

Implications of Anti-Ro/Sjögren's Syndrome A Antigen Autoantibody in Normal Sera for Autoimmunity

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Abstract

We have applied a sensitive assay to analyze lupus and Sjögren's syndrome autoantibodies in 40 normal sera. Seven of these bound Ro/Sjögren's syndrome A antigen (SSA). Although this binding was 1,000-fold lower than the highest anti-Ro/SSA level measured from patients, it was inhibited by human Ro/SSA. Positive normal serum-bound Ro/SSA in Western immunoblots and binding activity was demonstrated in the F(ab')₂ fragment of IgG. Affinity purification of normal anti-Ro/SSA IgG increased the specific anti-Ro/SSA binding by > 17-fold. This purified antibody formed a Ro/SSA precipitin and had a relative affinity for Ro/SSA identical to that of Ro/SSA precipitin-positive patients. These data demonstrate that the anti-Ro/SSA present in healthy normal donors is true autoantibody.

Anti-La/Sjögren's syndrome B antigen (SSB) autoantibodies were found in 3 of the 40 normal sera, while none bound nuclear ribonucleoprotein (Sm). Finding low levels of anti-Ro/SSA and anti-La/SSB among normals may indicate that anti-Ro/SSA and anti-La/SSB occur in disease by enhancement of a preexisting immune response.

Introduction

Since systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS)¹ are diseases acquired by those previously in good health, the characterization of autoimmunity in normals may define how the transition to disease occurs. Autoantibody to Ro/Sjögren's syndrome A antigen (SSA) and La/Sjögren's syndrome B antigen (SSB) commonly occur in patients with SLE and SS (1-3). The presence of anti-Ro/SSA can be associated with several patterns of disease expression, including nephritis (4), complement component C2 deficiency (5), and photosensitive cutaneous lupus lesions (6). This antibody has also been found in mothers of infants with either cutaneous neonatal lupus or congenital complete heart block (7, 8), and 0.1% of hospitalized patients (9), as well as some relatives of patients with autoimmune connective tissue disease (10, 11).

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1. Abbreviations used in this paper: CIE, counterimmunoelectrophoresis; nRNP, nuclear ribonucleoprotein; SS, Sjögren's syndrome; SSA and SSB, Sjögren's syndrome A and B antigens.

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Ro/SSA is a RNA-protein complex composed of a 60,000-D peptide noncovalently associated with certain small uridine-rich RNAs. In man, there are four RNAs that are associated with the Ro/SSA peptide (12). The antigenicity is found in at least the peptide since RNase digestion does not alter its reactivity with anti-Ro/SSA antibody and since the peptide alone reacts with anti-Ro/SSA autoimmune sera in immunoblots (13, 14). The RNA is termed Y RNA (cytoplasmic RNA) to signify that it is found in the cytoplasm in agreement with early work using autoimmune sera (13, 15), but more recent workers have claimed that the antigen is found in the nucleus (16). The role of this RNA-protein in cellular metabolism is not known. Nor is it understood why Ro/SSA is a relatively common autoantigen in man, much less what the factors are that lead this autoantibody in some individuals to be associated with particular disease states or clinical manifestations, while others with anti-Ro/SSA are asymptomatic.

Traditionally, anti-Ro/SSA has been detected by Ouchterlony immunodiffusion using tissue extracts as the antigen source (13). With the purification of Ro/SSA, much more sensitive solid-phase assays have become feasible (14). We have evaluated the normal sera with high binding levels to determine if they do indeed have anti-Ro/SSA autoantibody and contrasted their Ro/SSA binding to that of La/SSB (17) and nuclear ribonucleoprotein (nRNP)(Sm).

Methods

Sample collection. Serum samples from 13 patients with Ro/SSA precipitins and 40 normal donors were collected and stored at -20°C. Normal donors included lab workers, physicians, secretaries, and medical students whose mean age was 32.7 yr with a range of 22 to 54 yr. Subdivisions according to race and sex were as follows: 38, caucasian, 1, negroid, 1, oriental, 26 female, and 16 male.

