o60 316–335 was a dominant T cell epitope, whereas mR o60 441–465 was a cryptic T cell epitope. The possibility that the difference observed between the immune responses to the two immunodominant T cell peptides on hR o60 might be due to lack of presentation of the autologous mR o60 441–465 was suggested from data shown in Fig. 5 A. Peptide mR o60 311–330 (overlaps hR o60 316–335) was able to recall the LNC proliferative response in mice immunized with rmR o60, whereas peptides mR o60 441–430 and mR o60 431–470 failed to do so. This was further explored. Peptides mR o60 316–335 and mR o60 441–465 were made and used to recall the LNC proliferative responses in mice immunized with rmR o60. Fig. 13 shows representative results from one of three experiments. While peptide mR o60 316–335 could recall the proliferative response, mR o60 441–465 could not, indicating that the T cell epitope in the latter peptide is cryptic.

Despite the three amino acid differences between hR o60 316–335 and mR o60 316–335, the T cell epitopes in these two peptides were cross-reactive. Peptide mR o60 316–335 was able to recall proliferative responses in mice immunized with the homologous human peptide (Fig. 14 A), and vice versa (Fig. 14 B). In both cases, the proliferative responses were recalled by the recombinant R o60 proteins, further confirming the noncryptic nature of the auto T epitope in the peptide mR o60 316–335.

LNC proliferative responses were induced by immunization with both peptides hR o60 316–335 and mR o60 316–335 (Fig. 14, C and D). However, these responses were significantly lower than those observed for the peptides, hR o60 316–335 and mR o60 316–335. At best, very weak cross-reactivity was observed between the peptides hR o60 441–465 and mR o60 441–465. Immunization of mice with the mR o60 441–465 peptide induced a weak proliferative response with SI at 2–4. In addition, rmR o60 was not able to recall this response. The weak cross-reactivity between the mouse and human peptides is likely due to the more extensive differences in their amino acid sequences.

Homologous Peptide mR o60 316–335 Induced Antibody Diversification. Immunization of mice with peptide mR o60 316–335 resulted in high titers of anti-peptide antibodies, capable of immunoprecipitating native mR o60. Reactivity to La, SmD, and U1RNP associated 70-kD protein and DHFR was observed in slot blots (Fig. 15 A) indicative of intramolecular epitope spreading. The reactivity patterns of the immune sera were similar to those of sera of mice immunized with the human peptide (Figs. 7 and 15 B). With respect to intramolecular spreading, absorption of sera with the immunogen had little effect on the reactivity to R o60 although almost all reactivity to the peptide was abolished (Fig. 15 C). Absorption with control peptide JS7A had no effect, either on the reactivity with the peptide or with the whole antigen. It is of interest to note that these immune sera did not stain the Golgi complex. Similar results were obtained in an additional experiment.

Figure 13. Recall of in vitro LNC proliferative responses by rmR o60 (open bars) and peptides mR o60 316–335 (cross bars) and mR o60 441–465 (hatched bars) in mice immunized with rmR o60. Results are expressed as mean triplicate SI. An SI > 2.0 was considered positive.

Figure 14. Cross priming between human and mouse peptides. SJL/J mice were immunized with peptides: hR o60 316–335 (A), mR o60 316–335 (B), hR o60 441–465 (C), and mR o60 441–465 (D). 2 wk later, proliferative responses were recalled with peptides hR o60 316–335 (●), mR o60 316–335 (○), hR o60 (▲), mR o60 (△), hR o60 441–465 (■), and mR o60 441–465 (▲). Results are expressed as mean triplicate SI. y-axis scales for A, B and C, D are different. Peptide concentrations are in µM and those for R o60 in µg/ml as shown in the x-axis.

Discussion

R o60 (SS-A) was initially described by Clark et al. (17) as R o and by Alspaugh and Tan (18) as SS-A. Later it was documented that R o and SS-A were identical (19). R o (SS-A) is a 60-kD protein that is thought to be associated with La through their binding to a common set of hY-RNAs (20). Autoantibodies to R o52 are commonly found...
to reexamine some of the current impressions and hypotheses regarding the genesis of SLE-related autoantibodies. Specifically, the clinical impression that SLE-related Abs react to linked sets of autoantigens, i.e., Ro60 and La, Sm and U1-RNP (snRNPs), and histones and DNA (22, 23) should be revisited. The "particle hypothesis" (24), which was postulated to explain the occurrence of autoantibodies in linked sets and which states that chromatin, snRNPs, and Ro/La complexes are available, as intact particles to the immune system, should be scrutinized.

The identification of multiple T cell determinant containing peptides in both human and mouse Ro60 allows us to explore the immunogenicity of dominant and subdominant T cell determinants of this auto antigen. R60316–335 has both T and B cell determinants. Although there are three amino acid differences between the xenogeneic and autologous peptides, both peptides have dominant T cell determinants for multiple strains of mice. Both human and mouse peptides induce a T cell proliferative response, and the intact recombinant proteins recall the induced T cell responses (Fig. 14). These peptides have cross-reactive T cell epitopes and they recall T cell responsiveness induced by the recombinant Ro60 proteins (Figs. 2 and 13). By the criteria of Sercarz et al. (25), the T cell epitopes in the xenogeneic and the autologous peptides should be considered immunodominant. In addition, these peptides induce a diverse autoimmune response to multiple autoantigens. The targets for the diversification of the autobody responses include La, SmD, 70-kD U1-RNP, and other unidentified intracellular constituents (Figs. 9–11). Although only the data on SJL/J are presented here, we found similar but not identical data in A/J and BALB/c. Thus, a dominant T cell determinant of an autoantigen need not be tolerogenic. The hierarchical self-tolerance to T cell determinants demonstrated in La (SS-B) (6) may not be generalized to other autoantigens readily.

