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Evidence for Multiple Shared Antigenic Determinants within Ro60 and Other Lupus-Related Ribonucleoprotein Autoantigens in Human Autoimmune Responses

Rahul Pal,2* Umesh S. Deshmukh,* Yukiko Ohyama,3* Qiang Fang,† Carol C. Kannapell,* Felicia Gaskin,† and Shu Man Fu4**‡

Ab responses directed against several ribonucleoprotein (RNP) Ags are a characteristic feature of systemic lupus erythematosus (SLE). Previous work in our laboratory using mouse model systems had revealed that both epitope spreading and inherent cross-reactivity between ribonucleoproteins contributes to the observed multiple specificities in autoimmune sera. We have now extended these studies to human autoimmune responses. Using purified polyclonal and mAbs derived from SLE patients, cross-reactivity between Ro60 and SmD was demonstrated. The cross-reactive epitope was mapped to nonhomologous regions on Ro60s1.50 and SmD88–102. Five mAbs specifically recognized apoptotic cells, demonstrated variable levels of cross-reactivity toward other nonhomologous ribonucleoprotein targets and bound multiple, nonoverlapping and nonhomologous epitopes on Ro60. Our study demonstrates that cross-reactivity between frequently targeted autoantigens is an important aspect of human systemic autoimmune responses. The presence of multiple cross-reactive epitopes on Ro60 might be important for the generation of anti-Ro60 Ab in SLE patients and in normal individuals displaying no evidence of clinical disease. The Journal of Immunology, 2005, 175: 7669–7677.

Autoantibodies reactive with several self-Ags are a common feature in systemic lupus erythematosus (SLE). Immune responses to ribonucleoproteins (RNPs) such as Ro60/La and SmD, SmB/B′, and 70-kDa U1RNP of the small nuclear RNP particle are characteristic and are considered a diagnostic criteria as stipulated by the American College of Rheumatology (1–3). Abs to some of these Ags have been shown to appear far in advance of disease diagnosis and end-organ damage, enhancing the probability of their etiological involvement in pathology (4). These and other studies using sequential sera from human SLE patients as well as from either spontaneous or induced models of lupus consistently demonstrate an increasing diversification of autoantibody specificities with the passage of time (5–9). The “particle hypothesis” postulates the phenomenon of epitope spreading, wherein a focused response against one or a few autoepitopes diversifies initially to other epitopes on the same molecule and then to epitopes on molecules that are in physical association. However, several unexpected patterns of apparent diversification have been reported, indicating the need to revisit the issue of autoantibody diversification (10, 11). Previous work in our lab using murine models of lupus made it evident that, while epitope spreading was an important and widespread phenomenon in the diversification of the immune response, there also existed an extensive and unappreciated level of cross-reactivity between several seemingly nonhomologous RNP autoantigens (12–14). Cross-reactivity between Ro60 and SmD was particularly striking because it linked immune responses to proteins within two different antigenic systems. This study was designed to determine whether such cross-reactive Ab responses are present in SLE patients. Using polyclonal and mAbs from SLE patients, we demonstrate that cross-reactive Ab responses to Ro60 and SmD are evoked in SLE patients. The epitopes recognized by the cross-reactive Abs do not share sequence homology with each other. In addition, the Ro60 molecule harbors multiple nonhomologous cross-reactive epitopes within itself.

Materials and Methods

Cloning and expression of recombinant proteins, fragments of Ro60 and Der P2, and peptide synthesis

The cDNA encoding human Ro60, 70-kDa U1-RNP (gifts from J. Keene, Duke University, Durham, NC), and U1-RNP A protein, SmB and SmD (gifts from J. Craft, Yale University, New Haven, CT), were either expressed directly or first cloned into appropriate expression vectors and purified as described previously (12). Human Ro52 (a gift from E. Tan, The Scripps Research Institute, La Jolla, CA) was cloned into the pQE-70 expression vector (Qiagen) and expressed and purified essentially as described for Ro60 (12). Human La (from J. Keene) was expressed and purified as described previously (15). Der P2 was used as a recombinant protein negative control in these studies. The cDNA was a gift from A. Smith (University of Virginia, Charlottesville, VA). It was cloned by PCR into pQE30 vector (Qiagen) and expressed and purified essentially as described previously (12). Seventeen subfragments covering the entire coding region of human Ro60 (50 mer with an overlap of 20 aa) were generated

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‡ Abbreviations used in this paper: SLE, systemic lupus erythematosus; RNP, ribonucleoprotein; CDR, complementarity determining region; A-RNP, μ1 RNA-associated A protein.