Antigen purification. Ro/SSA and La/SSB were purified from calf thymus and human spleen extracts as described (14, 17). Parallel technology was used to purify the nRNP(Sm) antigen using relatively monospecific anti-nRNP IgG to construct an affinity column. Since anti-nRNP purified the U₁ particle, which contains both nRNP and Sm activities, the resulting preparation has been designated nRNP(Sm).

Enzyme-linked immunosorbent assay (ELISA). A solid-phase assay similar to that described previously (14, 18) was used to detect antibody binding. U-vinyl microtiter plates (Costar, Cambridge, MA) were coated overnight with purified bovine Ro/SSA, La/SSB, or nRNP(Sm) at 1-5 µg/ml in carbonate-coating buffer, pH 9.6. Remaining protein binding sites in the wells were blocked with 0.1% bovine serum albumin (BSA) in 0.02 M phosphate-buffered saline (PBS), pH 7.4. The plates were washed thrice with 0.05% Tween in PBS (Tween-PBS). Appropriate serum or antibody dilutions in PBS with 0.1% BSA and 0.05% Tween (diluent) were added to the plate and incubated 16-18 h. After a second Tween-PBS wash, 1:1,000 dilution of affinity-purified goat anti-human IgG conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) in diluent was added and allowed to incubate 6-8 h. All incubations were made at 4°C. The plates were washed five times with Tween-PBS

and developed at room temperature with *p*-nitrophenylphosphate (Sigma Chemical Co.). Optical densities were read at 405 nm with a MR580 Microelisa Autoreader (Dynatec Laboratories, Alexandria, VA). Antibody binding was expressed as units of anti-Ro/SSA activity, where 1 U was equal to the optical change observed in a 10⁻⁷ dilution of an anti-Ro/SSA precipitin-positive patient serum. Affinity purification of anti-Ro/SSA (18) and anti-nRNP showed that in each case 1 U of autoantibody activity was between 180 and 230 pg of the purified autoantibody.

All serum samples were also preincubated with 10 µg/ml purified bovine or human autoantigen for 4 h at room temperature before being added to the Ro/SSA-coated plate. Specific binding activity is reported as the units of anti-Ro/SSA inhibited by purified antigen. Affinity binding curves of anti-Ro/SSA were determined by inhibiting antibody binding with increasing concentrations of Ro/SSA antigen.

Western blot. Polyacrylamide gel electrophoresis was performed under reducing conditions where Ro/SSA was boiled in 5% β,2-mercaptoethanol and 2% sodium dodecyl sulfate (SDS) and electrophoresed in a 10% polyacrylamide gel with 0.1% SDS (14). Ro/SSA was transferred electrophoretically to nitrocellulose paper (19) after which the sheets were blocked for 1 h at 37°C with 3% BSA. The nitrocellulose strips were washed and incubated with serum dilutions for 6 h at 4°C. After a second wash, the strips were incubated in 1:300 dilution of anti-human IgG conjugated to alkaline phosphatase (Sigma Chemical Co.) overnight at 4°C. The strips were washed and developed with β-naphthyl acid phosphate (20).

F(ab')₂ purification. Sera from 13 of the 40 normal donors were collected 18 mo after the initial sampling along with plasma from an anti-Ro/SSA-positive patient. IgG was isolated from serum by DE52 chromatography (Whatman Chemical Separation, Inc., Clifton, NJ) and digested with pepsin (21). F(ab')₂ fragments were purified by passage through Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ). The ensuing fractions were coated on a U-vinyl plate and tested for purity with goat anti-human F(ab')₂ and Fc conjugated to peroxidase (Cappel Laboratories, Cochranville, PA) using *o*-phenylenediamine as the indicator substrate. All samples with evidence of Fc contamination were passed through a Staphylococcus protein A Sepharose 4B column (Pharmacia Fine Chemicals), which successfully removed all remaining Fc reactivity.

