The Importance of the Light Chain for the Epitope Specificity of Human Anti-U1 Small Nuclear RNA Autoantibodies Present in Systemic Lupus Erythematosus Patients

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*J Immunol* 1999; 163:3304-3312; 
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Abs to U1 RNA are frequently found in patients suffering from systemic lupus erythematosus overlap syndromes and Ab titers correlate with disease activity. We describe the isolation of the first human anti-U1 RNA autoantibodies from a combinatorial IgG library made from the bone marrow of a systemic lupus erythematosus patient. With the use of phage display technology, two anti-U1 RNA single-chain variable fragment (scFv) Abs were selected. Both high affinity anti-U1 RNA Ab fragments (K_{d} \sim \text{nM}) recognize stem II of U1 RNA and were derived from the same heavy chain gene (V3–11) and the same light chain gene (3\text{r}) although somatic mutations, predominantly present in the complementarity-determining regions, are different. Experiments, in which the heavy chain genes of both anti-U1 RNA scFvs were reshuffled with the original light chain repertoire of the patient resulted, after selection on stem loop II, in a large number of RNA-binding Ab fragments. All these stem loop II-specific RNA binding clones used a similar, but not identical, 3\text{r} light chain. When scFvs were selected from the reshuffled libraries by stem loop IV, representing the other autoantigenic site of U1 RNA, most selected Ab clones did react with stem loop IV, but no longer with stem loop II. The stem loop IV-reactive Ab clones contained different, not 3\text{r}-related, light chains. These results point to a major role for the light chain in determining the sequence specificity of these disease-related anti-U1 RNA Abs. The possibility that secondary light chain rearrangements are involved in this autoimmune response is discussed. The Journal of Immunology, 1999, 163: 3304–3312.

Patients with systemic lupus erythematosus (SLE) and SLE overlap syndromes often produce IgG autoantibodies directed to components of the U1 small nuclear ribonucleoprotein particle (snRNP) (reviewed in Ref. 1). Autoantibodies have been found recognizing the U1-specific proteins (U1A, U1C, and U1-70K) and the core or Sm proteins (B/B′, D1, D2, D3, and the EFG complex). Autoantibodies directed to the U1-specific proteins are often accompanied by Abs directed to the naked U1 RNA (2–5). The epitope regions on the U1 RNA recognized by these patient Abs are located within stem II and loop IV of U1 RNA (4, 6–8). Interestingly, the Ab titers to individual epitopes on U1 RNA appeared to correlate with disease activity (9), and Ab activity directed to U1 RNA was found in the more severely affected patient group (5), suggesting that this type of autoantibody has some intrinsic relationship with the disease. Both anti-stem II and anti-loop IV Abs have also been shown to interact with the native cellular U1 snRNP complex (10).

Although it is generally accepted that most nucleic acids are poor immunogens, Abs to DNA and RNA are found in several autoimmune diseases. In patients with SLE, Abs have been described to single-stranded and double-stranded DNA, as well as to 28S ribosomal RNA (11) and U1 RNA (2–4), whereas in myositis Abs have been described to tRNA^{Ala} (12), tRNA^{Met} (13), and tRNA^{His} (14). Ab titers to dsDNA, present in a subpopulation of SLE patients, often correlate with disease activity and can be used as a predictive marker for this disease (15). Most of these Abs contain a net positive charge in the complementarity-determining region (CDR) loops of heavy and light chain. In this respect the CDR3 of the heavy chain, the most variable loop of the Ab, is thought to play the most important role.

The reason why anti-nucleic acid Abs arise is not known. It has been proposed that the anti-RNA activity could be the result of accidental cross-reactivity with RNAs of other cellular or viral immunogens (4). Cross-reactivity between proteins and RNA is also a possible mechanism (7).

To study the phenomenon of anti-nucleic acid autoantibodies in more detail, we prepared combinatorial Ab libraries representing the IgG repertoire present in the bone marrow of four patients with SLE overlap syndromes. The genes encoding the V domains of heavy (V_{H}) and light (V_{L}) chains were cloned in a phagemid vector and expressed as a fusion protein of the minor coat protein pIII. Two Ab fragments, specifically recognizing stem loop II of U1 RNA were isolated and analyzed in more detail. Both are derived from the same heavy chain gene (V3–11 = DP–35) and the same light chain gene (3\text{r} = DPL–23), but somatic mutations and CDR sequences were different.
To study to what extent the light chain was important in determining the specificity of these two related anti-U1 RNA Abs, we reshuffled both original anti-U1 RNA heavy chains with the light chain repertoire from the same patient. These reshuffled libraries were then analyzed for their U1 RNA-binding capacities. Stem loop II-specific binders were found only when similar, but not identical, light chains were used. Combinations of V3-11 with other light chains produced anti-RNA or anti-stem loop IV binders. These results underscore the important role of the light chain in the RNA-recognizing specificity of these Abs.