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by PCR and directionally cloned in the 5′ BamHI, 3′ EcoRI restriction sites of the GST fusion vector pGEX-5X. Recombinant subfragments were purified by affinity chromatography as per the manufacturer’s instructions. Some of the fragments were further purified by preparative gel electrophoresis using the Bio-Rad Prep Cell Model 491. Thirty-one overlapping peptides (20 or 25 mer, with an overlap of 5 aa) covering the entire Ro60 sequence were synthesized using F-moc chemistry on an automated peptide synthesizer (AMS-422; Gilson). Peptides were analyzed and purified on a reverse phase HPLC column. Thirty-four overlapping peptides (15 mer, with an overlap of 12 aa) covering the entire SmD sequence were synthesized at the Biomolecular Research Facility at the University of Virginia. All peptides were obtained at >95% purity.

**ELISAs**

Direct binding ELISAs were conducted to determine the autoantigen specificity of the Abs. Recombinant human RNP s (U1-RNP A, SmB, SmD, Ro60, Ro52; L.a., U1-RNP 70 kDa) and Ro60 fragments (1 µg/well) were absorbed onto Immulon-2 (Dynatech) ELISA plates in 50 mM sodium carbonate-bicarbonate buffer (pH 9.6) by incubation for 18 h at 4°C. Der P2 was used as a negative control. All subsequent incubations were for 2 h at room temperature. After two washes with 50 mM PBS (pH 7.4), containing 0.05% Tween 20 (PBST), nonspecific binding sites were saturated by incubation with PBS containing 2% BSA. Plates were washed with PBST, and Abs (diluted to a concentration of 5 µg/well) and 0.1% Tween 20 and 3% BSA) were added and incubated. After extensive washings, an appropriately diluted goat anti-human Ig-alkaline phosphatase conjugate (Southern Biotechnology Associates) was added and another incubation was conducted. The enzymatic reaction was developed using p-nitrophenyl phosphate. Optical densities were determined at 405 nm. A similar protocol was followed for ELISAs with the Ro60 and SmD somytic peptides, except that the coating concentration was 2 µg coating/well, and the plates used were Immulon-4.

Ab specificities were further confirmed by absorption assays. Abs (1 µg/ml) reactive as well as nonreactive toward SmD were incubated with SmD-coupled Sepharose CL-4B beads for 2 h on an end-to-end shaker. Beads were separated using Spin-X centrifuge tube filters (Costar), and supernatants were analyzed by ELISA as described above.

Cross-reactivity studies were conducted to determine cross-reactivity. Diluted Abs were preincubated with varying concentrations of reactive peptide (from 0.5 to 50 µg/ml) for 2 h at room temperature. Reactivity to other peptides and proteins was then analyzed as described above. Nonreactive peptides were used as negative controls.

**EBV transformation, human mAb production, cloning, and sequencing of VL and VH genes**

All human studies were reviewed and approved by the University of Virginia, Human Investigation Committee. PBMCs were isolated from the blood of an SLE patient (P1) by Ficoll-Hypaque density gradient centrifugation as described previously (16). Isolated cells were washed three times with 50 mM PBS (pH 7.4) and resuspended to a concentration of 6 × 10^7 cells/ml in RPMI 1640 containing 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate. One day before the experiment, cells were fractionated by Ficoll-Hypaque centrifugation and cultured at 5 × 10^4 cells/ml. Apoptosis was induced by an 8-h (“early apoptosis”) or 24-h (“late apoptosis”) incubation in the presence of 2.5 µM camptothecin (Sigma-Aldrich). A total of 5 × 10^5 control or drug-treated cells was transferred to 24-well plates (BD Biosciences) at 1000 cells/well. A total of 10^5 PBMCs was added as a source of EBV. Growing cells were transferred to 24-well plates and then to 25-cm² flasks. Cells were then replicated at −153°C for future use, and supernatants were analyzed for anti-Ro60 reactivity by an ELISA as described above. Cells secreting Abs to Ro60 were further assayed for reactivity toward SmD and Ro60.