Anti-Ro/SSA purification. Purified Ro/SSA that showed a single band in an SDS-polyacrylamide gel was coupled by CNBr activation to Sepharose-4B according to the manufacturer's instructions (Pharmacia Fine Chemicals). Plasma from an anti-Ro/SSA-positive normal donor and patient were passed through the affinity column. Bound immunoglobulin was eluted with 3 M MgCl₂ and dialyzed into 0.02 M Tris-HCl, pH 7.3. Anti-Ro/SSA activity was evaluated by ELISA as described above and counterimmunoelectrophoresis (CIE) as described by Wasicek and Reichlin (4).

Autoantibody screen. All available normal sera from the original 40 donors were tested for antinuclear antibodies by indirect immunofluorescence on HEp-2 cells and mouse kidney substrate and for anti-native DNA on *Crithidia lucilliae* (22). Anti-single-stranded DNA activity was detected by radioimmunoassay (Farr Technique) and rheumatoid factor was assayed by latex agglutination.

HLA typing. Histocompatibility antigens were determined by the microcytotoxicity test using University of California, Los Angeles HLA and DR typing trays (Lamda One; Los Angeles, CA).

Data analysis. Unless otherwise stated, means are presented as the anti log₁₀ of the log₁₀ average of the original data. Fisher's exact test is used to compare categorical data.

Results

Anti-Ro/SSA antibody levels were assayed by ELISA in sera from 13 patients with precipitating levels of anti-Ro/SSA and 40 normal donors. Patients with anti-Ro/SSA precipitins had between 419,000 and 17,309,000 U of antibody activity specific for Ro/SSA, with an average of 6,022,470 U (Table I). Anti-Ro/SSA antibody activity in normal sera was ~ 1,000-fold lower

Table I. Specific Units of Anti-Ro/SSA Activity in Precipitation-positive Anti-Ro/SSA SLE Patients

Patient no.	Units of specific binding*	Inhibition		Patient no.	Units of specific binding	Inhibition	
		%				%	
1	15,815,800	91		8	7,028,010	99	
2	3,944,850	91		9	419,270	77	
3	1,153,000	91		10	1,323,000	98	
4	7,701,650	95		11	3,700,550	97	
5	11,492,700	87		12	6,694,080	96	
6	17,309,000	95		13	1,173,000	85	
7	537,230	62					

ELISA was used to measure anti-Ro/SSA in sera from patients with immunoprecipitating levels of autoantibody.

* Arbitrary expression of anti-Ro/SSA activity, where 1 U is equal to the optical density change in a 10⁻⁷ dilution of an anti-Ro/SSA precipitin-positive serum standard. The anti-Ro/SSA specific binding is expressed as the units of anti-Ro/SSA binding activity per milliliter of serum that is inhibited by 10 µg/ml Ro/SSA.

than patient sera and in most of these was not significantly inhibited by purified Ro/SSA. The log average specific binding was 2,484 anti-Ro/SSA units (Table II). 7 of the 40 normal sera

Table II. Units of Anti-Ro/SSA Activity in Normal Human Serum Donors

Donor no.	Units of specific binding	Inhibition		Donor no.	Units of specific binding	Inhibition	
		Bovine	Human			Bovine	Human
		Ro/SSA	Ro/SSA			Ro/SSA	Ro/SSA
		%	%			%	%
1	1,565	35		21	17,095*	81	68
2	2,204	44		22	12,572*	85	92
3	2,389	33		23	2,495	28	
4	16,686*	54	69	24	2,036	33	
5	1,966	42		25	23,690*	88	66
6	3,411	41		26	3,460	50	
7	1,587	39		27	1,041	30	
8	2,585	46		28	1,029	41	
9	3,553	55		29	1,277	15	
10	122	3	44	30	1,508	33	
11	1,117	37		31	1,357	41	
12	1,785	27		32	4,195	46	
13	664	29	0	33	3,515	52	
14	6,547*	52	72	34	3,824	22	
15	1,643	52		35	587	19	53
16	448	11		36	1,122	55	
17	2,353	34		37	5,524*	54	75
18	5,156	29		38	10,819*	84	79
19	2,981	46		39	4,592	40	
20	1,759	18		40	3,745	73	

* Identifies normal sera with anti-Ro/SSA binding over 4,800 U inhibited by > 50% with 10 µg/ml Ro/SSA. Log₁₀ averages of 3 to 9 separate determinations are presented.