Materials and Methods

Library construction

Patient libraries were made from bone marrow cells obtained from 4 SLE overlap syndrome patients (D18, HO, O11, and Z5) (as synonym for SLE overlap, these patients are in the literature also classified as mixed connective tissue disease or anti-RNP-positive connective tissue disease patients). Serum samples from these patients were able to immunoprecipitate naked U1 RNA (data not shown). The patient libraries were constructed essentially as described (16, 17). All libraries contained >10^6 individual clones, and >75% of all clones contained full length inserts. From each library, 96 clones were analyzed by PCR-fingerprinting (BstNI digestion) to confirm that the patterns were highly diverse.

Isolation of single IgG-positive B cells, amplification and sequencing of V_H/V_L regions

PBL were obtained from a healthy donor and two SLE patients, Z5 and D101, using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Single IgG-positive B cells were isolated using a Coulter Epics Elite flow cytometer (Coulter, Hialeah, FL) equipped with an automatic cell capture device unit set as previously described (18, 19). These individual B cells were cultured for 10 days after which isotype-specific Ab production was tested. From IgG-positive cultures (76% or more), RNA was isolated, heavy and light chain V regions were amplified with family-specific primers, and the sequences were determined.

Selection and purification of Ab fragments

The first round of screening was performed with in vitro T7 RNA polymerase-transcribed U1 RNA (4). Immunotubes (Maxisorp, Nunc, Roskilde, Denmark) were precoated with magic coating (2 h at 4°C) and blocked with 0.5% BSA for 2 h at 4°C. After 5 washings with RNase-free milliQ water, U1 RNA was coated (20 μg/ml) in PBS overnight at 4°C. The remaining binding sites on the coated tubes were blocked by a 2-h incubation at 4°C with a mixture of PBS containing 2% nonfat dry milk powder (Marvel, Food Store, U.K.), 0.5% BSA, 50 U/ml RNasin (Promega, Madison, WI), and 20 μg/ml total yeast RNA (MIX-1).

Phages were isolated from the libraries as described (18) and incubated in the Mix-1 mixture in an end-over-end rotator for 30 min at 4°C, after which the tubes were stored on ice for 90 min. Next the tubes were rinsed 15 times with RNase-free PBS containing 0.05% Tween 20 (PBS-T) and 15 times with RNase-free PBS. Elution of the phages (with 100 mM triethylamine), infection, and plating were performed as described (18).

The second round of selection was performed with biotinylated U1 RNA. First streptavidin (20 μg/ml) in 0.1 M NaHCO_3 (pH 8.6) was coated overnight at 4°C. After three washings with PBS, blocking was performed for 1 h at 4°C with biotin-free BSA in PBS. After those three washings with PBS, a mixture of Mix-1 containing biotinylated U1 RNA (10 μg/ml) was added and incubated in an end-over-end rotator wheel for 1 h at 4°C. Phage incubation, washings, elution, infection, and plating were performed as described (18).

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Light-chain shuffling libraries

The heavy chains of Z5scFv3 and Z5scFv7 were isolated by NcoI/SaI digestion of the full length-containing pHENIX clones. The DNAs were gel purified and used in ligation with NcoI/SaI-digested and gel-purified Z5Vα-pHENIX and Z5Vκ-pHENIX DNA (patient Z5) in the same way as described for the library construction. The four reshuffled libraries all contained >10^6 individual clones with >90% full length inserts. The libraries (1 round) were selected with biotinylated stem loop II and stem loop IV RNA in the same way as described above for the second round of selection. ScFv sequences were isolated from pHENIX as NcoI-NorI DNA fragments and subcloned into PUC119(His)_8 VSV (17). The light chain sequences of the reshuffled scFv clones (GenBank accession numbers AJ241379–AJ241419) were compared with the germline sequences in the V-base sequence directory (37).

