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Molecular Cloning of ANTI-SS-A/Ro 60-kDa Peptide Fab Fragments from Infiltrating Salivary Gland Lymphocytes of a Patient with Sjögren's Syndrome

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Anti-SS-A/Ro antibodies are commonly found in systemic autoimmune diseases such as Sjögren's syndrome and systemic lupus erythematosus, and some of these antibodies appear to be responsible for certain pathological lesions including congenital heart block in neonatal lupus. In this study, we generated three human antibody Fab fragments that specifically bind to SS-A/Ro 60-kd peptide from salivary gland lymphocytes of a patient with Sjögren's syndrome by using a phage-display technique. Sequence analysis demonstrated that two of the three Fab clones (E-42 and E-60) used homologous heavy chains derived from the germline VH gene DP73 in combination with different light chains which were derived from germline Vκ gene L6 and Vλ gene DPL23. The third Fab clone (E-56) used another heavy chain derived from the germline VH gene DP31 in combination with the identical light chain as that of E-42. All three Fab clones revealed a high number of somatic mutations that likely occurred in the context of antigen selection. These findings suggest the restricted usage of VH and VL genes of anti-SS-A/Ro antibodies in salivary gland lymphocytes of the patient.

Anti-SS-A/Ro antibodies are often observed in systemic autoimmune diseases such as Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE). With respect to the structure of the SS-A/Ro antigens, it has been reported that a 60-kd peptide that binds to Y RNAs inside eukaryotic cells and a recently recognized 52-kd peptide are antigenic [1,2]. Approximately half of anti-Ro positive sera recognize both the 60-kd and the 52-kd peptides and the other half recognize one of the two peptides [3].

Pathophysiological roles of anti-SS-A/Ro antibodies are obscure. However, some of these antibodies appear to be responsible for a congenital heart block in neonates from mother who have autoantibodies to Ro or to both Ro and SS-B/La, and for skin lesions in subacute cutaneous lupus erythematosus [4,5]. Information on the genes coding for anti-SS-A/Ro antibodies, as well as knowledge of their epitopes, would be important in understanding the pathogenesis of the antibody-mediated diseases.

Studies on the human autoantibody genes have been greatly hampered by the technical difficulties of establishing stable human monoclonal B cell lines. In most cases, only low-affinity IgM autoantibodies not representative of pathogenic IgG autoantibodies were obtained with EB virus transformation techniques. Antibody phage-display technique offers a valuable tool for generating human variable (V) domain antibody fragments recognizing self antigens from various lymphoid tissues containing these autoantibody-producing cells [6-8]. Increase of anti-SS-A antibody producing cells in salivary gland lymphocytes compared with peripheral blood lymphocytes (PBL) in SS, and ectopic expression of the autoantignes in SS salivary glands have been recently reported [9,10]. We report the molecular cloning of the genes for IgG class human antibody Fab fragments against SS-A/Ro 60-kd peptide from salivary-gland infiltrating B cells by the phage-display technique.

MATERIALS AND METHODS

Preparation of RNA. Total cellular RNA was extracted from minor (labial) salivary gland biopsies, obtained from three patients with SS (E.S., K.N. and S.T.) who had high titer of anti-SS-A/Ro antibodies in their sera. Diagnosis of SS was confirmed by histology of the

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biopsy samples and the presence of sicca complex. All three patients revealed positive for rheumatoid factor and anti-nuclear antibodies. The two patients, E.S. and S.T., were also positive for anti-SS-B/La antibodies. Antibodies against DNA, snRNP and Sm were all negative in these patients. They received no immunosuppressive drugs including glucocorticoids when biopsy was performed.

Library construction. RNA (10 μg) was reverse transcribed to single-stranded DNA using 0.05 μg oligo(dT) primer with 10 mM DTT, RNasin, 100 mM nucleotides (25 mM each), reverse trasncriptase buffer and 2 μl (25 U) AMV reverse transcriptase (Life Science, INC., St. Petersburg, FL) in 25 μl. The reaction mixture was incubated at 41°C for 60 min. Following treatment at 90°C for 5 min and on ice for 5 min, the cDNA was PCR amplified using family-specific variable region and isotype-specific constant region primers [11] to create Fd γ (IgG) DNA fragments and light chains (κ and λ chain) DNA fragments. PCR was performed with Perkin-Elmer 480 thermal cycler using 30 cycles of 94°C for 60 s, 52°C for 60 s, and 72°C for 90 s. Amplification was completed with a single cycle of 72°C for 10 min.

