# Molecular Characterization of Human Ro/SS-A Antigen

## Amino Terminal Sequence of the Protein Moiety of Human Ro/SS-A Antigen and Immunological Activity of a Corresponding Synthetic Peptide

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## Abstract

**The Ro/SS-A antigen was purified from an Epstein-Barr virus–transformed human B lymphoblastoid cell line. The amino terminal amino acid sequence of the 60-kD polypeptide bearing this antigenic epitope was determined to be:**

**5 10**
**glu/ala-pro-ala-val-tyr-phe-lys-glu-gln-phe-leu-asp-gly-asp-**

**15 20**
**gly-trp-thr-ser-arg-trp-ile-glu-ser-lys.**

**A peptide composed of residue 6-19 was synthesized by the solid-phase method. Immunodiffusion-defined monospecific autoimmune sera to Ro/SS-A reacted with this synthetic peptide in ELISA, whereas autoantibodies with other specificities such as anti-La/SS-B and anti-Sm, as well as normal human sera, were not reactive. In addition, rabbit anti-peptide 6-19 antisera reacted specifically with native human Ro/SS-A antigen in ELISA. Furthermore, this synthetic peptide inhibited the binding of rabbit anti-peptide antiserum to native human Ro/SS-A. An additional synthetic peptide corresponding to residues 7-24 partially inhibited the binding of a patient anti-Ro/SS-A serum to native Ro/SS-A. These results suggest that the amino terminal portion of the molecule represents a major epitope of Ro/SS-A. The determination of the amino acid sequence of Ro/SS-A and the availability of synthetic peptide(s) bearing this antigen should provide additional approaches to further characterize the autoimmune response to this antigen.**

## Introduction

An immune response to the Ro/SS-A antigen was originally described in patients with systemic lupus erythematosus (SLE) (1) and Sjogren's syndrome (SS) (2, 3). Subsequently, anti-Ro/SS-A autoantibodies were implicated in the renal disease associated with SLE (4) and with extraglandular disease such as vasculitis in Sjogren's syndrome (5). Recently, these autoantibodies have been found in a high percentage of patients with subacute cutaneous lupus erythematosus (SCLE) (6, 7), as well as in neonatal lupus erythematosus (NLE)[1]/congenital heart block (8–13). Because of the association of maternal anti-Ro/SS-A with the expression of NLE skin disease and the similarities between NLE and SCLE skin lesions, anti-Ro/SS-A antibodies have been implicated in the pathogenesis of these two disorders. Indeed, Ro/SS-A antigen has been detected in various types of human tissues, including skin and heart (14, 15).

Recent studies have begun to elucidate the molecular structure of the Ro/SS-A antigen. Ro/SS-A is present on a particle comprised of RNA and a single polypeptide. In humans, four low molecular weight RNAs, HY1, HY3, HY4, and HY5, have been found, while in the mouse only two (MY1 and MY2) have been detected (16, 17). The protein moiety of the Ro/SS-A complex is a 60,000-mol-wt polypeptide that bears the Ro/SS-A antigenic epitope(s) (17–19). Because of the wide implications of the Ro/SS-A antigen–antibody system in various human disease states, detailed information of the structure of this antigen is desirable. With pure Ro/SS-A in hand, one could study its interaction with autoantibodies, which would hopefully lead to a better understanding of the mechanism of autoimmune-related tissue injury.

The present investigation was initiated to further characterize the polypeptide component of the human Ro/SS-A antigen. Our studies have included the biochemical purification of the Ro/SS-A antigen, determination of the amino terminal amino acid sequence of the Ro/SS-A polypeptide, and an examination of the antigenic activity of synthetic peptides (SP) corresponding to a portion of the amino terminal sequence.

## Methods

*Antigen source.* The Ro/SS-A antigen was isolated from an extract of an Epstein-Barr virus transformed human B lymphoblastoid cell line (Wil-2). The cells were grown in Eagle's medium supplemented with 2 mM glutamine, sodium pyruvate, nonessential amino acids, 10% fetal calf serum, penicillin (10,000 U/ml), and streptomycin (10 mg/ml). The cells were centrifuged at 35 *g* for 12 min and washed with PBS (0.14 M NaCl, 0.01 M phosphate, pH 7.4) three times. The packed cells were mixed with the same volume of PBS containing 1 mM PMSF. The suspension was sonicated on ice with ten 15-s pulses using a heat system sonicator at a setting of 9. The sonicate was then centrifuged at 12,100 *g* for 1 h and the supernatant subjected to ammonium sulfate precipitation as previously described (18).