**Results**

**Cross-reactivity of anti-Ro60 and anti-SmD autoantibody responses in SLE subjects**

Total IgG and IgM Abs were purified from the sera of two SLE subjects (indicated as P1 and P2 in Fig. 1). ELISA was conducted to ascertain reactivity toward recombinant Ro60 and SmD. Both IgM and IgG fractions from these two patients reacted well with recombinant Ro60 and SmD. Absorption studies were conducted mAbs were assayed for reactivity on HeLa cells as previously described using methanol fixation (20). The bound Ab was detected with our goat anti-human F(ab’)_2 rhodamine conjugate.

**E6.1** cells (a human T cell line) were grown in RPMI 1640 containing 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate. One day before the experiment, cells were fractionated by Ficoll-Hypaque centrifugation and cultured at 5 × 10^4 cells/ml. Apoptosis was induced by an 8-h (“early apoptosis”) or 24-h (“late apoptosis”) incubation in the presence of 2.5 µM camptothecin (Sigma-Aldrich). A total of 5 × 10^5 control or drug-treated cells was suspended in FACS buffer (PBS containing 1% BSA and 0.2% sodium azide) and incubated with the mAbs for 30 min at 4°C on a plate shaker. After three washes in FACS buffer, appropriately diluted goat anti-human IgM-PE (Southern Biotechnology Associates) was added, and a further incubation for 30 min was conducted. Cells were washed twice with FACS buffer and once with PBS. They were resuspended in PBS and analyzed using a fluorescence microscope.

**Immunofluorescence**

Cross-reactivity of anti-Ro60 and anti-SmD autoantibody responses in SLE subjects. IgG and IgM Abs purified from the sera of two SLE subjects (P1 and P2) were either unabsorbed ( ), absorbed with blank beads ( ), or absorbed with SmD-coated beads ( ). ELISAs were then conducted to ascertain reactivity toward SmD and Ro60.

**FIGURE 1.** Cross-reactivity of anti-Ro60 and anti-SmD autoantibody responses in SLE subjects. IgG and IgM Abs purified from the sera of two SLE subjects (P1 and P2) were either unabsorbed ( ), absorbed with blank beads ( ), or absorbed with SmD-coated beads ( ). ELISAs were then conducted to ascertain reactivity toward SmD and Ro60.
using Sepharose beads coupled with recombinant SmD. SmD absorption not only reduced IgM reactivity toward SmD but also significantly diminished reactivity toward Ro60 (by 63 and 60% in P1 and P2, respectively). Reduction in IgG anti-Ro60 activity was more modest (29 and 4%, respectively). Thus, there appears to be a population of Abs that demonstrate cross-reactive recognition of these lupus-related Ags.

**Reactivity of IgG and IgM Abs purified from sera toward synthetic peptides from Ro60**

Fig. 2 show the reactivity pattern of IgG and IgM Abs purified from the two SLE patients toward synthetic overlapping peptides representing the Ro60 sequence. Reactivity to whole Ro60 is also shown. Although many common peptides were recognized by IgM and IgG, differences in reactivity and specificity were also observed. For example, IgG from P2 preferentially bound Ro60_{221–245} and IgG from both subjects exhibited superior recognition of Ro60_{456–475} compared with their respective IgM.