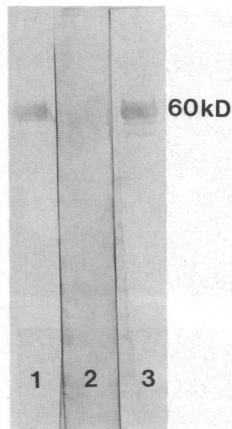


Figure 1. Western blot of purified Ro/SSA using SLE serum in lane 1, normal serum (No. 11) in lane 2, and normal serum (No. 37) in lane 3. SLE serum and normal serum No. 37 both identify the 60,000-mol-wt Ro/SSA polypeptide while normal serum No. 11 does not.

(17.5%) consistently had the highest binding activity, which was inhibited by > 50% with purified bovine Ro/SSA in competition assays. These seven sera were carefully evaluated to determine if this apparent antibody binding activity was Ro/SSA specific or due to nonspecific binding.

Human Ro/SSA was purified and used for inhibition experiments to establish whether or not the binding was potentially a true autoantibody as opposed to a heteroantibody against bovine Ro/SSA (Table II). Each of the high binding sera that were inhibited by bovine Ro/SSA were also inhibited by > 50% with the human antigen.

Table III. Anti-Ro/SSA Binding Activity in Serum and F(ab')₂ Fragments of Normal Donors

Sera	Donor No.	Whole serum		F(ab') ₂ fragments	
		Specific binding	Inhibition %	Specific binding	Inhibition %
Patient sera with anti-Ro/SSA precipitin		14,808,000	99	17,622,000	99
Normal sera with elevated anti-Ro/SSA activity	4	16,686	54	20,711	71
	14	6,547	52	4,043	38
	21	17,095	81	11,985	51
	25	23,690	85	49,946	68
	37	5,524	54	8,154	64
Normal sera with low anti-Ro/SSA activity	11	1,117	37	1,336	13
	13	664	29	850	12
	27	1,041	30	1,939	27
	28	1,029	41	2,800	35
	31	1,357	41	1,455	10
	35	587	19	4,037	39

F(ab')₂ fragments were purified from IgG of 1 patient and 11 normal donors, and tested by ELISA for anti-Ro/SSA activity. Four of the five normal sera with elevated anti-Ro/SSA demonstrated binding due to the F(ab')₂ fragments of IgG.