Heavy chain and light chain DNA fragments were separately pooled, electrophoresed on 2% agarose NA (Pharmacia), and then purified by electro-elution (Schleicher & Schuell Biotrap, Dassel, Germany). The purified heavy chain DNA was digested with *XhoI* and *SpeI*, and the light chain DNA with *XbaI* and *SacI*. Digested products were again gel purified and 0.45 μg of light chain libraries were ligated into 1.4 μg of digested phagemid DNA pComb3-H-SS (kindly provided by Dr. Carlos Barbas III, Scripps Research Institute, La Jolla, CA) [12] and DNA was precipitated. After washing of DNA with 70% ethanol, DNA was suspended in 15 μl of distilled water and electroporated into competent XL1 Blue cells (Stratagene, La Jolla, CA) with E. coli pulser (Bio-Rad, California) as described before [12]. κ, λ-pComb3-H-SS DNA was isolated (Qiagen, Chatsworth, CA) and digested with *XhoI* and *SpeI*, and 1.4 μg was ligated with 0.45 μg of digested heavy chain DNA. phagemid DNA was again electroporated into XL1 Blue, infected with 10^{12} VCM-M13 helper phages (Stratagene), and cultured overnight with SB medium containing 50 μg/ml carbenicillin and 70 μg/ml kanamycin. Fab-phages were precipitated with polyethylene glycol (M.W. 6000), centrifuged and suspended in 2 ml of Tris- NaCl buffer pH 7.5 (TBS).

Panning. Panning was performed as described before with modifications [12]. Recombinant 60-kd SS-A/Ro protein, kindly provided by MBL Lab. (Gifu, Japan), was prepared by expressing a cDNA for the 60-kd Ro in *E. coli* with a pUC expression vector, followed by further purification as previously described [13]. The recombinant 60-kd Ro protein possesses 16 amino acid residues at the amino terminal derived from a linker DNA. Purity of this material is at least 99%, judged by SDS-PAGE. The recombinant 60-kd Ro protein was coated on wells of a 96-well ELISA plate. The SS-A/Ro protein-coated plate was blocked for 2 h with 3% BSA (first 2 cycles of panning) or 5% skim milk (Difco Laboratories, Detroit, MI) (last 2 cycles of panning) in TBS. After removal of blocking solution, Fab-phages (250 μl) were added into wells and incubated for 2 h at 37°C. After washing, acid elution, and neutralization, phages were amplified by reinfecting XL1 Blue cells. Panning was repeated for total 4 times.

Preparation of soluble Fab. Phagemid DNA was isolated from the selected library and reengineered for soluble Fab expression [12]. Briefly, isolated phagemid DNA was digested with *SpeI* and *NheI* to remove the coat protein III gene segment, self-ligated and electroporated into XL1 Blue cells. Single colonies were picked up and cultured in 10 ml of SB medium containing 50 μg/ml carbenicillin, 10 μg/ml tetracycline, and 20 mM $MgCl_2$. After 6 h, bacteria cultures were induced with 1 mM IPTG and grown overnight at 30°C. Bacteria pellets were lysed by freezing and sawing, and centrifuged. The concentration of Fab molecules in the supernatants was determined and their binding to 60-kd Ro was examined by ELISA.

As a control, we developed a recombinant Fab clone essentially as described above from a B cell line (NE-1) producing monoclonal anti-DNA antibody, kindly provided by Dr. Sasaki[14].

ELISA. To detect the binding of Fab samples to SS-A/Ro and other antigens, ELISA plates were coated with 10 μg/ml of recombinant 60-kd Ro protein or other antigens (ssDNA, recombinant SS-B/La protein, β2-glycoprotein I, transferrin, ovalbumin) at 4°C for 12 h. Recombinant SS-B/La protein was kindly provided by MBL Lab. [13]. After blocking of wells with 1 % BSA in PBS pH 7.0 for 2 h at room temperature (RT), diluted recombinant Fab samples were added into wells and incubated for 2 h at RT. After washing with 0.05% Tween/PBS, plates were reacted with 1/1000 dilution of alkaline-phosphatase-labeled goat anti-human Fab (Pierce, cat. no. 31312), wahed 5 times with 0.05% Tween/PBS, and then developed with *p*-nitrophenyl phosphate at 5 mg/ml (Sigma Chemical Co., St. Louis, MO) in 10% diethanolamine, 1 mM $MgCl_2$, pH 9.8. Absorbance at 405 nm was measured after 20 to 30 min incubation.