It is evident that the IgM factions from both patients reacted well with Ro60_{101–125} and Ro60_{421–445}. This parallel in the reactivity pattern was observed in subsequent serial samples from these two patients and in other patients as well (data not shown). Competition studies were conducted to ascertain whether such persistent dominance was indicative of the presence of cross-reactive B cell epitopes in these two peptides; results are shown in Fig. 3. For both SLE patients P1 and P2, absorption with the nonreactive control peptide Ro60_{1–25} did not lead to significant decreases in reactivity toward Ro60_{101–125}, Ro60_{361–385}, Ro60_{381–405}, and Ro60_{421–445}. In contrast, absorption with beads coupled with Ro60_{421–445} decreased reactivity of IgM from both patients toward itself and Ro60_{101–125} while not affecting reactivity toward Ro60_{361–385} and Ro60_{381–405}. These results suggest that Ro60 harbors cross-reactive epitopes within itself.

**FIGURE 2.** Reactivity of human polyclonal IgG and IgM Abs toward synthetic overlapping peptides representing the entire Ro60 molecule. Reactivity to recombinant Ro60 is also shown. Many common peptides were recognized by the two isotypes but variations in reactivity and specificity were also observed.

**FIGURE 3.** Demonstration of peptidic cross-reactivity using IgM Abs purified from the sera of patients P1 and P2. Abs were absorbed with beads coated with control ( ), nonreactive peptide Ro60_{1–25} ( ), or with the reactive peptide Ro60_{421–445} ( ), and reactivities against Ro60_{101–125}, Ro60_{361–385}, Ro60_{381–405}, Ro60_{421–445} and Ro60 were analyzed.
To further explore the antigenic structures within Ro60 and among the lupus-related RNP autoantigens, mAbs to Ro60 were generated. Four hundred ninety-three EBV-transformed cell lines were generated from the peripheral blood of a patient (P1) with SLE. Supernatants were screened for reactivity against recombinant Ro60 by ELISA. Cells secreting such Abs were fused with the heteromyeloma K6H6/B5. After four successive subcloning procedures, six hybridoma cell lines (P1M48, P1M49, P1M79, P1M169, P1M256, and P1M398) secreting Abs reactive toward Ro60 were generated from the peripheral blood of a patient (P1) with SLE. Six hybridoma cell lines (P1M48, P1M49, P1M79, P1M169, P1M256, and P1M398) secreting Abs reactive toward Ro60 were established. All Abs were of the IgM isotype and used the λ L chain (GenBank accession nos. AY190818–AY190829 (www.ncbi.nlm.nih.gov/entrez/)). Table I summarizes the characteristics of these Abs. Four Abs used the V\_J family, with the closest germlines genes used being V3–48, DP-49, and DP-42. The other two Abs used V\_J (DP-27) and V\_J (V5–51). Four D regions were used (D3–10, D4–17, D6–19, and D6–19), and two JH regions were used (JH4b and JH6b). The six L chain sequences appeared to use two VA families and bore closest homology with five separate germline genes (HUMIGLVZG, V1–22, V2–1, V2–7, and V2–14); two JL regions were used (JL2/JL3a and JL3b). Among the 25 charged amino acids in the CDR3 regions of the six mAbs, we detected a replacement mutation in the CDR3 of P1M79 seven mutations were detected with one of them being a replacement mutation in the complementarity determining regions (CDRs) (Table I). The CDR3 lengths of the H chains were also analyzed (Table II). They were 15, 11, 10, 12, 11, and 13 aa in length for P1M48, P1M49, P1M79, P1M169, P1M256, and P1M398, respectively. There were only four positively charged residues in the six H chain CDR3 regions with R in P1M79 seven mutations were detected with one of them being a replacement mutation in the complementarity determining regions (CDRs) (Table I). The CDR3 lengths of the H chains were also analyzed (Table II). They were 15, 11, 10, 12, 11, and 13 aa in length for P1M48, P1M49, P1M79, P1M169, P1M256, and P1M398, respectively. There were only four positively charged residues in the six H chain CDR3 regions with R in P1M79, R in P1M256, and H and K in P1M398. These data further reinforce the recent finding that the lengths and charges of the H chain CDR3 are not characteristic of polyreactive autoantibodies (21).