Although human Ro60441-465 has a dominant T cell epitope for SJL (Fig. 2), the homologous mouse peptide cannot recall the anti-hRo60441-465 T cell response (Fig. 13). In addition, the autologous peptide appears to be cryptic in that it induces a weak T cell response to the immunogen and that the induced response cannot be recalled with the intact mouse Ro60 (Fig. 14). It is of interest to note that human Ro60441-465 induces only very limited autobody response to the immunizing peptides with little diversification to other autoantigens. This observation and the diverse autobody response induced by either human or mouse Ro60316–335 support the hypothesis that only immune responses to auto-T cell epitopes that can be generated by processing and presentation of the endogenous antigen lead to a diversified autobody response (6, 26). This hypothesis suggests a mechanism in which endogenous autoantigens play a significant role in the diversification of the autobody responses by relevant self-peptides and provides an explanation for the inability of certain self-peptides of SLE-related autoantigens such as Ro60441-465, certain La (6), and Sm (27) peptides to induce a diverse autobody response.

There are differences in the antibody specificities in the immune sera from mice immunized with the recombinant...
Ro60 proteins and those immunized with Ro60316–335. The immune sera of mice immunized with recombinant Ro60 are reactive with Ro52, SmA, and SmB, whereas those from mice immunized with Ro60316–335 are not reactive. Immune sera from mice immunized either with whole Ro60 protein or the peptide Ro60316–335 have Abs to native Ro60 as demonstrated by immunoprecipitation of the associated mRNAs. In addition, absorption experiments show that the immune sera from mice immunized with Ro60316–335 have Abs to determinants elsewhere in Ro60. These data are not in congruency with the particle hypothesis as discussed previously. If the particle hypothesis (24) is operative in our system, the differences in the fine specificities of the autoantibodies generated by immunizations with the intact protein and the peptide should not be significantly different. In addition, these data also suggest the presence of an antigenic peptide or peptides within the Ro60 molecule, which can induce autoantibodies reactive with Ro52, SmA, and SmB. Studies with peptides containing dominant T cell epitopes within either human or mouse Ro60 may provide data to support this thesis.

The generation of anti-Golgi Ab in mice immunized with human Ro60316–335, but not in mice immunized with human Ro60, mouse Ro60, or mouse Ro60316–335 requires some comments. The anti-Golgi Ab cannot be absorbed by mouse Ro60 or mouse Ro60316–335. In addition, human Ro60 cannot inhibit the staining. However, the human peptide blocks the staining completely. Further experiments to be presented elsewhere (28) indicate that there is a cross-reactive B cell epitope shared between the Golgi complex and the human peptide. These data indicate that the immunogen determines the specificity of the Ab generated even in the immune responses involving autoantigens.

From the above discussion, the nature of the immunogen is an important factor in determining the specificities of the autoimmune response in our system. This importance is further underscored by our early experience using synthetic peptides as the immunogen. During the early stage of our investigation, synthetic peptides were used as the immunogen without HPLC purification after they were made. These crude peptides induced Abs to Ro52 consistently, in addition to those autoantigens targeted by the immune response to the purified peptides. It is apparent that a contaminating peptide(s) was responsible for the induction of an antibody response with this specificity. This observation has led us to prepare our immunizing peptides carefully to assure the peptides are >95% in purity. In this regard, multiple antigenic peptides (MAPs) have frequently been used to induce autoantibodies and to study determinant spreading and autoimmunity (9, 29–31). In the construction of these MAPs, the purity of the peptides is not assured. More importantly, it is likely that these peptides may contain multiple T and B cell epitopes other than those specified by the amino acid sequences, the structure of which are not readily apparent. For example, recently the use of MAP of PPGP RPP, a B cell epitope of SmB/B’ as the immunogen, has led to the conclusion that B cell epitope spreading is not H-2 restricted in mice (30). Differential antigen processing and presentation of the immunogen MAP by two strains of mice sharing a similar H-2 region may result in T cell responses to different epitopes which may induce autoantibodies with differing specificities, providing an appearance of non-H2 restriction. Thus, experimental data involving MAP as the immunogen in the study of autoimmunity should be interpreted with caution.

In the present study, evidence has been presented that autoantibody diversification occurs through a process termed determinant spreading (32, reviewed in 10, 32–35). Both intramolecular and intermolecular epitope spreading occurs. Careful absorption experiments to be reported elsewhere (28) indicate that similar conformational epitopes are present in many of the SLE-related autoantigens. With peptide Ro60316–335 as the immunogen, considerable amounts of anti-Ro60 autoantibodies cannot be readily absorbed by the immunogen, indicating that intramolecular determinant spreading occurs by generating specific Ab to Ro60 determinants not present in the immunogen. In contrast, the immunogen can absorb almost completely the Ab to La, SmD, and 70 kD U1-RNP. The latter result indicates that intermolecular determinant spreading may be due to the generation of distinct populations of autoantibodies to the conformational epitopes shared in varying degrees among these autoantigens. Thus, there appears to be a qualitative difference between intramolecular determinant spreading and intermolecular spreading in our system. In addition, molecular mimicry may play an important role in the diversification of an autoimmune response in SLE.

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