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T Cell Immunity in Connective Tissue Disease Patients Targets the RNA Binding Domain of the U1-70kDa Small Nuclear Ribonucleoprotein

Eric L. Greidinger,‡ Mark F. Foecking,‡ Kim R. Schäfermeyer,‡ Craig W. Bailey,‡ Shannon L. Primm,‡ David R. Lee,‡ and Robert W. Hoffman 2

Although the T cell dependence of autoimmune responses in connective tissue diseases has been well established, limited information exists regarding the T cell targeting of self Ags in humans. To characterize the T cell response to a connective tissue disease-associated autoantigen, this study generated T cell clones from patients using a set of peptides encompassing the entire linear sequence of the 70-kDa subunit of U1 snRNP (U1-70kDa) small nuclear ribonucleoprotein. Despite the ability of U1-70kDa to undergo multiple forms of Ag modification that have been correlated with distinct clinical disease phenotypes, a remarkably limited and consistent pattern of T cell targeting of U1-70kDa was observed. All tested T cell clones generated against U1-70kDa were specific for epitopes within the RNA binding domain (RBD) of the protein. High avidity binding of the RBD with U1-RNA was preserved with the disease-associated modified forms of U1-70kDa tested. The high avidity interaction between the U1-RBD on the polypeptide and U1-RNA may be critical in immune targeting of this region in autoimmunity. The T cell autoimmune response to U1-70kDa appears to have less diversity than is seen in the humoral response; and therefore, may be a favorable target for therapeutic intervention. The Journal of Immunology, 2002, 169: 3429–3437.

T he mechanisms through which immunity to self proteins develops in patients with autoimmune diseases remain incompletely defined. One proposal suggests that in a genetically susceptible individual, ordinarily cryptic forms of self proteins act as immunogens that participate in escaping tolerance, contributing to a sustained anti-self response (1, 2). Proposed mechanisms to expose putatively ordinarily cryptic epitopes include apoptotic modification, granzyme B cleavage, and metal-catalyzed oxidative modification (3–5). However, little attention has been focused on the T cells that may be involved in autoimmu-

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Despite the diversity of Ag forms available, the only clones that we generated from CTD patients reacted exclusively against determinants within the RNA binding domain (RBD) of the U1-70kDa polypeptide, spanning residues 92–202 (16). Five distinct T cell epitopes were identified within the RBD, including two that were recognized by T cells from multiple patients. This focused T cell reactivity directed against a limited portion of the molecule was in contrast to the diversity of patterns of Ab reactivity that we observed. Binding studies confirmed that U1-RNA bound with high avidity to the intact form of U1-70kDa, as well as to the modified forms of the polypeptide that have been implicated in the pathogenesis of CTDs. Thus, the high avidity interaction between the U1-RBD on the polypeptide and U1-RNA may be critical in immune targeting of this region in autoimmunity.

Materials and Methods

Subjects

All samples and data for this study were obtained through protocols approved by the University of Missouri Institutional Review Board (Columbia, MO). Sera from patients seen in the Division of Immunology and Rheumatology at the University of Missouri (Columbia, MO) were characterized for the presence of autoantibodies reactive with the U1-70kDa polypeptide using immunodiffusion, immunoblotting, and ELISA as previously described (17, 18). Sera were also characterized for reactivity with U1-RNA using an assay for binding to radioactively labeled U1-RNA, as described previously (19). Patients (P) (P1 to P6) studied for select all had IgG Abs against U1-70kDa. P4 had IgG Abs to U1-RNA. All patients were female. The P1, P2, and P4 were classified as mixed CTD. P3 and P5 were classified as SLE, and P6 was classified as undifferentiated CTD (20, 21). The patients exhibited mild to moderate disease activity with disease activity indexes ranging from 0–6 and prednisone doses ranging from 0–20 mg per day (22). All of the patients were receiving 400 mg hydroxychloroquine per day. None of the patients were receiving additional immunosuppressive drugs during the study. HLA typing was done using PCR-based, and DNA-based typing methods as described previously (23). In brief, this was done using a combination of subfamily-specific PCR and direct DNA sequencing of PCR-amplified products (24, 25). The HLA-DRB1 genotypes of the patients were: P1, DRB1*0101; P2, DRB1*0401, 1502; P3, DRB1*0101, 1302; P4, DRB1*0401, 1303; P5, DRB1*0301, 1301; and P6, DRB1*0301, 1301. The HLA genotypes of the controls were HLA-DRB1*0101, 0401; HLA-DRB1*0401, 0401; and HLA-DRB1*0701, 1501.