**Human anti-Ro60 mAbs**

To determine the reactivities of the monoclonal anti-Ro60 Abs, two approaches were taken. In the first approach, a panel of overlapping 25mer peptides, representing the entire Ro60 molecule was used in ELISA (Fig. 4). Five of the six mAbs reacted with multiple peptides, whereas P1M398 reacted predominantly with a single peptide Ro60\_101–125. The antigenic determinants for all five multireactive Abs appeared to be clustered in five regions, as exemplified by the reactivity of P1M49. Keenly aware of the possibility that the peptides may not represent some conformational epitopes present in the whole Ro60 molecule, the second approach was taken. Recombinant Ro60 fragments (50 mer in length with an overlap of 20 aa), spanning the entire molecule, were generated as GST fusion proteins. These fragments were used as the substrates in ELISA (Fig. 5). With the exception of P1M398, the reactive patterns were very similar to those obtained upon synthetic peptides in that these mAbs were reactive with multiple fragments and corresponding regions representing the Ro60 molecule appeared to be recognized. P1M398 was nonreactive with any of the fragments, suggesting that the epitope in Ro60\_101–125 was not accessible in the fragments. Nevertheless, studies with both serum IgM as well as with human mAbs derived from SLE patients revealed that multiple B cell epitopes were present upon the Ro60 molecule. The multireactivity of the mAbs was further explored by absorption experiments. As shown in Fig. 6, absorption of the Ab P1M169 with peptide Ro60\_121–145 markedly diminished its reactivity to this peptide as well as to peptides Ro60\_316–335 and Ro60\_361–385, in addition to reducing reactivity to Ro60. Incubation with a nonreactive peptide (Ro60\_1–25) at the same concentration did not reduce Ab reactivity toward Ro60 or toward the three peptides of interest. Similar results were obtained in absorption experiments involving the four other mAbs which react with multiple peptides.

**Shared B cell epitopes present on lupus-related RNPs**

Although the mAbs were identified by their reactivity to recombinant Ro60, their reactivity toward other lupus related autoantigens was also investigated. μ RNA-associated A protein (A-RNP) and U1RNP70 proteins, SmB, SmD, Ro52, and La, were used as additional substrates. Recombinant Der P2, a mite allergen, was tested for reactivity. The multireactivity of the mAbs was further explored by absorption experiments involving the four other mAbs which react with multiple peptides.

**Table I. H and L chain variable region gene segments used by the six human Ro60–reactive mAbs**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Family Locus</th>
<th>Closest Germline</th>
<th>CDR3</th>
<th>FR</th>
<th>Family</th>
<th>Closest Germline</th>
<th>JH</th>
<th>FR</th>
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<tbody>
<tr>
<td>P1M48</td>
<td>VH3–48</td>
<td>V3–48</td>
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<tr>
<td>P1M49</td>
<td>VH3–30</td>
<td>DP–49</td>
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<tr>
<td>P1M79</td>
<td>VH3–53</td>
<td>DP–42</td>
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<tr>
<td>P1M169</td>
<td>VH3–30</td>
<td>DP–49</td>
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<tr>
<td>P1M256</td>
<td>VH2–70</td>
<td>DP–27</td>
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<tr>
<td>P1M398</td>
<td>VH5–51</td>
<td>V5–51</td>
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</table>

