THE Ro/SSA AUTOANTIGEN AS AN IMMUNOGEN

Some Anti-Ro/SSA Antibody Binds IgG

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Patients with connective tissue diseases produce autoantibodies to diverse cellular components including nucleic acids, mitochondria, cell-surface antigens, and ribonucleoproteins. A small RNA–protein complex known as Ro/SSA binds autoantibody from many patients with SLE and Sjögrens syndrome (1–4). Anti-Ro/SSA autoantibodies have been closely associated with the appearance of nephritis, vasculitis, lymphadenopathy, and leukopenia in SLE patients (5, 6). Furthermore, maternally acquired anti-Ro/SSA has been implicated in the immunopathogenesis of congenital complete heart block and with the dermatitis of neonatal lupus (7–10).

Ro/SSA was originally characterized as an antigen to which precipitating autoantibodies were detectable in gel double diffusion (11, 12) and has only recently been the subject of more definitive biochemical evaluation (13, 14). The Ro/SSA particle is composed of an acidic protein of 60 kD that in human cells is associated with four distinct uridine-rich RNAs ranging from 80 to 112 bases (15). The antigenic reactivity of Ro/SSA is apparently independent of the RNA since the precipitin activity is resistant to RNAse (11, 12, 16) and since the autoantibody binds the protein after denaturation and separation from the RNA (14).

Clinical studies have shown that 70% of patients with Ro/SSA-precipitating antibodies also have rheumatoid factor (17). In primary Sjögren's syndrome patients, the rheumatoid factor titer and the concentration of anti-Ro/SSA are closely correlated (18). In addition, previous data demonstrate that rheumatoid factor and antinuclear antibody contribute to Ro/SSA precipitation by double diffusion (19). In other disorders, selected rheumatoid factors (some restricted to particular idiotypes) have been shown to bind non-IgG cellular components including histones (20–22) and DNA (23, 24), as well as bacterial peptidoglycan and nitrophenyl groups (25, 26). This study compares the binding characteristics

of heteroimmune anti-Ro/SSA generated in a rabbit host with human autoim-
mune anti-Ro/SSA. In this study, the data clearly show that both autoimmune
and heteroimmune anti-Ro/SSA not only bind the Ro/SSA protein but also react
with IgG.

Materials and Methods

Ro/SSA Antigen Preparation.   Ro/SSA was purified according to a method adapted
from Yamagata et al. (14). Briefly, bovine spleen was homogenized in an equal amount of
(wt/vol) PBS (pH 7.2) supplemented with 2 mM DTT at 4°C. The cell lysate was
centrifuged at 10,000 g for 1 h. The supernatant was mixed with 40% (vol/vol) DE-52
(Whatman Co., Clifton, NJ) for 4 h at 4°C. DE-52 was then washed thoroughly with PBS,
pH 7.2, followed by extraction with 1.0 M NaCl in 0.02 M phosphate buffer, pH 7.2.
The extract was then applied to an anti-Ro/SSA affinity column. The affinity column was
made by coupling purified IgG from an SLE patient with precipitating anti-Ro/SSA to
CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) by conventional
methods (27). The patient IgG fraction was negative for rheumatoid factor by the latex
agglutination assay (28) but was additionally adsorbed with human IgG–Sepharose 4B
before the coupling reaction. Ro/SSA was eluted from the affinity column with 3 M
$MgCl_2$, pH 7.0, and the eluate was dialyzed against 0.02 M Tris, 0.15 M NaCl, pH 7.2,
and concentrated by Amicon filtration (Amicon Corp., Danvers, MA). Purified La/SSB
was similarly prepared by affinity chromatography using the anti-La/SSB IgG from an
SLE patient coupled to Sepharose 4B (13).

Enzyme-linked Immunosorbent Assay (ELISA).   The solid-phase assay for anti-Ro/SSA or
anti-La/SSB was modified from previously reported techniques (13, 14, 29). Immulon 96-
well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated over-
night at 4°C with 50 μl of antigen (human IgG, bovine IgG, or affinity-purified bovine
Ro/SSA or La/SSB) in carbonate buffer, pH 9.6. Our preliminary studies demonstrated
that 5 μg/ml of each of these antigens was optimal for detection of antibody. After
coating, microtiter plates were washed with 0.05% Tween in PBS and unbound sites were
blocked with 0.1% BSA in PBS. Sera were diluted in PBS containing 0.5% Tween and
0.1% BSA and incubated on the plate for 12 h at 4°C. In some experiments, dilutions of
sera were preincubated with several concentrations of bovine or human IgG (Cohn fraction
II, Sigma Chemical Co., St. Louis, MO), total histones (purified by the method of Rubin
et al. [30]), or affinity purified bovine Ro/SSA or La/SSB for 4 h at room temperature
before application to the wells. Following serum incubation, the plates were washed and
a saturating concentration of the IgG fraction of goat anti-human or anti-rabbit IgG
conjugated to alkaline phosphatase (Sigma Chemical Co.) was applied to the plate. The
plates were allowed to incubate overnight at 4°C and again washed free of unbound
conjugate. Paranitrophenylphosphate (PNPP)[1] was then added to the plates and chromo-
phore development was measured at 405 nm with a MR 580 Microelisa Auto Reader
(Dynatech Laboratories, Inc.)