To determine Fab concentration in the cell-lysates, ELISA plates were coated with 0.1 μg/ml of affinity-purified goat anti-human κ and anti-human λ IgG antibodies (Southern Biotechnology Associates, Birmingham, AL). After blocking with 1% BSA in PBS, Fab (IgG) standard solutions and diluted recombinant Fab samples were added into wells and incubated for 2 h at RT. After then, ELISA was performed as described above.

In some experiments, diluted recombinant Fab samples (1 μg/ml) were incubated with 10 μg/ml of 60-kd Ro or other antigens before adding them to wells of 60-kd Ro-coated ELISA plates (Competition ELISA).

Nucleotide sequence analysis. Phagemid DNA was prepared from the selected clones and nucleotide sequences were determined by an ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and the Model ABI PRISM 310 genetic analyzer (Applied Biosystems). To obtain heavy chain sequence, primers from the 5′ vector sequence (5′-CTATTGCCTACGGCAGCCGCTG-3′) (+) and the CH1 constant region (5′-GAAGTAGTCCTTGACCAG-3′) (−) were used. To obtain the light-chain sequence, the 5′ vector sequence (5′-GTGGAATTGTGAGCGGATAAC-3′) (+) and Cκ primer (5′-CACAACAGAGGCAGTTCC-3′) (−), or Cλ primer (5′-GAGACA-CACCAGTGTGGC-3′) (−) were used. The sequences of the heavy-chain V region (VH) genes were compared with the germline VH gene segments as published in Tomlinson et al. [15]. The light-chain V region (VL) genes were compared with the germline gene segments as described in Cox et al.[16] (Vκ segments) and Williams and Winter [17] (Vλ segments). Sequence homologies were also compared with previously reported human sequences in the GenBank database using the FASTA program.

RESULTS

In this study, Fab-phage libraries were constructed from lip biopsy samples, obtained from 3 patients with SS (E.S., K.N., S.T.). Mean library size was 2.2×10^7 transformants (0.5×10^6 to 3.2×10^7) after κ and λ light chain cloning and 1.4×10^7 transformants (0.6×10^6 to 2.3×10^7) after heavy chain cloning. These libraries were subjected to 4 rounds of panning against SS-A/Ro 60-kd protein. After 4 round pannings, individual clones were screened for their capacity to bind recombinant 60-kd Ro protein by ELISA. At least 20 Fab clones of each library were examined for their binding. A number of Fab clones from the 3 libraries (*ex.* K-10, S-46, and K-44) revealed detectable binding to 60-kd Ro protein at considerably high concentrations (several μg/

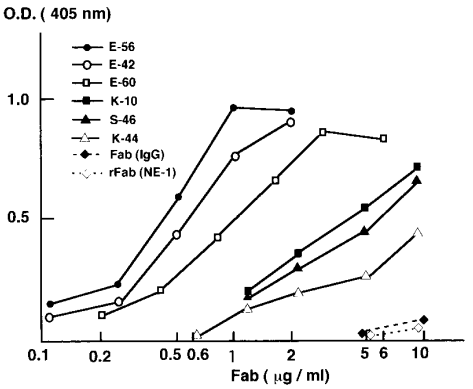

FIG. 1. 60-kd Ro-binding activity of rFab clones. 60-kd Ro-coated ELISA wells were reacted with rFab clones derived from the three patients with Sjögren's syndrome (E, K and S), and bound rFabs were detected with an alkaline phosphatase-conjugated anti-Fab reagent. As controls, Fab derived from serum IgG (a healthy subject) and rFab derived from an anti-DNA antibody producing B cell line (NE-1) were also reacted.

ml and more), however, their bindings did not reach plateau at 10 μg/ml (Fig. 1). Three Fab clones (E-42, E-56, and E-60) obtained from the library E.S. revealed significant bindings to 60-kd Ro protein at less than 1 μg/ml and their bindings reached plateau at 1 to 2 μg/ml (Fig. 1). As controls, recombinant Fab derived from anti-DNA antibody producing clone NE-1 [15] and Fab (IgG) derived from a normal serum did not bind the 60-kd Ro at as much as 10 μg/ml. These three Fab clones were selected for further analysis.