Recombinant Ag

Control unconjugated maltose-binding protein (MBP), U1-70kDa-MBP fusion protein, and C-terminal truncated forms of U1-70kDa-MBP fusion protein were expressed from the pMAL plasmid in Escherichia coli and affinity purified over amylose columns as previously described (14). For some experiments, forms of U1-70kDa-MBP were further purified by elution of the relevant band cut from 10% SDS-PAGE preparative gels as previously described (26). Caspase-cleaved U1-70kDa was produced by incubating intact U1-70kDa fusion protein with caspase-3 (R&D Systems, Minneapolis, MN) in buffer containing 10 mM HEPES (pH 7.4), 2 mM DTT, 5 mM EDTA, 5 mM DTT, and 1% Nonidet P-40 as previously described (6). To generate apoptotic samples, caspase-3 cleaved cell lysates or purified Ag forms were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 3% BSA, membranes were exposed to 1/10,000 dilutions of test sera, incubated with HRP-linked goat anti-human secondary Ab (Southern Biotechnology Associates, Birmingham, AL), and visualized with chemiluminescence (Pierce, Rockford, IL). Cell lysates were prepared from Jurkat cells using previously described protocols (4, 12). To generate apoptotic samples, samples were exposed to 1,650 J/m2 of UV-B light and incubated for 16 h before harvesting. To make oxidatively cleaved and granzyme B-cleaved samples, cell lysates were incubated with 100 μM iron (II) sulfate and 2 mM sodium ascorbate for 30 min, or with 42 μM granzyme B in the presence of 2 mM iodoacetamide for 1 h, respectively.

Peptide design and synthesis

Peptides were synthesized using N-(9-fluorenyl) methoxycarbonyl solid-phase chemistry on an Applied Biosystems Model 432A Peptide synthesizer (Applied Biosystems, Foster City, CA) or were purchased (Research Genetics, Huntsville, AL) (27). Peptides were analyzed for purity and sequence fidelity using HPLC and mass spectrometry. The peptides that were used were required to have at least 90% purity and unambiguous sequence fidelity. For experiments using pooled peptides, each peptide was synthesized and validated individually, and equal molar quantities of every peptide in the pool were used.

Generation of human T cell clones

T cell clones used in these studies were generated as described previously (28). In brief, cells were obtained by apheresis and then subject to density gradient centrifugation using Histopaque (Sigma-Aldrich, St. Louis, MO). Patient PBMC were used immediately or cryopreserved for use as APCs to restimulate clones. A total of 5 × 104 cells were cultured in RPMI (Life Technologies, Rockville, MD) with gentamicin 20 μg/ml, 2 ml·g−1 l-glutamine, 15% AB-positive human serum, and pooled peptides. As for Ags, a series of synthetic peptides 25 residues in length were used. Each peptide spanned the previous peptide in the series beginning at residue 13. The entire series of peptides was designed to span the U1-70kDa polypeptide. Cells in a final volume of 5 ml were placed in a 25-cm2 flask and incubated in 5% carbon dioxide at 37°C. Cells were restimulated with 5 × 104 autologous APCs irradiated with 30 Gray and Ag in fresh medium on day 7. On day 14, T cell blasts were separated by centrifugation on a density gradient (Percoll; Amersham Pharmacia Biotech, Piscataway, NJ) and cloned by limiting dilution in the presence of pooled peptides, fresh irradiated autologous APCs, and 10 U/ml IL-2 (R&D Systems) as described previously (13, 27, 28). Cloning was attempted twice with every donor. Cells were cloned by limiting dilution and those that were positive for growth, seeded at the lowest number of cells per well, were selected for expansion. Typically, 10–20 wells were expanded and tested for reactivity with U1-70kDa peptides. Those clones that exhibited strong reactivity with U1-70kDa and no reactivity with APCs alone were studied further. All these clones reactive with autologous APCs alone were identified, they were not characterized further. Cell surface phenotype of clones was determined by flow microfluorometry as described previously (13).

Proliferation assay

A total of 2 × 104 T cells in complete medium were cultured for 48 h in 96-well flat-bottom tissue culture plates and then pulsed for 18 h with 1 μCi/well ofTdR. Cells were harvested and TdR incorporation detected by liquid scintillation counting (13).