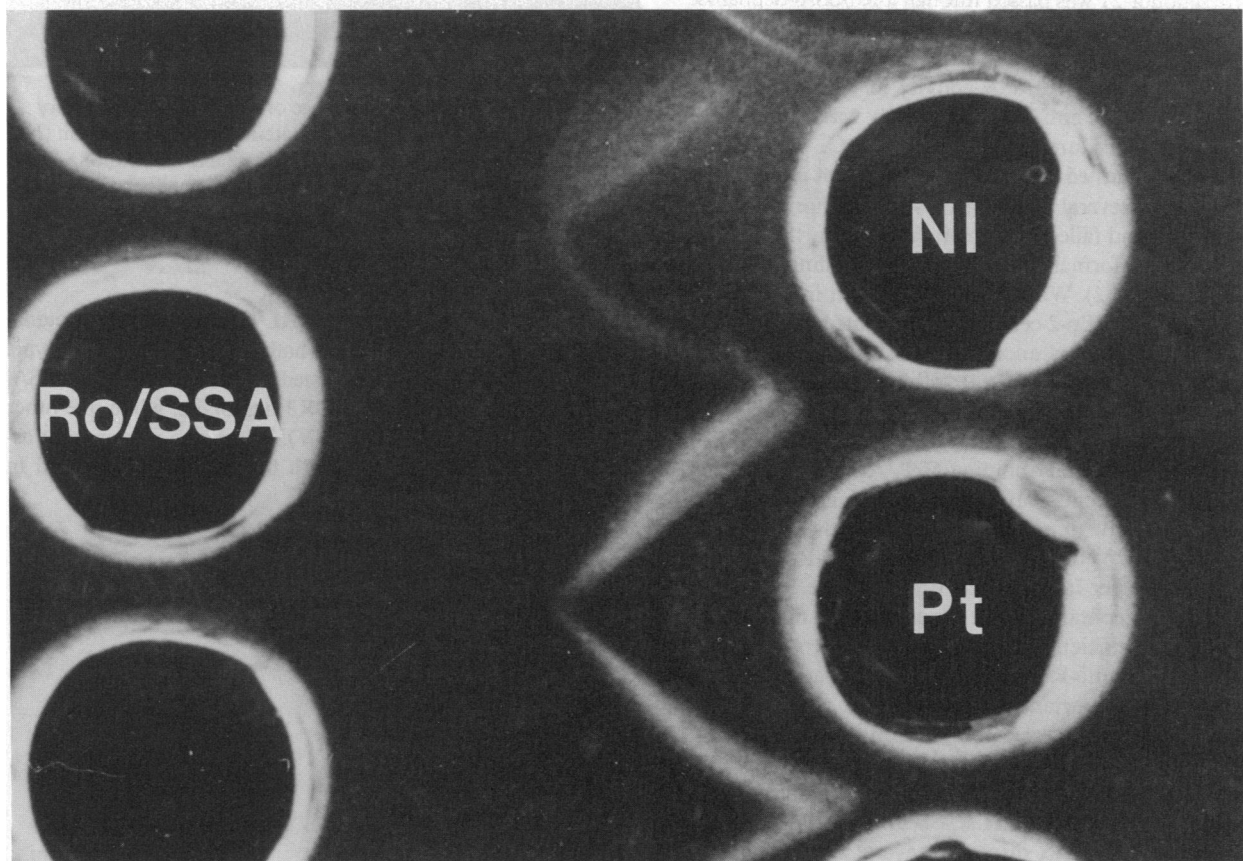


Figure 2. CIE of affinity-purified anti-Ro/SSA from a normal donor (NI) and an autoimmune patient serum with high levels of anti-Ro/SSA (Pt). Both precipitate purified Ro/SSA antigen (Ro/SSA).

Several of the normal sera were tested by Western blot to demonstrate binding to the 60,000-mol-wt antigenic Ro/SSA protein. Fig. 1 shows the binding of an anti-Ro/SSA-positive patient in lane 1, an anti-Ro/SSA-negative normal serum in lane 2 (donor 11), and a normal with elevated anti-Ro/SSA by ELISA in lane 3 (donor 37). The patient and donor 37 sera both bound the 60,000-D Ro/SSA polypeptide.

Sera from 13 of the normal donors were reassessed for anti-Ro/SSA activity after 18 mo. Five of the seven control sera with high anti-Ro/SSA continued to display elevated binding activity, while sera from two normal donors no longer bound Ro/SSA. Six of the normal controls that previously failed to bind Ro/SSA remained negative.

To determine whether the antigen-combining portion of IgG was binding Ro/SSA, F(ab')₂ fragments were prepared from normal sera, of which five bound Ro/SSA and six did not. Also included was one Ro/SSA precipitin-positive patient serum. Table III shows anti-Ro/SSA activity by ELISA due to purified F(ab')₂ compared with the anti-Ro/SSA activity in whole serum. Four sera had anti-Ro/SSA activity attributable to the F(ab')₂ segment of IgG that could be blocked by purified Ro/SSA in competition assays. Donor serum No. 14 failed to satisfy criteria for specific reactivity in the F(ab')₂ serum fraction and was not significantly different from the negative control group. Either this serum did not have specific binding when originally tested or the antibody binding was destroyed by pepsin digestion. Indeed, its whole serum reactivity only marginally satisfied the arbitrary criteria for binding to Ro/SSA.