Specificity of the bindings of the three Fab clones to SS-A/Ro 60-kd protein was examined. At 1 μg/ml, E-42 and E-56 clones revealed significant bindings to 60-kd Ro protein, whereas almost no or only little binding to SS-B/La (Fig. 2). Both Fab clones did not bind β2-glycoprotein I, ssDNA, transferrin or ovalbumin (Fig. 2). E-60 clone revealed significant, but slightly weak

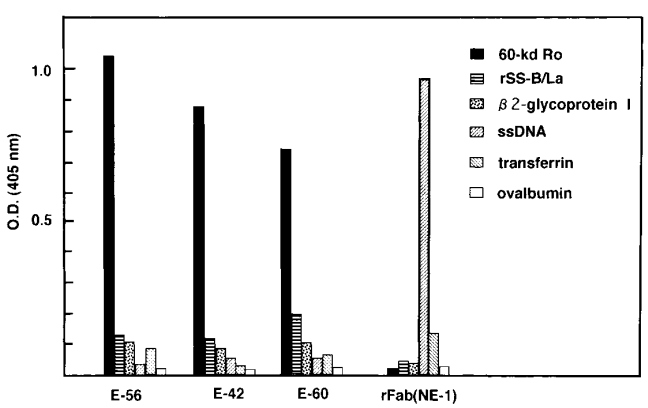

FIG. 2. Cross-reactivity of rFabs to a panel of solid-phase antigens tested by ELISA. rFabs were added at 1 μg/ml.

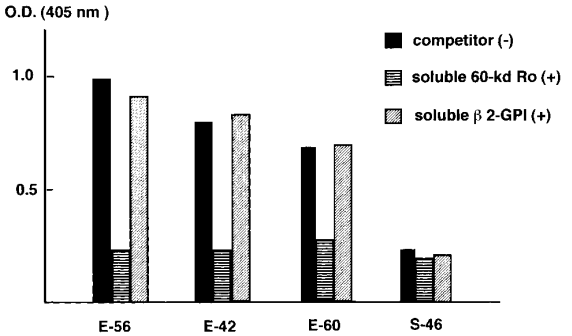

FIG. 3. Inhibition of binding of rFabs (1 μg/ml) to solid-phase 60-kd Ro protein by preincubation with soluble 60-kd Ro (10 μg/ml) before adding them to wells. As controls, preincubation was also performed with BSA (no competitor) or β2-glycoprotein I (β2-GPI)(10 μg/ml).

binding to 60-kd Ro protein compared with E-42 clone at 1 μg/ml and some cross-reactivity to SS-B/La protein. However, E-60 did not bind β2-glycoprotein I, ssDNA, transferrin and ovalbumin at 1 μg/ml (Fig. 2). rFab clone derived from NE-1 failed to bind 60-kd Ro protein, but revealed significant binding to ssDNA at 1 μg/ml (Fig. 2). The specificity of the bindings of the Fab clones to 60-kd Ro protein was further determined by a competition ELISA. The preincubation of the three Fab clones (E-56, E-42, and E-60) at 1 μg/ml with recombinant 60-kd Ro protein (10 μg/ml) before adding them significantly inhibited the binding of the Fab clones to 60-kd Ro coated on an ELISA plate, whereas no significant inhibition by preincubation with β2-glycoprotein I (an unrelated autoantigen) was observed (Fig. 3). The inhibition of the binding of S-46 Fab clone to 60-kd Ro by preincubation with soluble Ro was unclear (Fig. 3).