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1. *Abbreviations used in this paper:* CIE, counterimmunoelectrophoresis; KLH, keyhole limpet hemocyanin; NLE, neonatal lupus erythematosus; N-PAGE, native polyacrylamide gel electrophoresis; PBE, polybuffer exchange; SCLE, subacute cutaneous lupus erythematosus; SP, synthetic peptide.

*Human antisera.* Anti-Ro/SS-A sera were selected by the presence of a single precipitin line in Ouchterlony analysis having complete identity with prototypic sera used in previous studies (18). The absence of antibodies to other nuclear antigens such as La/SS-B, U1-RNP, and Sm was also confirmed by ELISA. Monospecific anti-Sm and anti-La/SS-B human autoimmune sera were originally obtained from the laboratory of Dr. E. M. Tan (Scripp Research Institute, La Jolla, CA). Each formed a single precipitin line in double immunodiffusion analysis with the appropriate antigens and did not react with purified Ro/SS-A in the ELISA.

*Polybuffer ion exchange column chromatography.* Polybuffer exchange (PBE) (PBE 94; Pharmacia Fine Chemicals, St. Louis, MO) was equilibrated with 24 mM borate buffer, pH 7.6, and packed into a 1.5 × 30-cm column. Ro/SS-A antigenic activity was eluted from the column by a previously described procedure (18).

*SDS-PAGE and Western blots.* SDS-PAGE was performed according to the method of Laemmli (20). The proteins were separated using 12.5% polyacrylamide gel in the presence of SDS. Western blot analyses were performed as described by Towbin et al. (21). Ro/SS-A obtained by native PAGE (N-PAGE) was electrophoresed in 12.5% SDS-PAGE. The resolved protein bands were transferred electrophoretically from the gel to a nitrocellulose sheet in buffer consisting of 192 mM glycine, 25 mM Tris, and 20% methanol vol/vol, pH 8.3, at 60 V, 200 ma for 4 h at room temperature followed by 100 mA for 16 h at 4°C. To ensure that the protein was properly transferred, one of the sheets was stained with 0.025% amido black in 20% ethanol/5% acetic acid and compared with the original gel-staining pattern. The remaining binding sites on the nitrocellulose sheet were blocked with 1% BSA in 10 mM Tris-HCl saline, pH 7.4, containing 0.05% Tween. The blots were then incubated with sera diluted with 1% BSA in 10 mM Tris buffer for 15 h. After washing three times with Tris buffer, $^{125}$I-labeled goat anti-human IgG or horseradish peroxidase-conjugated goat anti-human IgG (Tago Inc., Burlingame, CA) was added. The blots were rinsed three times with Tris buffer followed by a distilled water wash. After air drying, the blots were exposed to XRP film for 3–4 d at −20°C. In experiments with horseradish peroxidase goat anti-human IgG, diaminobenzidine tetrahydrochloride (12.5 mg/100 ml Tris buffer, pH 7.6) was used.

*ELISA.* The native Ro/SS-A antigen (5 μg/ml) or SP (10 μg/ml) in PBS was added to wells of a microtiter plate and incubated 16 h at 4°C. The plates were washed with PBS-Tween and the remaining sites were coated with 1% BSA for 1 h. After washing three times with PBS-Tween, sera diluted with 1% BSA, 0.5% bovine gamma globulin in PBS-Tween were added and incubated for 2 h. The plates were then washed three times with PBS-Tween. Peroxidase-conjugated goat anti-human IgG diluted 1:3,000 in PBS-Tween containing 1% BSA and 0.5% bovine gamma globulin was added and incubated for 2 h at room temperature. The plates were washed in a similar manner. The color was developed by adding a peroxidase substrate solution containing 1 mg/ml of 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) and 0.005% $H_2O_2$ in 0.1 M McIvaine's buffer, pH 4.6. The optical density was measured by a Titertek Multiskan Reader.