To purify Ro/SSA-specific IgG from normal serum, plasma from normal donor 21 was passed through a Ro/SSA-Sepharose 4B affinity column and the bound immunoglobulin was eluted. Anti-Ro/SSA activity in the eluted Ro/SSA-specific IgG was compared with the anti-Ro/SSA activity in the donor's total IgG by ELISA. Specific anti-Ro/SSA activity was concentrated 17.7-fold by this procedure relative to whole IgG.

Anti-Ro/SSA purified from normal and patient plasma were then compared in several assay systems. While normal donor 21 whole plasma had failed to precipitate Ro/SSA, the purified anti-Ro/SSA from normal plasma precipitated affinity-purified Ro/SSA in CIE (Fig. 2). When tested by indirect immunofluorescence on cultured HEp-2 cells, the concentrated normal anti-Ro/SSA showed a speckled nuclear pattern similar to anti-Ro/SSA from patient serum (Fig. 3). Relative binding affinities of purified anti-Ro/SSA from patient and normal plasma were compared in ELISA by determining the concentration of Ro/SSA that inhibited antibody binding 50%. Anti-Ro/SSA from precipitin-positive patient serum and normal donor 21 had virtually identical relative binding affinities, as shown in Fig. 4. The relative affinity of the patient's purified anti-Ro/SSA was identical to that of her IgG and was similar to the IgG of five other anti-Ro/SSA containing sera. Thus, we have no evidence that purified and sufficiently concentrated anti-Ro/SSA from normal plasma and anti-Ro/SSA purified from patient plasma are different in any important respect.

All normal sera were tested for additional autoantibodies, and two of the seven sera with elevated anti-Ro/SSA demonstrated further serologic abnormalities. Donor 37 was a 25-yr-old white female who also bound La/SSB by ELISA (15). Donor 4, a 22-yr-old white female, displayed several additional links to autoimmunity, including low titer antinuclear antibody, anti-single-strand DNA, intermittently positive rheumatoid factor at

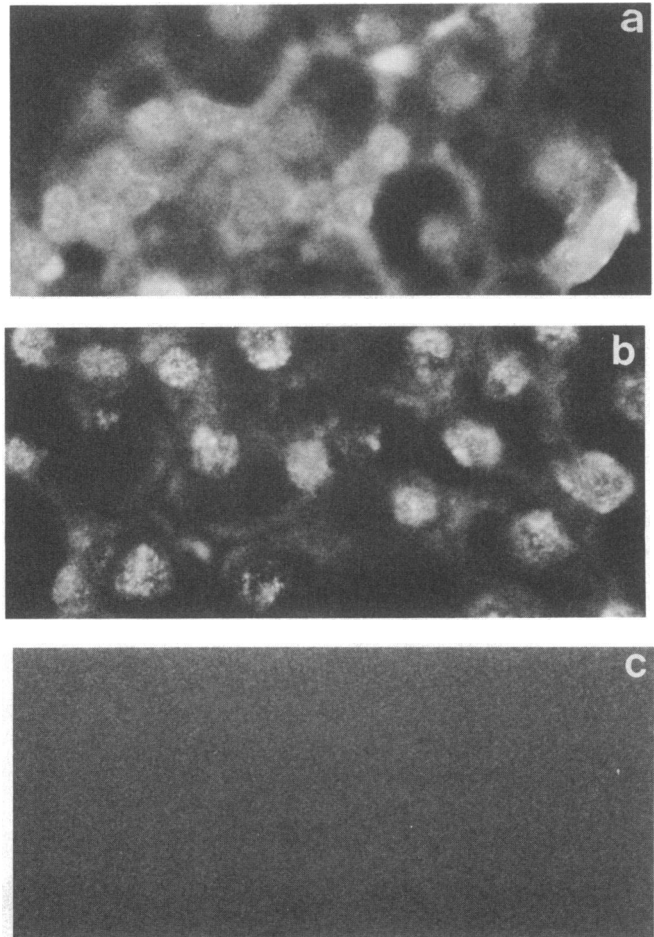


Figure 3. Indirect immunofluorescence of affinity-purified anti-Ro/SSA from a normal donor (a) and an autoimmune patient serum (b) compared with a normal serum with no detectable anti-Ro/SSA activity (c). Affinity-purified anti-Ro/SSA from a normal donor and an autoimmune patient both displayed a speckled nuclear binding pattern on cultured HEp-2 cells.