Western Blot.   Western immunoblots were performed by a procedure modified from
Towbin et al. (31). Purified Ro/SSA was denatured and reduced and then applied to
7.5% (wt/vol) polyacrylamide gels containing SDS and electrophoresed at 30 mA for 4 h
at 10°C. The proteins were either silver stained (Bio-Rad Laboratories, Richmond, CA)
or electrophoretically transferred to nitrocellulose sheets in a Trans-Blot cell (Bio-Rad
Laboratories) at 200 mA for 4 h at 10°C. The nitrocellulose was blocked with 4% BSA
in PBS for 4 h at room temperature. Appropriate dilutions of human au. nmune anti-
Ro/SSA, rabbit heteroimmune anti-Ro/SSA, or IgG adsorption column eluates were
incubated with the nitrocellulose for 4 h at room temperature. The nitrocellulose was
washed with PBS for 30 min followed by a 3-h incubation with the IgG fraction of goat
anti–human or anti–rabbit IgG conjugated to alkaline phosphatase. Nitrocellulose was
again washed and then soaked in substrate solution consisting of 2.0 mM β-naphthyl acid

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[1] *Abbreviation used in this paper:*   PNPP, paranitrophenylphosphate.

phosphate (Sigma Chemical Co.), 1.0 mM o-diansidine (Sigma Chemical Co.), and 4.0 mM MgSO$_4$ in 0.07 M borate buffer (32). The substrate reaction was then stopped with methanol, water, and acetic acid (5:5:1).

Preparation of Rabbit Anti-Ro/SSA.	Heteroimmune rabbit anti-Ro/SSA antisera was made by repeated immunization of New Zealand White rabbits with purified Ro/SSA preparations. Rabbits were initially immunized intramuscularly and subcutaneously with 200 μg purified Ro/SSA in 1.0 ml CFA. They were boosted at 2 wk with 200 μg Ro/SSA in 1.0 ml IFA and by ear vein at 4 wk and 200 μg Ro/SSA. They were bled 10 d later. Subsequent sera were collected after repeating the intravenous boosting.

Adsorption of Rabbit and Human Anti-Ro/SSA with IgG-Sepharose.	Bovine or human IgG (Cohn fraction II, Sigma Chemical Co.) was covalently bound to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) by the method of Axen et al. (27). Columns were equilibrated in PBS and rabbit heteroimmune anti-Ro/SSA serum was passed sequentially through the bovine and human IgG columns. The columns were washed in 0.02 M Tris, 0.15 M NaCl, pH 7.4 (Tris-NaCl), until no evidence of protein was apparent in the wash (OD at 280 nm was <0.01). The prototype human anti-Ro/SSA serum was similarly passed through a human IgG affinity column. The columns were eluted with 3 M MgCl$_2$, pH 7.2, and eluates were dialyzed against Tris-NaCl and concentrated. Both the rabbit and human sera were passed through control affinity columns consisting of ethanolamine-blocked Sepharose 4B to eliminate interference from nonspecific binding. Similarly, nonimmune rabbit and normal human serum were passed through bovine and human IgG affinity columns as controls.

Anti-Ro/SSA-specific Antibody Purification of Immune Sera.	Affinity-purified Ro/SSA antigen was covalently attached to CNBr-activated Sepharose 4B and the heteroimmune rabbit or autoimmune human anti-Ro/SSA serum was passed through at 0.5 ml/min. The column was washed (Tris-NaCl), eluted with 3 M MgCl$_2$, dialyzed, and concentrated as described above.

Specific Activity Determination.	Rabbit and human anti-Ro/SSA and selected affinity-purified isolates were examined for specific anti-Ro/SSA or anti-human IgG activity. IgG concentrations were determined by ELISA (33). Goat anti-rabbit IgG or anti-human IgG (Sigma Chemical Co.) was adsorbed to 96-well microtiter plates and unbound sites were blocked with 0.1% BSA in PBS. Rabbit or human IgG standard dilutions and test sera were added to the wells and detected by goat anti-rabbit IgG or anti-human IgG conjugated to alkaline phosphatase (Sigma Chemical Co.). Upon addition of substrate (p-nitrophenylphosphate), IgG concentrations were calculated from IgG standard curves and correlated with anti-Ro/SSA or anti-human IgG ELISA OD values. Values are expressed as ELISA activity (OD$_{405}$)/μg IgG.