To determine the proportion of the total native Ro/SS-A antigenic activity that is present on the amino terminal SP, increasing amounts of keyhole limpet hemocyanin (KLH)–coupled SP ($SP_{7-24}$) were preincubated with a monospecific patient anti-Ro/SS-A serum. After a 16-h incubation at 4°C, the inhibited serum aliquots were diluted to the appropriate concentrations and added to a native Ro/SS-A antigen-coated plate. The degree of inhibition was then calculated by comparison with the reactivity of untreated anti-Ro/SS-A serum.

*Protein sequencing.* The purified Ro/SS-A antigen preparations, either from N-PAGE or from SDS-PAGE, were subjected to automated Edman degradation using either the gas-phase sequencer (model 470; Applied Biosystems, Inc., Foster City, CA) with the on line HPLC PTH amino acid identification system (model 120; Applied Biosystems, Inc.) or a spinning cup sequencer (model 890M; Beckman Instruments, Inc., Palo Alto, CA). In the later case, the PTH amino acids were identified using a NovaPac column in an HPLC system (model 840; Waters Assoc., Millipore Corp., Milford, MA).

*Peptide synthesis.* A peptide ($SP_{6-19}$) consisting of amino acids 6-19 with a cysteine added to the amino terminus was synthesized on a solid-phase support using a peptide synthesizer (model 430A; Applied Biosystems, Inc.). The peptide was deprotected by hydrogen fluoride cleavage. The peptide was sequenced as above to confirm the sequence after synthesis. A portion of the peptide was conjugated to KLH. A longer peptide ($SP_{7-24}$) corresponding to ammino acid 7-24 was also synthesized and conjugated by the same method.

*Immunization protocol.* Female rabbits (New Zealand White) were immunized subcutaneously in the neck region with 0.5 mg of peptide $SP_{6-19}$ conjugated to KLH emulsified in Freund's complete adjuvant. At 1 wk, the animals were similarly immunized with 1 μg of the unconjugated peptide alone. At monthly intervals thereafter the rabbits were boosted with the KLH-conjugated peptide emulsified in Freund's complete adjuvant.

## Results

*Purification of the Ro/SS-A molecule.* Previous studies had established that the Ro/SS-A antigen was present in the 33–80% saturated ammonium sulfate fractions of lysates from the B lymphoblastoid cell line. These fractions were pooled and dialyzed against 24 mM borate buffer, pH 7.6, and applied to a polybuffer ion exchange column. After washing the column with 24 mM borate buffer (eluate with $OD_{280} < 0.1$), a stepwise sodium chloride gradient was applied to the column. The majority of La/SS-B antigen was eluted in the 0.1 and 0.2 M sodium chloride fraction, whereas all the Ro/SS-A antigen was recovered in the 0.5 and 1 M sodium chloride fractions (Fig. 1). After concentration by ammonium sulfate precipita-

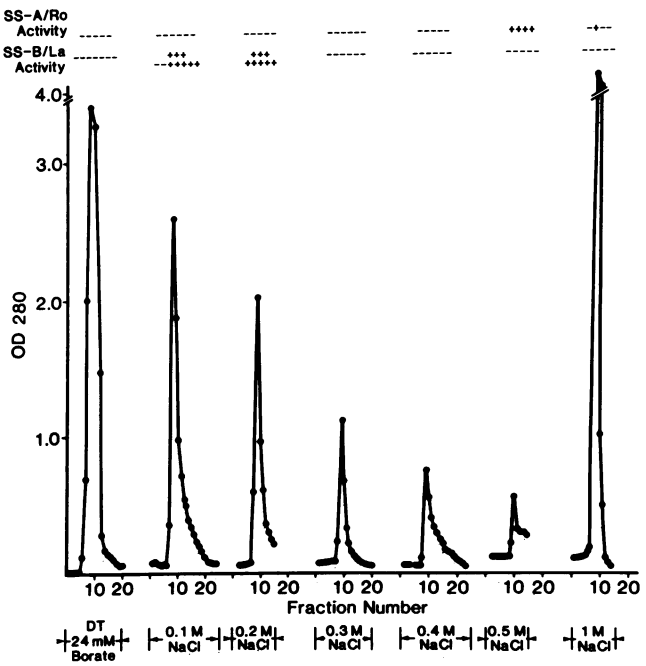


*Figure 1.* PBE chromatography of ammonium sulfate precipitated material from Wil-2 cell extract. The column (1.5 × 30 cm) was eluted with a stepwise gradient of increasing sodium chloride concentration from 0.1 to 1.0 M. The Ro/SS-A and La/SS-B antigenic activities were detected by CIE. The La/SS-B antigenic activity was eluted by 0.1 M and 0.2 NaCl, whereas Ro/SS-A antigen was recovered in the 0.5 and 1 M NaCl eluates.