MHC blocking of Ag-induced proliferation and MHC restriction element mapping

A series of HLA-homozygous lymphoblastoid cell lines (LCLs) of known HLA genotypes and HLA-DQ, and DP-specific mAb were used in Ag stimulation and mAb blocking experiments to define the restriction elements used by individual T cell clones, as described previously (28).
Searches for similarity with known DNA and protein sequences

Searches for similarities between T cell epitopes identified in the present study and all DNA and protein sequences in GenBank Database were done using basic local alignment search tool (National Center for Biotechnology Information, Bethesda, MD). Searches for similarity were done using the core T cell epitopes identified and then repeated using the information derived from alanine substitution analysis to distinguish residues that tolerate amino acid substitution (e.g., searches of sequences HMVYSKRSSGP R and XXVYXXRXXGPR against GenBank Database for homology).

Determination of U1-RNA binding avidity to U1-70kDa polypeptide

U1-RNA was produced and purified as previously described (19), except 35S-labeled UTP (NEN, Albany, MA) was used in the preparation of the labeled product. Briefly, the RNA was produced in vitro transcription from the corresponding DNA contained in linearized SP64 (a kind gift from Dr. S. Deutscher, University of Missouri), followed by template digestion with DNase I and purification by Sephadex spin column chromatography.

Saturation binding experiments were modified from Query et al. (16) and Kessler (29). In each experiment, a constant amount of unlabeled U1-RNA was used to dilute different amounts of 35S-labeled U1-RNA of known specific activity in NET-2 buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl (pH 7.4) with 0.05% Nonidet P-40). This was allowed to interact with 1 pmol of test protein for 20 min at room temperature, after which the mixture was immunoprecipitated by a high-titer anti-U1-70kDa positive antisem previously documented to have no reactivity to U1-RNA. This was precipitated with washed Pansorbin (Calbiochem, San Diego, CA), and the complex washed four times with ice-cold NET-2 after removal of supernatants for liquid scintillation counting. The washed pellets were also counted, and total bound and free fractions were then calculated. This was used to determine the $K_d$ using a one-site binding nonlinear regression model with Prism 3.0 (GraphPad Software, San Diego, CA).

Results

**U1-70kDa specific human T cell clones were generated from CTD patients**

U1-70kDa-specific human T cell clones were successfully generated from six patients (P1 to P6) against a pool of peptides, each 25 residues in length, spanning the entire U1-70kDa molecule. Each peptide overlapped the previous peptide in the series by 13 residues. Representative U1-70kDa reactive T cell clones that were generated and studied from three patients are shown in Fig. 1. As shown in Fig. 1, all clones demonstrated a strong proliferative response to a pool of U1-70kDa peptides or PHA, but did not respond to APCs alone or APCs with an irrelevant peptide. All clones examined had a CD4-positive cell surface phenotype. These clones and others from P1 to P6 were subsequently characterized to determine more precisely which region of U1-70kDa they were responding against. Sera from the patients studied were noted to be diverse with regard to immunoblot recognition of the apoptotic, oxidatively cleaved, and granzyme B-cleaved forms of U1-70kDa (Table I).

**U1-70kDa-specific human T cell clones could not be generated from HLA-matched healthy blood donors**

Three separate attempts were made to generate T cell clones from three normal control subjects using U1-70kDa pooled peptides. These normal control subjects were matched for the presence of the HLA-DRB1*0401 or HLA-DRB1*1501 responder genotype. Although we could generate T cell clones from these donors specific for a control Ag (tetanus toxoid or heterogeneous nuclear ribonucleoprotein), no U1-70kDa-specific clones could be generated from normal controls.

*Table 1. Immunoblot recognition of modified forms of U1-70kDa by sera of study patients*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Intact</th>
<th>Apoptotic</th>
<th>Oxidative</th>
<th>Granzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P2</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>P3</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>P4</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P5</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>P6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$ P1–P6 indicates anti-U1-70kDa-positive study subjects. Apoptotic indicates Abs recognizing the 40-kDa caspase-cleaved fragment of U1-70kDa. Oxidative indicates Abs recognizing the 30-kDa oxidatively cleaved form of U1-70kDa. Granzyme indicates Abs recognizing the 60-kDa granzyme B-cleaved fragment of U1-70kDa.

$^b$ +, Abs detected; −, Abs not detected.