Determination of the K_d

The K_d of Z5scFv3 was determined by a nitrocellulose filter binding assay. A constant amount of purified scFv Ab was used with a series of stem loop II RNA concentrations (fixed amount of ^32_P-labeled stem loop II RNA (20,000 cpm) mixed with varying amounts of unlabeled stem loop II RNA). First, a saturation curve was made to determine the scFv concentration in which a linear fit was found between the concentration of stem loop II RNA and the scFv concentration. Secondly, the maximum amount of bound scFv stem loop II RNA was determined. Using a constant amount of scFv, the amount of U1 RNA was increased, and the bound U1 RNA (B) was quantified with the nitrocellulose-binding assay counting the ^32_P activity (9). Assuming a 1:1 complex, the K_d was calculated (in triplicate) by Scatchard analysis.

Radiolabeled RNA and immunoprecipitation

Radiolabeled RNA (^32-P-labeled) was prepared either by deproteinization of extracts of HeLa monolayer cells, cultured overnight in the presence of radiolabeled orthophosphate (3), or by in vitro transcription. For in vitro transcription the T7 RNA polymerase system was used as previously described (3). All immunoprecipitations (unless stated otherwise) were performed at 150 mM NaCl as described previously (21). DNA templates encoding wild-type U1 RNA wt, stem loop II, stem loop IV,ΔDE (4), U3 RNA (22), and RNase MRP RNA (25) have also been described previously.

Results

Selection of U1 RNA-specific Ab fragments

Four combinatorial Ab libraries (all containing >10^6 individual clones) were made from bone marrow cells of four SLE overlap syndrome patients and screened for the presence of anti-U1 RNA activity. In the first round of selection U1 RNA was directly coated to the immunotubes, while in the second round biotinylated U1 RNA was used (see Materials and Methods). Both selections were conducted in the presence of an excess of yeast RNA to avoid selection of general nucleic acid-binding Abs and to prevent degradation of U1 RNA. The two different selection methods were used to avoid nonspecific binding of phages to biotin or streptavidin. After two rounds of selection, a 100-fold enrichment for binding phage compared with the first round was obtained for all 4 libraries. At this stage, 96 individual clones of each library were screened for binding to ^32-P-labeled U1 RNA with the nitrocellulose filter binding assay (4). In addition, scFv expression levels were determined with a dot blot assay, and fingerprint patterns were determined using BstNI digestion. Twelve U1 RNA-binding clones were subsequently selected and characterized (four from D18, one from HO, and seven from Z5).

We also tried to isolate anti-U1 RNA Abs from semisynthetic Ab libraries (23, 24). Although we succeeded in isolating Abs directed to nucleic acids from these libraries, they were not specific for U1 RNA. Libraries derived from PBLs from patient Z5 and another SLE patient (D101) were analyzed as well, but possibly because of the low number of plasma cells in these preparations, no V_H/V_L combinations able to recognize U1 RNA were obtained from these libraries.
Specificity of anti-U1 RNA Ab fragments

To determine the specificity of the Ab fragments, competition experiments were performed in which radiolabeled stem loop II or IV of U1 RNA (the two major autoepitopes of U1 RNA) was incubated with the scFvs in combination with an excess of either unlabeled stem loop II or unlabeled stem loop IV as competitor and analyzed in a nitrocellulose filter binding assay. Most clones reacted with both stem loop II and IV of U1 RNA except Z5 scFv3, scFv5, and scFv7, which showed only reactivity and competition with stem loop II (Fig. 1A).

To further analyze the specificity of these Ab fragments (scFv3, scFv5, and scFv7) competition experiments were performed with poly(A), poly(G-C), poly(G-U), poly(G), poly(C), HeLa cell ribosomal RNA, dsDNA, and U1 RNA. With scFv3 and scFv7, none of the competitors (except U1 RNA) showed inhibition of binding to \(^{32}\text{P-}U1\) RNA, even when a 500-fold molar excess of competitor was used, whereas scFv5 binding was strongly competed by ribosomal RNA (data not shown).