Sequence analysis of the VH and VL genes of the three clones (E-42, E-56, and E-60) was conducted and compared with the sequences of putative germline V genes (Table 1 and Fig. 4). The sequences of the VH gene segments of E-42 and E-60 clones were highly homologous and differed in only one nucleotide residue (one amino acid) (Fig. 4). Both clones appeared to be derived from the same putative germline VH gene DP73 (VH5 family), and used identical CDR3 sequence and JH5 gene (Fig. 4). The two highly homologous heavy chain V region sequences represent the products either of somatic mutations on the same original heavy chain sequence or PCR-generated mutations from the same progenitor sequence. E-56 Fab appears to be derived from a different putative germline gene DP31. The usages of VL genes in the three clones appeared to be also restricted. Two of the three clones (E-42 and E-56 clones) used identical light chains derived from a putative germline Vκ gene L6 (Vκ3) and the other clone E-60 used an unrelated light chain derived from a puta-

TABLE 1

Comparison of Gene Usage and Structural Homologies for Heavy- and Light-Chain V Regions (VH and VL) of Anti-60-kDa Ro Peptide Fabs

	VH usage					VL usage				
			% Homology with germline					% Homology with germline		
Fab	VH family	Nearest VH	DNA	Protein	JH	VL family	Nearest VL	DNA	Protein	JL
E-42	VH5	DP73/VH251	92.4	88.2	JH5	κ3	L6/Vg	94.3	90.3	Jκ4
E-56	VH3	DP31	93.2	89.2	JH3	κ3	L6/Vg	94.3	90.3	Jκ4
E-60	VH5	DP73/VH251	92.8	89.2	JH5	λ3	DPL23	95.6	92.3	Jλ2/3

tive Vλ gene DPL23 (Vλ3). Interestingly, E-42 and E-60 Fab clones which used highly homologous heavy chains in combination with different light chains revealed only slight differences in the binding activity to 60-kd Ro protein (Fig. 1 and Fig. 4). A number of amino acid replacements from the germline genes were observed in both heavy and light chains of the three Fab clones. (Table 1 and Fig. 4).

DISCUSSION

In this study, we have used a phage-display technique to isolate anti-SS-A/Ro Fab clones from lip biopsy samples of patients with SS. We could obtain three Fab clones which specifically bound to SS-A/Ro 60-kd protein from one patient with high titer of anti-SS-A/Ro antibodies. From other two patients, polyreactive Fab clones which showed significant bindings to 60-kd SS-A/Ro protein only at high concentrations were obtained. The reason why specific Fab clones were obtained from lip biopsy samples of the one patient (E.S.) remains to be determined. However, an important factor may be the concentration of anti-SS-A/Ro antibody producing cells in the lymphocyte populations. A recent study reported an ectopic expression of autoantigens in salivary glands of Sjögren's syndrome [10]. In addition,