Table II. *CDR3 and JH encoded amino acids usage by the Abs*

<table>
<thead>
<tr>
<th>Clone</th>
<th>Amino Acid Sequence</th>
<th>CDR3</th>
<th>JH</th>
<th>CDR3 Length</th>
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<tr>
<td>P1M48</td>
<td>VPNYGYGFPRD</td>
<td>GMDV</td>
<td>WQGQTTVTSS</td>
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<tr>
<td>P1M49</td>
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<td>YGMDV</td>
<td>WQGQTTVTSS</td>
<td>11</td>
</tr>
<tr>
<td>P1M79</td>
<td>VGIAGAG</td>
<td>FDY</td>
<td>WQGQTTLTVSS</td>
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<td>P1M169</td>
<td>GPWGDSL</td>
<td>YFDY</td>
<td>WQGQTTLTVSS</td>
<td>12</td>
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<tr>
<td>P1M256</td>
<td>IDYSGETGL</td>
<td>DY</td>
<td>WQGQTTLTVSS</td>
<td>11</td>
</tr>
<tr>
<td>P1M398</td>
<td>LPHSSGGMGL</td>
<td>DY</td>
<td>WQGQTTLTVSS</td>
<td>13</td>
</tr>
</tbody>
</table>

a Positively charged amino acids in the CDR3 regions are underlined.
was used as a control. The results of these experiments are shown in Fig. 7. With the exception of P1M398, all the mAbs reacted with more than one recombinant protein. A similar trend was observed when reactivity of these Abs was analyzed by Western blotting, using HeLa cell extracts as substrates (data not shown).

For additional evidence that these SLE-related autoantigens share multiple epitopes, absorption experiments were conducted. A representative experiment is summarized in Table III. Absorption with SmD beads simultaneously reduced the reactivity of Ab P1M79 toward SmD as well as toward other six recombinant proteins, while the control beads had no effect. Because P1M48 was nonreactive toward SmD, it was used as an additional index of specificity; the reactivity of the Ab toward Ro60 and Ro52 was not affected upon absorption with SmD beads. To explore further the basis of cross-reactivity between Ro60 and SmD, competitive inhibition assays were conducted. Upon a panel of overlapping peptides representing SmD, multiple peptides at the C terminus of SmD were shown to be reactive with the Ab, with SmD88-102 being the most reactive (Fig. 8A). As shown above, P1M79 reacted best with Ro60481-505 on Ro60 (Fig. 4). Two experiments revealed cross-reactivity: first, Ro60481-505 inhibited the binding of P1M79 to Ro60 as well as to SmD in a dose-dependent manner (Fig. 8B). Second, at the peptide level, Ro60481-505 inhibited the binding of P1M79 to itself as well to SmD88-102, although the inhibition was more efficient for the Ro60 peptide (Fig. 8C). Although not shown, similar results were obtained with P1M49 and P1M169, both of

FIGURE 5. Elucidation of the intramolecular specificity of Ro60-reactive human mAbs. The reactivity of six human mAbs (P1M48, P1M49, P1M79, P1M169, P1M256, and P1M398) was assessed against a panel of recombinant subfragments of Ro60. Reactivity to the entire molecule is also shown for comparison. P1M398 failed to recognize any fragment.
which are also reactive with SmD. Thus, cross-reactivity between Ro60481–505 and SmD88 –102 may be at least partially responsible for the shared recognition of Ro60 and SmD by these mAbs.

Differential staining of HeLa cells and preferential reactivity with apoptotic cells by anti-Ro60 mAb

Five of the six mAbs were reactive toward HeLa cells by indirect immunofluorescence (Fig. 9). Once again, each Ab demonstrated an unique staining pattern. Both P1M48 and P1M49 (Fig. 9, A and B) stained the cytoplasm, with P1M49 additionally staining strands (or “threads”) overlaying the nuclei. P1M79 (Fig. 9C) showed staining over both the nucleus and the cytoplasm. P1M169 (Fig. 9D) showed an accentuated perinuclear reaction compared with the cytoplasm, while P1M256 (Fig. 9E) predominantly stained just the perinuclear region. No staining was detected with P1M398 (Fig. 9F).

Several of the Ags recognized by these Abs are packaged into bleb-like structures as cells undergo apoptosis (22). To obtain additional evidence of the recognition of native Ags/epitopes by the Abs, FACS analysis was conducted on healthy and apoptotic E6.1 cells. The mAbs did not bind to the plasma membrane of healthy cells. However, they did react with cells undergoing apoptosis; Fig. 10 shows results obtained with P1M169. Upon cells treated for 8 h with camptothecin (“early apoptosis”), Annexin VFITC uniformly bound the entire plasma membrane, whereas the Ab only bound at discreet sites, corresponding to putative blebs (seen as red in the figure). Upon cells at a late stage of apoptosis/secondary necrosis (“late apoptosis”), cytoplasmic staining was also apparent as cells became permeable, as expected. Similar data were obtained with the other four mAbs. These results were confirmed by FACS analysis (data not shown).