tion, a modest amount of La/SS-B activity was also detected in the 1 M NaCl fraction by counterimmunoelectrophoresis (CIE) against prototypic antisera.

The antigenically active material from the PBE column (0.5 and 1 M NaCl fraction) was further purified by electrophoresis in a 5.6% N-PAGE. After electrophoresis, the gel was divided into a series of 10-mm slices and the material was eluted from the gel with distilled water. By CIE, maximal Ro/SS-A antigen activity was located in the fraction with a retardation factor ($R_f$) of 9.0 (Fig. 2), whereas the maximal La/SS-B activity was recovered in the region with an $R_f$ of 0.7. When the antigenically active fraction from the N-PAGE was subjected to SDS-PAGE, it contained a single-stained band of 60,000 mol wt whose identity was confirmed by Western blot analysis (Fig. 3).

*Absorption spectrum of purified Ro/SS-A antigen.* It has previously been demonstrated that the Ro/SS-A antigen is a complex of low molecular weight RNAs and a 60-kD polypeptide. Enzymatic digestion and immunoblot analysis has also indicated that the protein moiety is essential for the immunologic activity. Since in our purification scheme we used human autoantibodies to trace the Ro/SS-A antigenic activity, it was of interest to determine whether the purified material still contained low molecular weight RNA. Ultraviolet absorbance analysis performed on highly purified Ro/SS-A exhibited maximal absorption at 270 nm (Fig. 4), indicating that this preparation still contained some RNA.

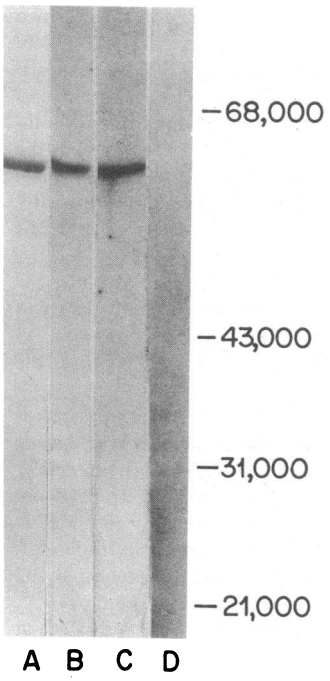


*Figure 3.* Western blot analysis of Ro/SS-A antigen. Ro/SS-A purified by N-PAGE was resolved on an SDS polyacrylamide gel, transferred to nitrocellulose, probed by human autoantibody to Ro/SS-A, and $^{125}$I-labeled goat anti-human IgG. (Lane *A*) Staining pattern of purified Ro/SS-A antigen fraction used in the transfer. (Lanes *B* and *C*) Autoradiographs of electrophoretically transferred Ro/SS-A antigenic fraction on a nitrocellulose sheet probed by the double antibody procedure with two human anti-Ro/SS-A sera (1:150). (Lane *D*) Control using normal human serum.