low titer, and a false-positive VDRL 6 mo before the original serum sampling. This individual had the HLA DR3 haplotype. Of the five normal individuals tested whose sera bound Ro/SSA, only this donor had the HLA-DR3 haplotype, while one other was HLA-DR2 positive (Table IV). There was no apparent association of an HLA antigen with the anti-Ro/SSA found in these normal sera.

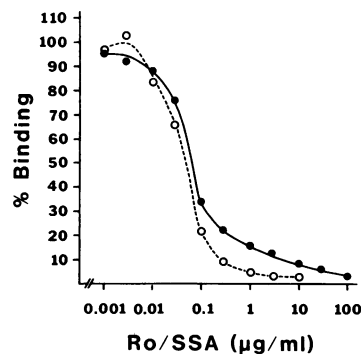


Figure 4. Relative affinity of affinity-purified anti-Ro/SSA from a normal donor (solid circles) and from an autoimmune patient serum (open circles).

Table IV. HLA Typing, Age, Sex, and Race of Anti-Ro/SSA-positive Normal Donors

Donor no.	A	B	DR	Age	Sex	Race
4	1, w32	8, 40	3, 4	22	F	C
14	3, 29	7, w44	2, 7	25	F	C
21	28, -*	44, 62	5, 8	41	M	C
25	1, 3	7, 14	1, 6Y	49	M	C
37	2, -*	w44, w62	1, 4	25	F	C

F, Female; M, male; and C, Caucasian.

* Indicative of homozygous alleles or a rare antigen without available typing sera.

All 40 normal sera were screened by ELISA for anti-La/SSB and anti-nRNP(Sm) reactivity. Three sera (7.5%) showed elevated anti-La/SSB levels, which proved to be autoantigen specific as shown previously (15). None of the 40 normal sera bound nRNP(Sm) (Fig. 5).

Discussion

We have established that certain normal individuals have elevated levels of anti-Ro/SSA autoantibody. The antibody can be demonstrated by binding to purified Ro/SSA in a solid-phase ELISA and Western immunoblot as well as precipitation in CIE after concentration. Binding in the anti-Ro/SSA ELISA was inhibited by both human and bovine Ro/SSA in competition assays. The antigen binding was found in the F(ab')₂ segment of IgG and therefore can not be attributed to a nonspecific Fc interaction. Anti-Ro/SSA IgG was concentrated 17.7-fold when purified from normal plasma by affinity chromatography.

While 17.5% of normal sera bound Ro/SSA, none of the 40 normal sera had anti-nRNP(Sm) activity. In SLE precipitating antibodies to Ro/SSA and La/SSB tend to occur together, while anti-Sm is associated with anti-nRNP (12). The finding that anti-Ro/SSA commonly occurs at low levels in the normal population while anti-nRNP(Sm) do not ($P = 0.007$, Fisher's exact test using