Discussion

Systemic lupus erythematosus is characterized by the presence of autoantibodies to several protein Ags and to dsDNA (1, 2, 23). Ab titers to many components arise ahead of pathological sequelae (4), exhibit increased complexity with the passage of time, and display disease-related fluctuations. Epitope (or determinant) spreading has been investigated as a possible mechanism by which this diversity may arise. There are several reports of intra- and intermolecular diversification of the immune response upon immunization of experimental animals with Ags implicated in SLE (6–9, 12–14). Although the extent and consequences of such diversification have been a subject of some debate (24, 25), it has been theorized that increasing complexity in autoimmune responses could result in additional pathology.

The “particle hypothesis” has been proposed to explain the phenomena of epitope spreading, which states that the breaking of tolerance to one protein leads to the generation of immune responses to other proteins that associate with it to form a multimeric

FIGURE 6. Absorption studies with human mAb P1M169. The Ab was incubated with buffer alone ( ), with nonreactive peptide Ro601–25 ( ), or with reactive peptide Ro60121–145 ( ), and reactivity was assessed against reactive peptides Ro60121–145, Ro60316–335, and Ro60361–385 and against Ro60.

FIGURE 7. Reactivity of human monoclonal Ro60-reactive Abs against other RNP autoantigens; Der P2 was used as a negative control.
complex. Although physical association may be required for autoantibodies of different specificity to develop, it is becoming more apparent that the hypothesis cannot fully account for all autoimmune phenomena. For instance, although no physical association of Ro52 and the Ro60/La complex has been convincingly demonstrated, responses to Ro52, Ro60, and La appear to be linked (10, 11). Our lab has made some observations in this regard, which also indicate that additional mechanisms may contribute toward Ab diversification (12, 13). Immunization of inbred strains of mice with human and mouse Ro60, as well as with selected peptides derived from these autoantigens, elicited cross-reactive IgG Ab responses to La, SmD, and the U1 RNP 70-kDa protein (12). Critically, similar cross-reactive Abs were observed in spontaneously autoimmune NZB/NZW F1 mice, ruling out the possibility that the observations were an artifact of immunization (13). Subsequent studies revealed that cross-reactive IgG Ab responses to polypeptides within the small nuclear RNP complex can be readily induced in mice immunized with SmD, SmB, and A-RNP (14). These results demonstrate that some of the Ab diversity seen in lupus sera can be contributed by cross-reactivity.

The results reported in the present study add crucial evidence to this debate. Both the polyclonal and mAbs used here were obtained from humans, adding relevance to human disease; additionally, monoclonals by their very nature introduce an increased stringency, thus making the observations of multireactivity more reliable. Though all monoclonals were of the IgM isotype, none of them (with the possible exception of P1M79) exhibited across-the-board, polyspecific binding; distinctly variable specificities were apparent in ELISAs upon recombinant proteins in Western blots and in immunofluorescence assays. Cross-reactivity of human mAbs to multiple proteinic and nonproteinic Ags has been documented previously. Although more data exist for Abs of the IgM isotype in this regard (26, 27), monoclonal IgG Abs reactive to multiple ribonucleoprotein autoantigens have also been reported (28), further discounting the possibility that such interactions are nonspecific and the result of ill-defined “stickiness” often ascribed to the IgM isotype.