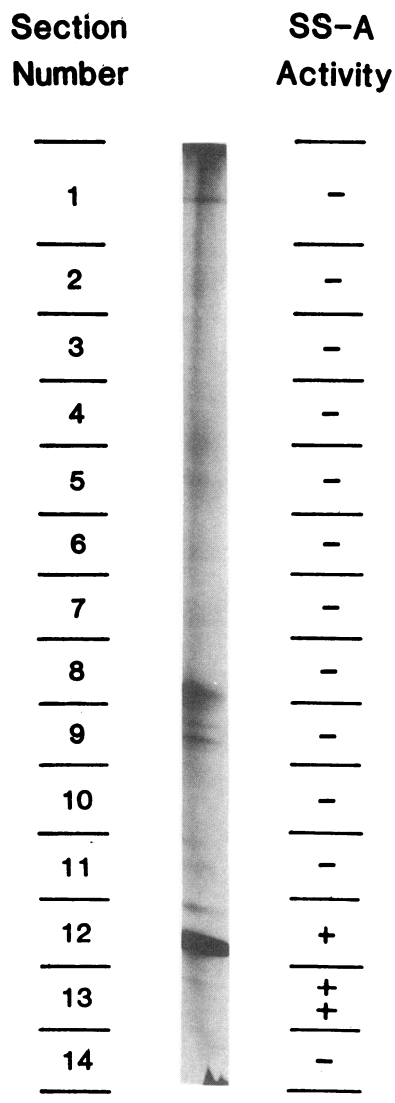


*Figure 2.* Profile of Ro/SS-A antigen containing fractions from PBE column on N-PAGE. The gel was divided into 10-mm slices and the material was eluted out of the gel by distilled water. The Ro/SS-A antigenic activity was detected by CIE analysis at sections 12 and 13 representing $R_f$s of 0.86–0.93.

*Aminoterminal amino acid sequence of human Ro/SS-A antigen.* Two Ro/SS-A antigenic preparations were sequenced: (*i*) the CIE-positive fraction from N-PAGE, and (*ii*) the 60-kD, immunoblot-active protein eluted from SDS-PAGE. 40 μg of the protein was analyzed each time and the following sequence was obtained:

5 10
glu/ala-pro-ala-val-tyr-phe-lys-glu-gln-phe-leu-asp-gly-asp-

15 20
gly-trp-thr-ser-arg-trp-ile-glu-ser-lys.

Two amino acids, glutamic acid and alanine, were found at the amino terminus of the molecule. This finding implies that multiple forms of this molecule might exist, an idea which has been suggested by others (22). The amino terminal sequence of

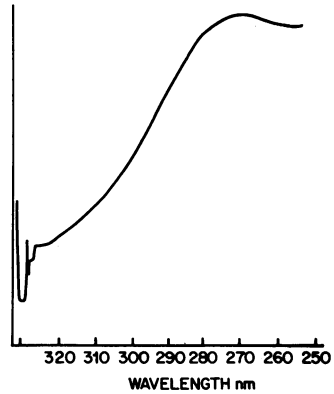


*Figure 4.* Wavelength scan of purified Ro/SS-A antigen. The purified Ro/SS-A was dissolved in Tris-HCl buffer, pH 8.8. The scan was carried out on a gel scanner in a Varian DMS-70. The Ro/SS-A antigen has a maximum absorption at 270 nm.

Ro/SS-A appears to be unique among 4,000 proteins so far searched by computer analysis. However, it does have limited sequence similarity to the human $C_{4a}$ (25%) (amino acids 511–538) (23), lipoprotein signal peptidase (33%) (amino acids 51–73) (24, 25), epidermal growth factor–binding protein type B precursor (29%) (amino acids 39–58) (26), human low density lipoprotein receptor precursor (17%) (amino acids 10–27) (27), and B-2 glycoprotein (21%) (amino acids 264–282) (28).

*Antigenic activity of Ro/SS-A amino terminal SP (residues 6-19) ($SP_{6-19}$).* A computer analysis of the amino terminal sequence of Ro/SS-A antigen using the method of Hopp and Woods (29) suggested that a region from amino acid residue 6 to 19 might be antigenic. A peptide corresponding to this region was synthesized. A human autoimmune serum to Ro/SS-A diluted 1:100 reacted with both the $SP_{6-19}$ and the $SP_{6-19}$ conjugated to KLH (Table I). Human autoimmune sera specific for other antigens such as Sm, La/SS-B, and normal human sera did not produce significant binding. Furthermore, the binding of anti–Ro/SS-A sera to $SP_{6-19}$ was completely blocked by preincubating the sera with native Ro/SS-A antigen. Finally, anti–Ro/SS-A sera which show strong binding to $SP_{6-19}$ did not react in ELISA with other control polypeptides such as ribonuclease A or an unrelated SP conjugated to KLH.

The inhibition of binding of a monospecific human anti–Ro/SS-A serum to native Ro/SS-A antigen in ELISA by amino terminal $SP_{7-24}$ is shown in Table II.