Next, the Ab fragments that seemed most specific for U1 RNA, scFv3 and scFv7, were used in immunoprecipitations in which the scFvs were indirectly bound via their VSV tag to protein A-agarose and incubated with a radiolabeled RNA mixture containing stem loop II RNA, stem loop IV RNA, U1 RNA, and RNase MRP-RNA. The result shows that only stem loop II RNA is recognized by these two scFv Abs (Fig. 1B). Using various stem loop II mutants, we were able to show that both scFv3 and scFv7 recognized the stem of stem loop II of U1 RNA. A detailed analysis of the epitope has been published elsewhere (25). The \(K_d\) of the scFv3 for stem loop II was determined with a nitrocellulose filter binding assay (Scatchard analysis) to be 1.0 ± 0.2 nM. Although the affinity of scFv7 could not be determined as accurately, because of its lower expression levels the estimated affinity of this scFv for stem loop II appeared to be of the same order of magnitude.

Recognition of the native U1 snRNP particle

Because of the low expression levels of scFv7, these experiments were performed only with scFv3. With \(^{32}\text{P-}U1\) RNA isolated from HeLa cells, scFv3 was shown to specifically immunoprecipitate U1 RNA (Fig. 1C). The Ab was also tested on Western blots containing HeLa nuclear extract and by ELISA using recombinant U1A and U1C autoantigens as Ag. No reactivity of scFv3 with proteins could be detected. Using a [\(^{35}\text{S}\)]methionine-labeled cell extract in immunoprecipitation experiments, we have shown recently that scFv3 is able to immunoprecipitate the U1 snRNP complex from a HeLa S100 extract (25). The U1 snRNP proteins precipitated by scFv3 were similar to the pattern of proteins precipitated by an anti-Sm Ab which was used as a positive control and showed that the U1A protein, which binds to the loop of stem loop II of U1 RNA, is not interfering with the binding of scFv3 to the stem of stem loop II (25).

V gene usage of anti-U1 RNA Ab fragments

To determine the V gene usage of the two U1 stem loop II-specific scFvs, the sequences of both heavy chain (\(V_H\)) and light chain (\(V_L\)) were analyzed (Table I). Both \(V_H\) genes align best with the VH3-11 (DP-35) gene, and both contain a relatively large number of somatic mutations (21 aa for scFv3 and 16 aa for scFv7), the majority being located in CDR2. The CDR3 of the heavy chain of both Ab fragments contains a number of positively charged amino acids at identical positions (for scFv3 lysines at positions 3, 6, and 9 and for scFv7 lysines at positions 3, 6, 8, and 9). Also in the CDR1 and CDR2 some replacement mutations introducing basic amino acids (arginine, lysine, and histidine) are found. No significant homology to any D segment could be detected. Both light chains of the two Abs align to the same \(\lambda\) germline gene 3r (DPL-23), and also here in both cases a relatively large number of somatic replacement mutations are found (for scFv3, 12 aa, and for scFv7, 21 aa). In this case, the CDR1 is the main target. In all light chain CDR sequences, except for the CDR1 of scFv7, basic amino acids are found, in part resulting from germline gene sequences and in part introduced by somatic mutations. In scFv3, as a result of somatic mutations, also two acidic amino acids are replaced by either a neutral (D→N) or a basic (D→R) amino acid.

To compare the frequency of the selected \(V_H/V_L\) genes with the total \(V_H/V_L\) use in this patient, we isolated IgG-positive B-cells from the peripheral blood of this patient by FACS selection (18). Individual IgG-positive B cells were cultured for 10 days, and the IgG isotype of the B cells was confirmed by ELISA. Next we amplified and determined the sequences of 100 \(V_H/V_L\) pairs of patient Z5 (19). In the IgG-positive B cell population of patient Z5, 4% of the Abs contained \(V_H\) gene VH3-11, which is not different from the frequency found in normal individuals (19). We also investigated the frequency by which the 3r (DPL-23) light chain gene is expressed in IgG positive B cells from a normal individual and from patient Z5. In only 1% of the normal IgG repertoire could 3r light chain-related clones be identified, whereas sequencing of the light chains of 100 IgG-positive B-cells from patient Z5 failed to detect any clone expressing this particular light chain.

Light chain reshuffling

It is generally accepted that the heavy chain determines in most cases the major part of the specificity of an Ab, although the light chain can also influence the affinity or specificity. Because both anti-U1 RNA Abs aligned to the same light chain germline sequence, we wondered whether, in this case, the light chain is important in determining the specificity of the anti-stem loop II activity.