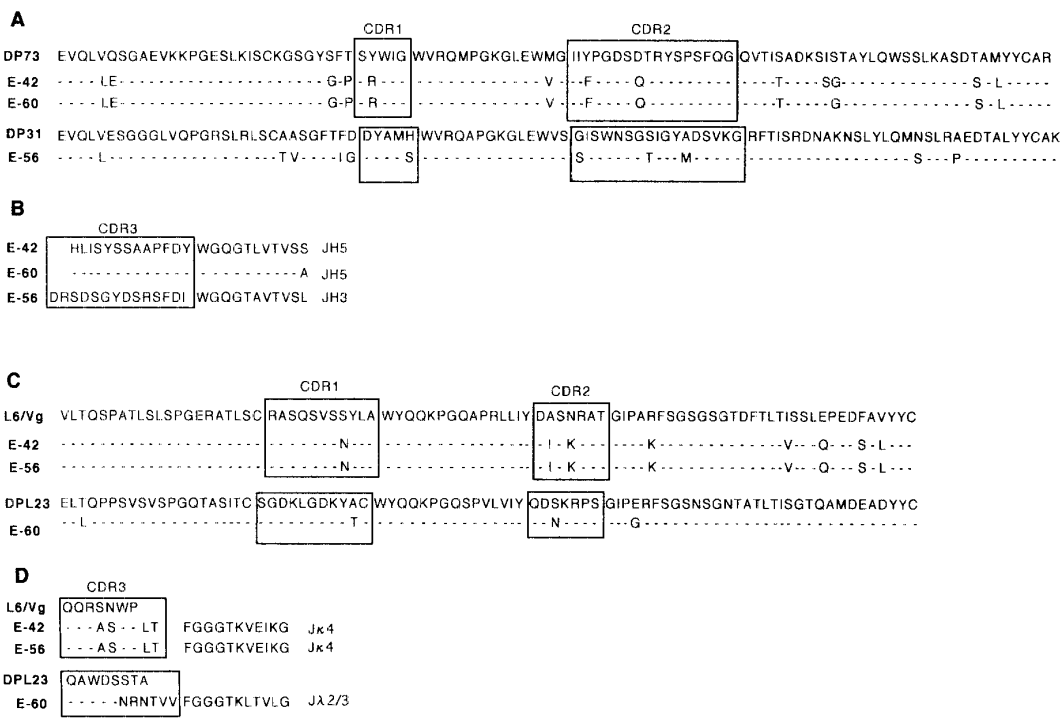

FIG. 4. Translated amino acid sequences of VH regions of the three rFab clones compared to the nearest germline gene segments (A). Amino acid sequences of CDR3 regions of the clones displayed in A (B). Translated amino acid sequences of the light chain variable regions of the rFab clones compared to the nearest germline sequences (C and D).

increased frequency of anti-SS-A/Ro antibody producing cells in salivary gland lymphocytes from patients with Sjögren's syndrome compared with PBL has been reported [9]. Histological examination showed more aggressive infiltrations of lymphocytes in minor salivary glands of the patient, E.S. compared with the samples from other two patients. Accordingly, a possible increase of autoantibody-producing cells including anti-SS-A/Ro antibody producing cells in salivary glands of the patient, E.S. may be one reason for successful isolation of specific anti-SS-A/Ro Fab clones.

We examined the specificity of the three anti-SS-A/Ro Fab clones by ELISA and a competitive binding assay. By ELISA, these Fab clones revealed significant binding to SS-A/Ro at as low as 0.1 to 0.3 μg/ml and reached plateau at 1 to 2 μg/ml, indicating high affinity antibodies. These Fab clones did not react with some other autoantigens including ssDNA, β2-glycoprotein I, but one of the three clones slightly cross-reacted with recombinant SS-B/La antigen. By a competition ELISA, the binding of the Fab clones to recombinant 60-kd Ro protein was significantly inhibited by preincubation with 60-kd Ro protein. From these findings, we conclude that the isolated Fab clones are specific to 60-kd Ro protein.

The information on nucleotide sequences of human monoclonal anti-SS-A/Ro antibodies is very limited. To our knowledge only one study on nucleotide sequence of an IgM monoclonal anti-Ro/La autoantibody has been reported [18]. The VH of the IgM monoclonal antibody was 100 % identical to a germline VH gene VH418, which was different from the VH genes in our study [18]. However, this monoclonal IgM antibody was developed by transformation of PBL with EB virus from a healthy donor and was polyreactive with both purified Ro and La proteins. Accordingly, our study is the first report on the nucleotide sequences of IgG anti-Ro antibodies derived from a patient with Sjögren's syndrome. The nucleotide sequences of the three Fab clones demonstrated that the two of them (E-42 and E-60) used highly homologous heavy chains derived from DP73 in combination with different light chains (L6 (Vκ3)- and DPL23 (Vλ3)-derived light chains). E-42 Fab revealed slightly higher binding activity to 60-kd Ro than E-60 Fab. This finding suggests a predominant role of the heavy chain for the binding to 60-kd Ro protein. The third clone, E-56 used different DP31-derived heavy chain in combination with the light chain (L6-derived) identical as that of E-42. These findings may suggest the restricted usage of VH and VL genes of anti-SS-A/Ro antibodies in salivary glands of this patient. In this respect, restricted usage of VH and VL genes has been reported in combinatorial Fab fragments against other autoantigens [19-20]. More than half of anti-thyroid peroxidase Fab fragments isolated from thyroid-infiltrating B cells used homologous HV1L1-derived heavy chain in combination with similar light chains belonging to the same Vκ family (KL012) [19]. The heavy and light chain restriction evident in our study and in others may have several causes. The outgrowth of the highest affinity colonies may be a potential drawback of the combinatorial library method [21]. Alternatively, the restriction may reflect genuine heavy and light chain restriction of the autoantibodies present in the tissue used to produce the libraries. The possibility of in vitro biased selection of particular heavy and light chain combinations in the recombinant anti-60-kd Ro library may not be denied. Further studies will be necessary to elucidate these possibilities. Finally, all three Fab clones revealed considerable substitutions of nucleotides from the germline genes, suggesting the generation of these antibodies by an antigen-driven mechanism.

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