To further characterize the amino terminal region of the Ro/SS-A antigen, an antiserum to $SP_{6-19}$ was raised by immunizing a rabbit with KLH-$SP_{6-19}$. The antibody level in rabbit serum (diluted 1:100) was measured by ELISA against native human Ro/SS-A antigen as well as the SP. Elevated binding to Ro/SS-A antigen ($OD_{405}$, 0.9365 for first bleeding; $OD_{405}$, 1.6875 for second bleeding, compared with $OD_{405}$, 0.1565 for preimmune rabbit sera) was detected. The binding of rabbit anti–peptide 6-19 to native human Ro/SS-A antigen was quantitatively inhibited by KLH $SP_{6-19}$ (Table III). The results of these experiments indicate that residues 6 to 19 from the amino terminal portion of the Ro/SS-A molecule are on the outer surface of native Ro/SS-A. It is possible, however, that distortion might occur when the native Ro/SS-A protein is coated to the ELISA plate, allowing the 6-19 epitope to become relatively more exposed. This is also consistent with the finding that some prototypic human autoimmune sera to Ro/SS-A antigen reacted with the SP.

*Table I. Reactivity of Ro/SS-A Amino Terminal SP (Residues 6-19) with Human Autoimmune Sera*

| Immobilized antigen | Antibody | ELISA $OD_{405}$ |
|---|---|---|
| KLH-$SP_{6-19}$ | Anti-Ro/SS-A | 1.823 |
| | Anti-Sm | 0.288 |
| | Anti-La/SS-B | 0.355 |
| | Normal serum | 0.263 |
| $SP_{6-19}$ | Anti-Ro/SS-A | 1.059 |
| | Anti-Sm | 0.236 |
| | Anti-La/SS-B | 0.158 |
| | Normal serum | 0.118 |

*Table II. Inhibition of the Binding of a Monospecific Human Anti–Ro/SS-A Serum to Native Ro/SS-A Antigen by Ro/SS-A Amino Terminal SP, KLH-$SP_{7-24}$*

| KLH-$SP_{7-24}$ | Percent inhibition |
|---|---|
| μg | |
| 2 | 11.9 |
| 6 | 17.0 |
| 8 | 27.3 |
| 12 | 28.4 |
| 16 | 39.2 |
| 24 | 29.0 |
| 30 | 32.0 |

## Discussion

This study provides the first information pertaining to the primary structure of the human Ro/SS-A antigen. We devised a purification scheme that allowed the isolation of this molecule from an Epstein-Barr virus transformed human B lymphoblastoid cell line. Enough material was isolated to obtain a partial amino terminal amino acid sequence, which revealed no striking homology to known proteins. We utilized the sequence data to synthesize two peptides, which allowed us to confirm that we had isolated authentic Ro/SS-A, since rabbit anti–peptide 6-19 antibodies reacted with native human Ro/SS-A antigen and monospecific anti–Ro/SS-A patient sera reacted with both SP. The chemical purification and partial primary structure of this autoantigen should provide an important link between a well-known, clinically relevant autoantibody and its antigen.

In contrast to other abundant autoantigens such as Sm, RNP, histone, and La/SS-B, Ro/SS-A is present in only small amounts in normal tissue. In addition, the antigenic activity is known to be labile. We therefore developed a purification scheme that did not expose the antigen to chaotropic agents in order to minimize losses of antigenic activity. The combination of polybuffer ion exchange and N-PAGE allowed us to obtain highly purified Ro/SS-A with immunological specificity identical to that of the starting material. The purified Ro/SS-A molecule has a molecular weight of 60,000 which is consistent with the results obtained by several investigators (17–19).