To investigate this, we reshuffled the heavy chains of both Abs with the original light chain repertoire of the same patient. The four sublibraries (each \(>10^7\) in size, referred to as VHscFv3\(_k\), VHscFv3\(_l\), VHscFv7\(_k\), and VHscFv7\(_l\)) were first analyzed without selection. Forty-eight clones were randomly picked from each sublibrary, and scFv production was induced using isopropyl \(\beta\)-d-thiogalactoside. Ab fragment expression levels were analyzed with a dot blot probed with an anti-VSV Ab. Consistent with the reproducibly low expression levels of scFv7 observed before, the scFv expression levels of most VHscFv7-derived clones were found to be very low compared with the expression by the scFv3-derived clones. The culture supernatants were also tested in a nitrocellular filter binding assay for reactivity to \(^{32}\text{P-}U1\) RNA. One positive clone was found for VHscFv7 (V17A) and 3 for VHscFv7 (V18A, V19A, and V16c). These four clones were sequenced (Table II, V1–V15). Only one of these four RNA-binding clones (V17) used a 3r (DPL-23)-related light chain. Also 15 nonbinders (7 \(\lambda\) and 8 \(\kappa\) clones) that showed scFv expression on dot blot were analyzed by DNA sequencing (Table II, V16–V19). None of these nonbinders used a 3r-related light chain.

Then the four libraries were subjected to one round of selection on biotinylated stem loop II RNA. After selection, again 48 clones derived from each library were randomly chosen and tested for reactivity to U1 RNA using the filter binding assay. For scFv3 ~50% of the clones were positive, whereas for scFv7 95% of all selected clones were reactive with U1 RNA. Fingerprinting was performed for all positive clones, and 17 different clones were analyzed in more detail by DNA sequencing (named II-3.1–II-3.9 and II-7.1–II-7.8). DNA sequencing revealed that a large number of the stem loop II-selected clones (seven of nine derived from VHscFv3, and three of eight derived from VHscFv7) used a light
FIGURE 1. Specificity test of patient-derived anti-U1 RNA-selected scFvs. A, Filter binding assay using radiolabeled domains of U1 RNA. $^{32}$P-stem loop II RNA (top, 20,000 cpm) or $^{32}$P-stem loop IV RNA (bottom, 20,000 cpm) were incubated with scFvs (2 µl culture supernatant) in the presence of 10 µg yeast RNA and different amounts of competitor (stem loop II or stem loop IV) RNA. After incubation (2 h, 4°C), samples were transferred to a dot blot manifold and washed three times with PBS as described previously (9). Bound RNA was visualized by autoradiography. Twelve scFvs were analyzed, seven derived from patient Z5, one from patient HO, and four from patient D18. (In the bottom panel of Z5, 6 (0 ng stem loop IV) no signal is detected; repeated experiments (not shown) resulted in a signal with the same intensity as that of Z5, 6 (0 ng stem loop II), indicating that this point is an artifact in this particular experiment). B, Immunoprecipitation of radiolabeled domains of U1 RNA by patient-derived scFvs. scFvs were indirectly coupled to protein A-agarose (using anti-VSV tag Abs) and incubated with a mixture of radiolabeled in vitro transcribed RNAs (stem loop II, stem loop IV, U1 RNA, RNase MRP-RNA). Immunoprecipitation was performed as described previously (21). Samples were analyzed on 10% polyacrylamide-8.3 M urea gels. The immunoprecipitations were performed with: Z5scFv3 (lane 2), Z5scFv7 (lane 3), and control anti-U1A protein scFv (lane 4). Lane 1, 10% of the input RNA mixture. C, Immunoprecipitation of U1 RNA from total HeLa cell RNA. Abs (serum or scFv) were coupled to protein A-agarose and incubated with $^{32}$P-labeled HeLa cell RNA. Immunoprecipitation was performed as described previously (21). Samples were analyzed on 10% polyacrylamide-8.3 M urea gels. The immunoprecipitations were conducted with: patient serum Z5 (lane 3), normal human serum (lane 4), Z5scFv3 (lane 5), control scFv (lane 6). Lane 1, 5% input RNA; lane 2, 1% input RNA. The positions of the most abundant RNAs are indicated on the left.
Table I. Amino acid sequences of heavy and light chains of scFvs binding to stem loop II of U1 RNA (Z5scFv3 and Z5scFv7), aligned to their most homologous germline sequencea