**FIGURE 1. Human T cells reactive with U1-70kDa Ag. Human T cell clones from P1, P3, and P5, reactive with U1-70kDa peptides. Representative proliferative responses of T cell clones are shown. Clones were stimulated with PHA, a pool of peptides spanning U1-70kDa protein in the presence of autologous APCs, clone with APCs and no peptide, or clone, APCs, and an irrelevant peptide. The results shown represent the mean of an experiment done in triplicate. The SEM between the results presented for each triplicate was <5%.

Five T cell epitopes were identified on the U1-70kDa polypeptide by T cell clones derived from patients

An iterative process was used to define the regions of the U1-70kDa polypeptide recognized by human T cell clones. Clones were initially analyzed for proliferation against a pool of peptides 25 residues in length, overlapping by 13 residues with the previous peptide in the protein, which taken as a set span the entire U1-70kDa polypeptide. Clones were subsequently tested against smaller pools of peptides and eventually against individual peptides 15 residues in length. Finally, select clones were tested against additional peptides truncated from either the amino or C terminus to define the minimal epitopes recognized (Fig. 2). Five T cell epitopes were identified by U1-70kDa-specific T cell clones on the U1-70kDa protein using the 25-residue and 15-residue synthetic peptides. Peptides encoding these five epitopes (E1) 1–5 reside, respectively, between residues 97–111, 112–136, 133–147, 151–165, and 173–187 of the full-length U1-70kDa protein. As shown in Table II, clones that recognized E1 were generated from P1, clones that recognized E2 were generated from P2, clones that...
recognized E3 were generated from P2 and P3, clones that recognized E4 were generated from P4, and clones that recognized E5 were generated from P1, P2, P3, P5, and P6.

Minimal T cell epitopes were identified from U1-70kDa using overlapping peptides, truncated peptides, and alanine scanning

The three T cell epitopes against which T cell clones were most frequently generated (E1, E3, and E5) were analyzed further to determine the minimal T cell epitope recognized by the clones. These minimal epitopes are summarized in Fig. 2. To determine the minimal region within the U1-70kDa-E1 required for T cell recognition, truncated variants of the 15 residue peptide AQG-DAFKTLFVARVN were synthesized and tested. As shown in Fig. 3A, stimulation of clones was observed only when the truncated peptides contained the core sequence DAFKTLFVARVN (contained in the full-length U1-70kDa polypeptide at residues 100–111). To determine the minimal region within the U1-70kDa-E3 required for T cell recognition, truncated variants of the 15 residue peptide HMVYSKRSGKPRGYA were synthesized and tested. As shown in Fig. 3B, stimulation of clones was observed only when the truncated peptides contained the core sequence HMVYSKRS GKPR (contained in the full-length U1-70kDa polypeptide at residues 133–144). To determine the minimal region within the U1-70kDa-E5 required for T cell recognition, truncated variants of the 15-residue peptide GRRVLVDVERGRTVK were synthesized and tested; stimulation of clones was observed only when the truncated peptides contained the core sequence VLVDVERGRTV (contained in the full-length U1-70kDa protein at residues 174–184; data not shown). These minimal epitopes are shown in Fig. 2; all reside within the RBD.

To define residues critical for MHC binding or T cell contact, peptides with sequential alanine substitutions of the core peptide sequences for minimal E1, E3, and E5, peptides were synthesized and tested for reactivity with the original T cell clones. Shown in Fig. 4A, alanine substitutions of the core peptide DAFKTLFVARVN for minimal E1 were synthesized and tested for their ability to induce proliferation of T cell clones. Substitution of the residues at positions 1, 3–6, 8, and 10–12 with alanine abolished T cell reactivity with DAFKTLFVARVN (E1). Positions 2 and 9 (underlined in Fig. 4A) that already contained alanine could not be evaluated. Alanine substitutions of HMVYSKRSGKPR (E3) revealed that alanine substitution at positions 3, 4, 7, and 9–12 abolished the ability of the peptide to stimulate the clone (Fig. 4B).

Finally, serial alanine substitutions of VLVDVERGRTV (E5) revealed that substitutions at positions 2, 3, and 5–11 abolished the ability of the peptide to stimulate the T cell clone (Fig. 4C). Alanine substitution at position 4 was partially inhibitory, while substitution of valine to alanine at position 1 was well tolerated.