Serum from rabbits immunized by KLH-$SP_{6-19}$ showed antibody activity toward native human Ro/SS-A antigen, confirming that the 6-19 peptide possesses the combination of

*Table III. Inhibition of the Binding of Rabbit $SP_{6-19}$ Antiserum to Native Human Ro/SS-A Antigen*

| KLH-$SP_{6-19}$ | Percent inhibition |
|---|---|
| μg | |
| 8 | 79 |
| 10 | 88 |
| 16 | 98 |

properties that are essential for antibody binding. The sites most frequently recognized by antibodies form three-dimensional superassemblies characterized by high local mobility, convex surface shapes, and negative electrostatic potential (30). The sequence data indicates that the 6-19 peptide carries a negative charge. Delineation of the exact location of the epitope(s) on $SP_{6-19}$ which binds to human autoantibody and to antibodies raised in rabbits will be interesting conceptually. In this way, the influence of the microenvironment on antigenic sites within peptides can be approached. In addition, the antiserum to KLH-$SP_{6-19}$ will be useful in determining the tissue distribution as well as cellular localization of native human Ro/SS-A antigen. Also, this rabbit antiserum could be used as a probe for identifying the amino terminal fragment of the native Ro/SS-A molecule after proteolytic cleavage. Such studies could provide further insight regarding the structure of the native molecule.

Relatively few amino acid sequences of "autoantigens" that react with autoimmune sera from patients with rheumatic disease have yet been elucidated. The carboxyl-terminal 55 amino acids of the La/SS-B antigen was recently identified by analyzing overlapping cDNA clones (31). There is no apparent sequence homology between the amino-terminal region of Ro/SS-A and the carboxyl terminal of La/SS-B.

SP have been widely applied as probes for the study of DNA-binding sites on protein (32), T and B cell recognition sites on protein antigens (30, 33–36), and peptide binding sites on Ia molecules (37). The results of recent studies using SP in combination with crystallographic studies indicate that initial binding to solvent-exposed amino acid residues may promote local side-chain displacements and thereby allow the participation of other, previously buried residues (38). That $SP_{6-19}$ reacted with monospecific antibodies to Ro/SS-A in human autoimmune sera indicates that a primary contact amino acid residue(s) is located in this region. Indeed, our preliminary data using synthetic analogues indicate that residues 14 to 16, -asp-gly-trp-, probably are involved in initial antibody recognition.

Immobilizing SP onto microtiter plates could provide a superior method for detecting anti-Ro/SS-A antibodies in the sera of patients with autoimmune disease. The advantages provided by the SP include the availability of a quality-controlled antigen in large amounts, the ability to automate the procedure, and the lower background and higher sensitivity of a SP-based ELISA technique. Present anti-Ro/SS-A assays are complicated by the recent observation that suggests that some epitopes on the Ro/SS-A antigen are crossreactive with IgG (39). The use of SP to mimic Ro/SS-A epitopes could provide advantages since the two different types of epitopes (Ro/SS-A only vs. Ro/SS-A plus IgG) could be separately analyzed. In fact, recent preliminary results from our laboratory suggest that sera from patients with different clinical disorders such as SCLE and Sjogren's syndrome, and children with congenital heart block, show different frequencies of reactivity to the $SP_{6-19}$ epitope (unpublished observations). The idea that variability might exist in the autoimmune response to different epitopes on the same molecule is supported by recent studies which have indicated that rabbits actively immunized against myohemerythrin also demonstrate a variable antibody response to different epitopes on this polypeptide (30).

The findings of the present investigation might help clarify the conflicting results of earlier studies pertaining to the role played by anti-Ro/SS-A antibodies in the pathogenesis of photosensitive forms of lupus erythematosus skin lesions. Some investigators have suggested that ultraviolet light exposure results in the translocation of intracellular Ro/SS-A antigen to the plasma membrane of cultured human epidermal keratinocytes, a position where this antigen would be more accessible to autoantibody binding. An antibody-dependent cellular cytotoxicity mechanism might then be responsible for eliciting the pattern of keratinocyte injury seen in NLE and SCLE (40). Other workers, however, have not been able to identify keratinocyte cell surface Ro/SS-A under similar experimental conditions (41). If the Ro/SS-A molecule has more than one epitope, perhaps a single epitope would be more exposed to antibody binding in the plasma membrane than others. The ability to identify cell surface Ro/SS-A with a monospecific human serum would then be highly dependent upon the presence in that particular serum of antibody activity toward the exposed epitope. Since our preliminary studies have suggested the existence of different degrees of reactivity toward the amino terminal Ro/SS-A epitope in sera from SCLE and Sjogren's syndrome patients, selection of an individual test serum might greatly influence the outcome of attempts to identify cell surface Ro/SS-A.

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