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<th>CDR 2</th>
<th>FR 3</th>
<th>CDR 3</th>
<th>FR 4</th>
<th>Mutationsb (aa/nt)</th>
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<td>WIRAPGKGLEWS</td>
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* Alignments using V-base homology search (37).

a The number of amino acid (nucleotide) mutations are given.

epitope recognized by SLE patients’ sera including this particular patient serum (Z5). The selection was again conducted in the presence of a large excess of yeast RNA. After selection, 20% of the clones originating from VHscFv3 and 50% of the clones from VHscFv7 appeared to bind U1 RNA in the nitrocellulose dot blot assay. Five clones were then analyzed for binding specificity by immunoprecipitation, and their sequences were determined. Most of the immunoprecipitation data are shown in Fig. 2B, and the overall results are summarized in Table III (stem loop IV-selected VHscFv clones are designated IV-3.x and IV-7.x depending on the library from which they originated).

As expected, most stem loop IV binders showed no or only very weak precipitation of stem loop II and much stronger precipitation of stem loop IV. However, the Abs were not specific for stem loop IV because U3 RNA, used as a control, was immunoprecipitated in all cases as efficiently as stem loop IV. In subsequent control experiments, it was found that some other RNAs (e.g., RNase MRP-RNA) were often recognized by these scFvs, whereas other small RNAs (e.g., hY RNAs) were not recognized (data not shown). One stem loop IV-selected scFv Ab (scFv IV-7.3) immunoprecipitated both stem loop II and stem loop IV RNAs at 150 mM NaCl, but lost stem loop IV reactivity, and not stem loop II reactivity, at 350 mM NaCl (data obtained at 250 mM NaCl are shown in Fig. 2B). These results underline the idea that the selected scFvs are not stem loop II specific. Nevertheless, the four stem loop IV binders (IV-3.1, IV-3.2, IV-7.1, and IV-7.2) all used different light chains, whereas IV-7.3, which also showed high affinity binding to stem loop II, again used a 3r-related light chain. Interesting, but as yet unexplained, was the observation that all 3r-related clones, in series II as well as series IV, contained a serine at position 34 instead of a cysteine (see Discussion).

The latter results are thus in complete agreement with the results from the stem loop IV selections and show that the stem loop II specificity of these anti-U1 RNA Abs is very much dependent on the identity of the light chain used.

In Fig. 3, all sequenced clones are classified according to their most homologous germline gene. Fig. 4 illustrates that within the
group of stem loop II-specific scFvs there is a dramatic overrepresentation of 3r-related light chains, whereas this particular light chain is not used by 15 randomly selected nonbinders. As noted above, the 3r light chain gene in IgG-positive B cells of the normal repertoire and in patient Z5 was used in 1% of the cases or less (19), indicating that there certainly is no general overrepresentation of this light chain product. Our results thus indicate strongly that in this SLE patient there is a clear light chain restriction for anti-stem loop II reactivity.

Discussion

We have isolated a number of patient autoantibodies with specificity for the U1 small nuclear RNA molecule. In patient sera, the anti-U1 RNA population of autoantibodies mostly recognizes the stem of stem loop II and/or the loop of stem loop IV of the RNA. The scFvs selected by us are specifically directed to the stem of stem loop II (see also Ref. 25). Both scFvs were derived from the same heavy (VH3-11) and light chain (λ3r) germline genes but differ because of a number of somatic mutations.

Reshuffling experiments, using the VH3–11 gene of the Abs and the complete light chain repertoire of the patient, resulted in a second group of anti-U1 RNA Abs with different specificities. Abs recognizing stem loop II, however, always contained a 3r-related light chain, while Abs with different specificities did not. This indicates that, at least in this patient, the choice of the light chain strongly influences target RNA specificity.

The two original stem loop II specific scFvs are characterized by a number of somatic mutations (K, R, H) are underlined.