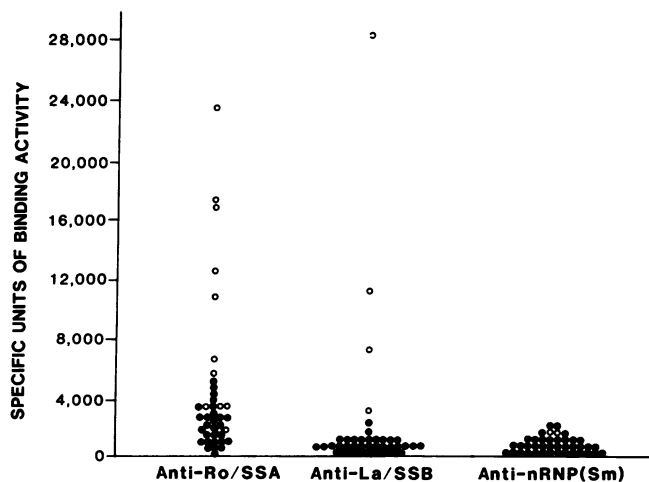


Figure 5. Specific units of anti-Ro/SSA, anti-La/SSB, and anti-nRNP(Sm) binding activity in 40 normal sera as determined by ELISA. (Solid circles) Represent < 50% inhibition with purified antigen; (open circles) represent > 50% inhibition.

data from Fig. 5) is consistent with the observed separation of these autoantibodies. This may indicate that different mechanisms of autoantibody generation are responsible for the presence of these autoantibodies in SLE. Anti-Ro/SSA and anti-La/SSB may occur in SLE by some phenomenon that enhances the production of antibody already present in some normal individuals. On the other hand, the initiation of anti-nRNP or anti-Sm would apparently require some primary immune response, as these autoantibodies are rare or absent in the normal population.

The presence of autoantibodies in low frequencies among normal subjects has been reported from time to time, including antinuclear antibodies and rheumatoid factor (23, 24). When autoantibodies were assessed in normal females according to age, the highest incidence occurred in the older age group (24). Less frequently precipitating levels of autoantibody to ribonuclear proteins have been documented (9, 24, 25). The highest incidence of autoantibody in normal individuals has been reported in mothers of neonatal lupus infants. More than 80% of these asymptomatic mothers have anti-Ro/SSA and/or anti-La/SSB with no clinical manifestations of autoimmune disease (7, 8). These mothers, however, seem to be at risk for future development of the clinical symptoms of connective tissue disease (26). With the application of solid-phase assays for more sensitive detection of autoantibodies to Ro/SSA, La/SSB, nRNP, and Sm, it may be possible to better identify another population of normals at risk for developing autoimmune disease. This would be especially relevant in the case of donor 4, who had the HLA B8, DR3 haplotype associated with increased risk of developing SLE (27), and had been associated with anti-Ro/SSA precipitins in lupus (28).

An increased prevalence of SLE in females over males has been documented (29), as well as an increase in autoreactivity among the elderly (24). 24 females and 16 males participated in this study, which is equal to the ratio of anti-Ro/SSA-positive females and males, 3:2 (Table IV). When the average age of our normal donors was assessed, the anti-Ro/SSA-positive donor's mean age was 32.4 yr, which was not significantly different from the group average of 32.7 yr. Thus, in our study, neither sex nor age accounted for the high incidence of Ro/SSA binding in the normal sera. When the mean ages of anti-Ro/SSA-positive females versus males were considered, however, the average female age was 24 yr, compared with the average age of males, which was 45 yr. This becomes interesting in light of the observation that the largest proportion of female SLE patients have disease onset at a young age (29).

Low levels of autoanti-Ro/SSA in normal donor plasma, similar to patient anti-Ro/SSA in binding characteristics, establishes the presence of autoreactive B cell clones in the repertoire of normal individuals. Recently there have been other reports of autoreactive immunoglobulin-secreting cells in normals. Hoch and Schwaber have shown that human hybridomas from splenic lymphocytes of a nonautoimmune individual secrete anti-DNA antibodies equivalent to those derived from SLE patients (30). Mumford et al. detected antibodies to La/SSB, RNP/Sm, and DNA in supernatant cultures from peripheral blood lymphocytes of healthy controls (31), which confirms the results of Mach et al., who reported anti-DNA antibodies produced by normal peripheral blood mononuclear cells (32). These findings all support the hypothesis that autoreactive cells are controlled by suppression rather than clonal deletion. The transition from a normal state to the expression of disease may then occur by loss of T

cell suppressor function, which results in activation and expansion of autoreactive clones under the appropriate stimulus.

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