**FIGURE 2.** T cell epitopes on U1-70kDa protein. The minimal T cell epitopes present on the U1-70kDa polypeptide are shown, labeled as epitopes 1–5. Also shown is the RBD of the polypeptide (residues 92–202), the caspase-cleavage site (residue 341), and the granzyme B cleavage site (residue 409).

**Table II. Epitopes recognized by human T cell clones specific for U1-70kDa**

<table>
<thead>
<tr>
<th>Clones Reactive with Epitope</th>
<th>Patient U1-70kDa-E1</th>
<th>U1-70kDa-E2</th>
<th>U1-70kDa-E3</th>
<th>U1-70kDa-E4</th>
<th>U1-70kDa-E5</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td></td>
<td>X</td>
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<td>P3</td>
<td></td>
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<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td></td>
<td></td>
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<td>X</td>
</tr>
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<td>P5</td>
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<td>X</td>
</tr>
<tr>
<td>P6</td>
<td></td>
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<td>X</td>
</tr>
</tbody>
</table>

**HLA-DR is the MHC restriction element used in T cell recognition of U1-70kDa peptides**

The MHC restriction element used by T cell clones was determined using Ag-induced proliferation in the presence of blocking concentrations of mAb specific for HLA-DR, HLA-DQ, or HLA-DP, or using a series of well-characterized homozygous LCL of known HLA genotypes as APCs. The HLA class II blocking mAb used were specific for either HLA-DR, DQ, or DP framework determinants. These were used to determine which HLA class II molecule was the restriction element for T cell recognition. It was found that all clones tested used HLA-DR as a restriction element for Ag recognition. A panel of LCL was then used to determine the specific HLA-DR allele used by the T cell clone. It was found that HLA-DRB1*0401 was used as the restriction element by clones from the two patients analyzed in detail. The complete HLA genotypes of all six of the donors were determined, and these are given in Materials and Methods. Interestingly, five of the six patients shared the closely related genotypes HLA-DRB1*0401 (P2, P4, and P5) and HLA-DRB1*0101 (P1 and P3).

**U1-70kDa polypeptides show homology with other spliceosomal proteins but not with other sequences in GenBank Database**

As a possible explanation for the limited targeting of anti-U1-70kDa T cell clones, we considered whether anti-U1-70kDa T cells evolved due to molecular mimicry with a microbial Ag. However, comparisons of the five T cell epitopes and their minimal peptides from truncation experiments with GenBank Database revealed homology only with other functionally related snRNPs from humans and other vertebrate species. Similar results were obtained when searches were performed with only the essential contact residues retained in the backbone of the peptide derived from alanine scanning experiments (e.g., searches of sequences HMVYSKRSGKPR and XXVYXXRXGKPR).
U1-RNA binds with similarly high avidity to the intact U1-70kDa polypeptide and to multiple modified forms of U1-70kDa implicated in the development of autoimmune diseases

As shown in Table I, the patients exhibited a broad spectrum of profiles of Abs reactive with different modified forms of U1-70kDa (caspase-cleaved, granzyme B, or oxidatively cleaved), but had a limited T cell response directed solely at the RBD (Fig. 2). As a possible explanation for the predominance of T cells reactive against the RBD of U1-70kDa among anti-U1-70kDa positive CTD patients, we considered whether similar T cell clones could be generated against the endogenous forms of the U1-70kDa Ag implicated in autoimmune disease pathogenesis. To assess for conserved structure of the RBD in modified forms of the molecule, we investigated whether the RBD demonstrated conserved U1-RNA binding affinity between the intact, caspase-cleaved, and oxidatively cleaved forms of the molecule. The caspase-cleaved and oxidatively cleaved forms of the molecule were generated by exposing the purified full-length form of U1-70kDa to caspase-3 and iron/ascorbate, respectively, following standard protocols (4, 6). Using C-terminal truncation mutants, we identified the site(s) of oxidative cleavage of U1-70kDa to be distal to residue 205, preserving the RBD sequence (Fig. 5). Thus, all modified forms retained the RBD.

We next determined if there were detectable differences in binding affinity to U1-RNA by the different forms. Using saturation binding experiments, the affinity of binding of U1-RNA was determined for the intact, caspase-cleaved, and oxidatively cleaved forms of U1-70kDa. A typical saturation binding curve from these experiments is shown, demonstrating excellent fit of the data with the model curve (coefficient of determination = 0.9999) (Fig. 6). The $K_d$ of U1-RNA with each of the forms of U1-70kDa tested (Table III) falls in the 10 pmol range. These values are similar to the dissociation constant reported for the homologous and highly avid interaction of the U1-A protein with U1-RNA (30). Thus, all forms of modified U1-70kDa bound to U1-RNA with similar high affinities.