### Table II. Amino acid sequences of the λ (A) and κ (B) light chains of scFvs obtained after light chain reshuffling of clones Z5scFv3 and Z5scFv7

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* Sequences are given of randomly chosen scFvs with no affinity for U1 RNA (V1–V4: VHscFv3κ; V5–V7: VHscFv7κ; V8–V12: VHscFv3λ; V13–V15: VHscFv7λ), nonselected scFvs with affinity for U1 RNA (V6–V19), scFvs selected on stem loop II of U1 RNA (II-3.1–II-3.9 and II-7.1–II-7.8), and scFvs selected on stem loop IV of U1 RNA (IV-3.1, IV-3.2, and IV-7.1, IV-7.2, IV-7.3). U1 RNA binding clones are bold. Primer-encoded amino acid mutations are shown in lower case letters. Basic amino acid mutations (K, R, H) are underlined.
Because the U1 RNA is contained in the human U1 snRNP particle, and because the Abs are directed to a free, accessible part of this complex (25), it is reasonable to assume that the patient’s U1 snRNP complex somehow became the target of the autoimmune response. Although the underlying cause for this is still unknown, there are indications that the group of patients with anti-U1 RNA autoantibodies is immunogenetically and clinically distinct from the anti-U1 RNA-negative patient group (5). Anti-U1 RNA Abs have been reported to be present in the more severely affected patient group (5) and, as is the case with anti-dsDNA Ab, the anti-U1 RNA Ab titer appears to correlate with disease activity (9). Also HLA-DR2/DR4, Raynaud’s phenomenon, and synovitis are significantly increased in the anti-U1 RNA-positive patient group (5). It is clear that further studies are needed to explain why these Abs develop.

**V H gene restriction**

Both anti-U1 stem loop II specific Abs use the same germline V H gene (DP-35, VH3-11).

Although several previous reports suggested an overexpression of particular V genes coding for autoantibodies (26, 27), more recent studies seem to support the idea that the frequency of V H gene usage in autoantibodies is comparable with that of the normal repertoire (19). For example, in the case of anti-DNA autoantibodies no particular overexpression of V H genes could be found (28). Nevertheless, when one focuses on one particular autoantigen, preferential V H gene usage sometimes seems to occur (as in the case of the U1A and U1C autoantigens (17, 21, 29)), which could be explained by the fact that these V H genes probably have a natural fit for the immunodominant epitopes present on these autoantigens, resulting in their selection by the immune system for affinity maturation.

**V L gene restriction**

Both anti-stem loop II Abs were derived from the same light chain (3r). This light chain gene is used in 1% of the cases in the normal IgG repertoire, and after sequencing the light chains of 100 IgG-positive B cells from patient Z5 this particular light chain was not found at all. This means that there is no general overexpression of this light chain V gene product, neither in normal individuals (see also Ref. 19) nor in this patient.

It is generally accepted that the heavy chain plays a major role in Ag recognition, although there are several reports that indicate that the light chain is important for Ag binding as well. Collet et al. (30) reported that when a number of anti-HIV p120-positive V H genes were reshuffled with different V L s, 43 to 100% of the V L s supported binding to p120, and this strongly depended on the par-
IV-3.1, IV-3.2, IV-7.1, IV-7.2, and IV-7.3 are positive clones obtained via selection on stem loop IV. a filter binding (dot blot) assay (9); IP, immunoprecipitation; Anti-VSV, relative expression levels of scFv was determined via anti-VSV reactivity.