To our knowledge, in previous instances of documented multireactivity of human mAbs, epitope analysis has not been conducted. In this study, the use of Ro60 recombinant fragments as

<table>
<thead>
<tr>
<th>Recombinant Proteins</th>
<th>P1M48 Unabsorbed</th>
<th>P1M48 Control absorbed</th>
<th>P1M48 SmD absorbed</th>
<th>P1M79 Unabsorbed</th>
<th>P1M79 Control absorbed</th>
<th>P1M79 SmD absorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ro60</td>
<td>0.46</td>
<td>0.37</td>
<td>0.44</td>
<td>1.10</td>
<td>0.96</td>
<td>0.02</td>
</tr>
<tr>
<td>SmD</td>
<td>0.01</td>
<td>0.10</td>
<td>0.10</td>
<td>0.96</td>
<td>0.84</td>
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<td>0.60</td>
<td>0.59</td>
<td>0.65</td>
<td>0.49</td>
<td>0.08</td>
</tr>
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<td>1.05</td>
<td>0.92</td>
<td>0.03</td>
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<td>0.10</td>
<td>0.21</td>
<td>0.16</td>
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<tr>
<td>SmB</td>
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<td>0.10</td>
<td>0.18</td>
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<td>0.01</td>
<td>0.56</td>
<td>0.44</td>
<td>0.01</td>
</tr>
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</table>

*P1M48 and P1M79 were incubated with either control beads or SmD-coupled Sepharose beads, and reactivities against seven recombinant proteins were determined by ELISA. Data are representative of two independent experiments and are shown as mean duplicate OD at 490 nm.
Studies using polyclonal anti-Ro60 Abs isolated from the sera of SLE patients have previously localized Ro60 to the nucleus (29, 30), the cytoplasm (31), or to both regions (32, 33). In this regard, patterns of localization we obtained deserve some mention, in the context of the differential antigenic recognition of the mAbs. P1M79, which appeared to be the most polyreactive Ab, demonstrated the most complex staining pattern. A second case in point is P1M49, which too was relatively more polyspecific, as determined both by Western blots as well by specific ELISA; its localization pattern was also observed to be more intricate with a “thread”-like pattern, as observed by other investigators as well (30). P1M256, which, in contrast, predominantly recognized Ro60 and Ro52, uniquely stained just the perinuclear region. We speculate that the extent of intermolecular cross-reactivity determines Ab localization.

The analysis of variable region genes encoding Abs of autoantigenic specificity has been the focus of investigation. Most previous work has focused on the study of anti-dsDNA Abs (34–37). In almost all instances, somatic mutations were preferentially localized within the CDRs, suggestive of Ag-driven selection. Mutations toward asparagine, arginine, and glutamine predominate at certain residues (35, 36). These amino acids have been shown to be present in DNA binding proteins such as transcription factors (38). Genetic analysis of autoantibodies reactive toward ribonucleoproteins has been more limited. Although some reports indicate that germline-encoded Abs are capable of binding the Sm Ag (39–42), there is also indication that Abs of this reactivity may be somatically mutated (43).

We propose that the observed reactivity patterns reflect the recognition of conformational epitopes by endogenously generated Abs, according to the following scenario. Anti-Ro60 T cells are activated via as yet undefined mechanisms. B cells expressing surface receptor specificities against tertiary epitopes of Ro60 (which have escaped deletion and can be activated upon appropriate stimulation) internalize and process the molecule that may become available as cells undergo apoptosis (22). Subsequently, these B cells receive help from the activated Ro60-specific T cells. These cells then secrete Abs that are capable of binding tertiary epitopes reflected by their ability to bind discrete Ro60 peptides. Preliminary data that inferred the existence of conformational epitopes on Ro60 has also been obtained using polyclonal Abs and recombinant fragments (44) and synthetic peptides (45). It is clear that, because mAbs are capable of such reactivity, data regarding increasing intra- and intermolecular diversification of the Ab response during disease progression (or indeed during immunization in experimental animals) would need to be analyzed more carefully.

Further experimentation is needed to ascertain if human anti-Ro60 Ab responses constitute a special case, or if the observations reported here are true for other autoreactive Abs as well. If the former were true, it might explain why anti-Ro60 reactivity is frequently seen in healthy individuals.

Disclosures
The authors have no financial conflict of interest.