The presence of U1-RNA does not directly influence the response of T cell clones to U1-70kDa

Because double-stranded and stem-loop RNAs have been shown to have direct immunostimulatory effects (unrelated to TCR recognition of Ag), and since the U1-RNA possesses a stem-loop structure, we examined whether U1-RNA acted as an immune adjuvant (31). To test for this possibility in vitro, we tested the proliferative response of U1-70kDa-specific human T cell clones to U1-70kDa
peptides presented by autologous irradiated APCs in the presence or absence of equimolar quantities of U1-RNA. No increase in the proliferative responses to the U1-70kDa peptides could be observed with the addition of U1-RNA compared with peptides alone (data not shown). The kinetics of survival of U1-RNA was not examined and these experiments could have failed due to RNA degradation. Studies are now in progress to examine the mechanism(s) by which U1-RNA may influence T cell epitope selection and/or T cell activation. Possible mechanisms could include influences on Ag processing or costimulation (31, 32).

FIGURE 4. Alanine scanning of T cell epitopes.
A, Alanine scanning of E1. Using serial alanine substitutions of the U1-70kDa minimal E1 core peptide sequence, DAFKTLFVARVN, it was possible to define those residues in the peptide which were critical for HLA binding or TCR interaction. The top line is the peptide without substitution. The next lines are serial alanine substituted peptides. Alanine substituted residues are in bold. Note that the core peptide contained alanine at positions 2 and 9 (underlined); and therefore, these positions could not be assessed. Alanine substitutions for native residues in the peptide at positions 1, 3–6, 8, and 10–12 resulted in the loss of the T cell stimulatory capacity of the peptide.

B, Alanine scanning of E3. Using serial alanine substitutions of the U1-70kDa minimal E3 peptide sequence, HMVYSKRSGKPR, it was possible to define those residues in the peptide which were critical for HLA binding or TCR interaction. The top line is the peptide without substitution. The next lines are serial alanine substituted peptides. Alanine substitutions for native residues in the peptide at positions 3, 4, 7, and 9–12 resulted in the loss of the T cell stimulatory capacity of the peptide.

C, Alanine scanning of E5. Using serial alanine substitutions of the U1-70kDa minimal epitope core peptide sequence, VLVDERGRTV, it was possible to define those residues in the peptide which were critical for HLA binding or TCR interaction. The top line is the peptide without substitution. The next lines are serial alanine substituted peptides. Alanine substitutions for native residues in the peptide at positions 2, 3, and 5–12 resulted in the loss of the T cell stimulatory capacity of the peptide.
Discussion

This is the first study to explore the diversity of T cell clones against the U1-70kDa protein in human autoimmune disease by raising clones against a complete pool of antigenic peptides. Consistent with our previous results describing highly restricted complementarity-determining region 3 usage by anti-U1-70kDa clones, a limited number of T cell epitopes were observed in the anti-U1-70kDa response (14). Remarkably, this response was exclusively limited to epitopes within the RBD of the molecule (residues 92–202), consistent with results previously reported in an animal model of systemic autoimmunity. There, Muller and colleagues (32) found that murine T cell responses in unprimed 7-wk-old MRL/Mp-Ipr mice are directed against residues 131–151 of U1-70kDa. This region is within the RBD and corresponds with the second most prevalent epitope identified in the present study among CTD patients (E3 in Fig. 2).

The finding that there are a limited number of T cell epitopes on U1-70kDa is similar to results of T cell epitope mapping of another antigenic spliceosomal polypeptide, the Sm Ag. We have recently reported that there were three T cell epitopes on the Sm-B polypeptide and two on the Sm-D polypeptide (33). We have also observed RBD-specific T cell clones directed against the heterogeneous nuclear ribonucleoprotein A2 autoantigen (34). The findings of the present study are also consistent with T cell epitope mapping of other autoantigens, including myelin basic protein in multiple sclerosis and topoisomerase I in systemic sclerosis, where only limited numbers of T cell epitopes were found to be present on the autoantigen (35, 36).