Light chain V gene usage of reshuffled scFvs. The light chain could be of influence for the fine specificity of an Ab directed to cytomegalovirus Gb. Murine anti-DNA Abs have been shown that a combination of VH3-11 with another light chain than 3r-derived light chains was found, all having different somatic mutations (Table II), and most of them also containing several positively charged amino acids in their CDRs. All of the clones also contained a serine at position 34 instead of a cysteine. This may be an indication for a genetic polymorphism. Alternatively, this might be a somatic mutation important for stem loop II RNA-specific binding. This possibility could not be verified because this light chain was not found to be used in the 100 IgG-positive B cell clones from this patient that were analyzed (19). The striking finding that a combination of VH3-11 with another light chain than 3r resulted in an Ab with another RNA recognition specificity was confirmed when selections were performed with the other autoepitope of U1 RNA, i.e., stem loop IV. Most clones that were obtained reacted with stem loop IV, although other RNAs like U3 RNA were recognized as well. In contrast to the situation for stem loop II, the stem loop IV epitope has not been characterized well. Correct folding of the stem loop structure is necessary for antibody recognition, and the upper part of the stem and the whole loop are the main regions targeted by patient autoantibodies (4). The stem loop IV epitope contains only four nucleotides (UUGC), which are stacked and form an extremely stable structure, which is also present in several unrelated RNAs. However, U3 RNA does not contain this particular heavy chain sequence. Ohlin et al. (31) reported that the light chain could be of influence for the fine specificity of an Ab directed to cytomegalovirus Gb. Murine anti-DNA Abs have been extensively studied in MRL/lpr mice, and although the heavy chain plays in most cases a major role the light chain is important as well (see, e.g., Refs. 32–36). To study the influence of the light chain in the anti-U1 RNA response, we performed reshuffling experiments with the total light chain repertoire of the patient. When selecting the reshuffled libraries with stem loop II RNA as Ag, we obtained only stem loop II specific binders when the VH chain had been combined with a 3r-derived light chain. A large number of different 3r-derived light chains were found, all having different somatic mutations (Table II), and most of them also containing several positively charged amino acids in their CDRs. All of the clones also contained a serine at position 34 instead of a cysteine. This may be an indication for a genetic polymorphism. Alternatively, this might be a somatic mutation important for stem loop II RNA-specific binding. This possibility could not be verified because this light chain was not found to be used in the 100 IgG-positive B cell clones from this patient that were analyzed (19). The striking finding that a combination of VH3-11 with another light chain than a 3r resulted in an Ab with another RNA recognition specificity was confirmed when selections were performed with the other autoepitope of U1 RNA, i.e., stem loop IV. Most clones that were obtained reacted with stem loop IV, although other RNAs like U3 RNA were recognized as well. In contrast to the situation for stem loop II, the stem loop IV epitope has not been characterized well. Correct folding of the stem loop structure is necessary for autoantibody recognition, and the upper part of the stem and the whole loop are the main regions targeted by patient autoantibodies (4). The stem loop IV epitope contains only four nucleotides (UUGC), which are stacked and form an extremely stable structure, which is also present in several unrelated RNAs. However, U3 RNA does not contain this

![FIGURE 3. Light chain V gene usage of reshuffled scFvs. The light chain V gene usage is shown for nonselected scFvs (random reshuffled light chains; V1–V15 in Table II), for U1 RNA binding scFvs (II-3.1–II-3.9, II-7.1–II-7.8, V16–V19), for scFvs specifically binding to stem loop II of U1 RNA and of scFvs binding to stem loop IV but not to stem loop II (designated stem loop IV binders).](http://www.jimmunol.org/)
sequence, suggesting that this element is not important for the recognition by the single-chain Abs selected by stem loop IV. Because the Abs selected from the reshuffled libraries are derived from scFVs (scFv3 and scFv7) recognizing an epitope composed of mainly double-stranded RNA (25), it is more likely that they target the double-stranded region of stem loop IV of U1 RNA and similar structures in other RNAs, e.g., U3 RNA.

In conclusion, the light chain seems to play a very important role in the recognition of RNA by the Ab, and the specificity of anti-U1 RNA Abs can be altered dramatically by replacement of the light chain. Recently, we reported that secondary light chain rearrangements are likely to occur relatively frequently in peripheral organs during or after the process of hypermutation (19). It might be possible that autoreactive B cells, once they have been triggered to proliferate, attempt to prevent autoreactivity by changing their light chain. This could, however, also lead to reactivity to other closely related proteins or nucleic acids. Therefore, it seems possible that via such secondary light chain rearrangements U1 RNA reactive B cells can change not only the affinity but also the specificity for its anti-RNA Ab. Our in vitro light chain reshuffling experiments show that combining a highly mutated VH gene with another light chain indeed can abolish binding to its original RNA target (e.g., stem II of U1 RNA) and enhance the affinity for other RNAs (e.g., loop IV of U1 RNA). It is tempting to speculate that secondary light chain rearrangements might be a way for the immune system to accomplish the so-called “epitope spreading” phenomenon. Autoantibodies to stem loop II of U1 RNA could via light chain rearrangements evolve into anti-stem loop IV or non-specific anti-RNA autoantibodies or vice versa. The fact that many SLE patients produce autoantibodies to both stem loops II and IV is even possible that such a mechanism is responsible for the development of Abs that could play a role in the pathogenesis of the disease.

Acknowledgments

We thank Dr. T. van Kuppeveld (Department of Biochemistry, University of Nijmegen, The Netherlands) for providing us with “RNA-magic coating” patients Z5, D18, HO, and O11 for donating bone marrow samples that were essential for this study; and Dr. W. Ouwehand (Blood Transfusion Centre, Cambridge, U.K.) for providing us with the pHENIX vector.

References