To further define the minimal epitope recognized by T cells and to identify the essential contact residues on the Ag, truncated peptides and alanine substitutions of the core peptides were synthesized and tested for their ability to stimulate T cell clones. These studies revealed substitutions of most residues were not well-tolerated, although there were specific exceptions. The findings were consistent with the presumed MHC binding motifs for the peptides, and were similar to those recently reported for the Sm-B and Sm-D polypeptides, where alanine substitutions were well-tolerated in selected positions of the peptide, but not tolerated in other positions (33). If the results of the current study accurately reflect the diversity of T cells involved in the pathogenesis of anti-U1-70kDa autoimmunity, epitope-specific T cell immunomodulatory therapies may be plausibly envisioned. This is in contrast to B cell immunity to U1-70kDa, where multiple, widely scattered epitopes have been identified (37).

Experiments using blocking mAb against HLA framework determinants and LCL homozygous for HLA demonstrated that anti-U1-70kDa clones studied were restricted in Ag presentation by HLA-DRB1*0401. Based upon the patients’ genotypes (see Materials and Methods), other restriction elements presumably are used in patients lacking HLA-DRB1*0401; although notably five of six patients share the HLA-DRB1*0401 or the closely related genotype, *0101. Studies directly testing HLA binding and TCR interactions, using the combination of HLA-transfected L cells with site-directed mutagenesis of cloned TCR genes expressed in TCR-deficient Jurkat cell lines are underway to more precisely define the molecular interactions between U1-70kDa self-reactive peptides, HLA, and the TCR (38, 39). However, the fact that T cell clones exclusively targeting the RBD of U1-70kDa were isolated from a patient (P6) without HLA-DRB1*0401 or HLA-DRB1*0101 suggests that a single restrictive HLA genotype is unlikely to fully explain the preferential targeting of CTD patient T cells to the RBD.

The properties of the RBD of the U1-70kDa polypeptide that preferentially target it for immune recognition have not been identified. Because the pooled peptides used to select the clones did not have RNA bound to them, it is unlikely that bound RNA plays a direct role in T cell recognition of Ag. However, RNA binding could influence the shaping of the initial anti-U1-70kDa T cell repertoire. For example, U1-70kDa bound to U1-RNA may be processed differently than other parts of the polypeptide. For example, such an effect could potentially occur in peripheral APCs relevant to the initiation of an autoimmune response if RNA binding protects this region of the protein from proteolysis. Alternatively, preferential T cell targeting of the RBD could be due to relative inefficient thymic deletion of autoreactive T cells against this region, potentially because the thymic Ag-processing pathway is inefficient in proteolyses of the RBD epitope or if it remains intact in a subset of peripheral APC. The uptake of RNA-complexed modified Ag as dead cell fragments in the periphery (such as in the case of apoptotically or granzyme B-exposed cells) may also contribute to differential central vs peripheral tolerance induction. Finally, the proposed target of the RBD could be due to effects entirely unrelated to the binding of the RNA itself; for example, the RBD could have promiscuous binding to MHC class II
molecules, similar to that described for superantigen or the HLA class II-associated invariant chain-derived peptide (40, 41).

Because the peptide ligand against which T cells were screened did not include posttranslationally modified peptides, we cannot exclude the existence of additional T cell epitopes on the U1-70kDa molecule. Potentially relevant posttranslational modifications of this and related snRNP polypeptides have been reported including glycosylation, phosphorylation, and arginine methyl-

ation (42–44). However, many autoantibodies targeting unmodified U1-70kDa peptides have been identified from diverse sections of the molecule both within and outside of the RBD (45).

In summary, we found that there was a highly restricted set of U1-70kDa self peptides recognized by T cells from CTD patients, with T cell recognition limited to five epitopes. All T cell epitopes were found to be located within the RBD of the U1-70kDa polypeptide. These findings, in conjunction with our recently reported findings that T cells against the Sm proteins preferentially target the areas of highly avid protein-protein interactions between these molecules (33) raise the question whether protein-nucleic acid or protein-protein interactions may be a general feature of T cell targets in autoimmunity. The T cell autoimmune response to U1-70kDa in CTDs appears to have less diversity than is seen in the humoral response and therefore may be a favorable target for therapeutic intervention.

References

bodies specificity in sequential autoimmune human antisera follows a character-

istic order that implicates the U1-70 kd and B and D polypeptides among patients with systemic lupus erythematosus and mixed connective tissue disease. Arthritis Rheum. 40:1493.