Isolation of 7S-anti-Ro/SSA IgG.	The 7S IgG from the heteroimmune rabbit and autoimmune patient anti-Ro/SSA was isolated by sucrose density gradient centrifugation adapted from established methods (34). 0.3 ml of serum was applied to a gradient consisting of 1.5 ml each of 16, 20, and 26% sucrose. Control gradients included 7S-I^{125}-IgG and 11S catalase were also applied in 0.3 ml. Gradients were centrifuged for 18 h at 100,000 g. The 7S fraction was separated from the 11S fraction and used for competitive inhibition assays.

Results

Evaluation of Immune Rabbit Serum for Anti-Ro/SSA Antibody.	The Ro/SSA immune rabbit serum formed a precipitin line of identity with prototype patient anti-Ro/SSA serum when tested against purified Ro/SSA (Fig. 1). Normal human serum and nonimmune rabbit serum do not precipitate purified Ro/SSA. Western blot analysis revealed binding of the 60-kD Ro/SSA protein by both the prototype human anti-Ro/SSA and the immune rabbit serum at 10^{-4} dilutions (Fig. 2). Control lanes of normal human and nonimmune rabbit serum fail to
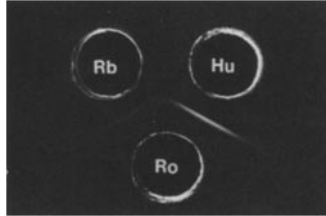
FIGURE 1.   0.6% agarose double immunodiffusion gel of affinity-purified Ro/SSA against undiluted rabbit anti-Ro/SSA (Rb) and prototype human anti-Ro/SSA (Hu).
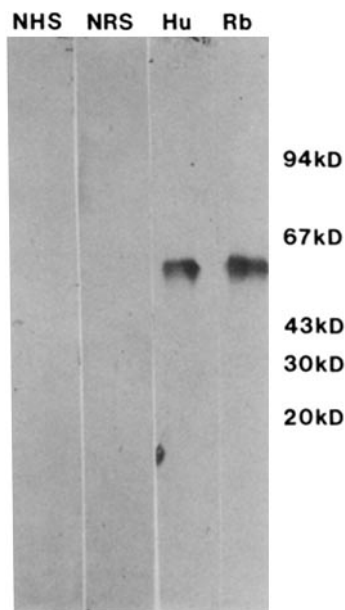

FIGURE 2.   Western immunoblot analysis of normal human serum (NHS), normal rabbit serum (NRS), prototype human anti-Ro/SSA (Hu), and rabbit anti-Ro/SSA (Rb). Affinity-purified Ro/SSA was electrophoresed under reducing conditions on a 7.5% SDS-polyacrylamide gel, and was transferred to nitrocellulose strips. All samples were tested at a 10^{-2} dilution.

detect this protein by Western blotting, confirming the binding specificity of the immune sera.

A sensitive solid-phase immunoassay (ELISA) for the detection of anti-Ro/SSA antibody was used to characterize the immune rabbit serum. Titrations of the rabbit serum were positive to a 10^{-6} dilution and subsequent assays used a 10^{-4} dilution for analysis. At this dilution, preincubation of the serum with 10 μg/ml purified Ro/SSA inhibited 92% of the binding activity of the serum (Table I). The specificity of the heteroimmune response to Ro/SSA was evaluated along with other autoantigens and proteins. Preincubation of the immune rabbit serum with purified La/SSB, histones, or albumin failed to competitively inhibit binding to the Ro/SSA protein (Table I).

Loss of Anti-Ro/SSA Activity with IgG Adsorption.   The rabbit anti-Ro/SSA antiserum bound both human and bovine IgG in ELISA (Table II). We per-

TABLE I

Anti-Ro/SSA Activity of Heteroimmune Rabbit Serum

Competitive inhibitor	OD_{405}	Percent inhibition
—	0.74	—
Ro/SSA (10 µg/ml)	0.06	92
La/SSB (10 µg/ml)	0.77	0
Total histones (10 µg/ml)	0.79	0
BSA (10 µg/ml)	0.80	0

A 10^{-4} dilution of rabbit anti-Ro/SSA was preincubated for 4 h at room temperature with selected inhibitors. Inhibition was calculated as 100 × (1 − inhibited sample OD_{405})/(uninhibited sample OD_{405}).

TABLE II

Rabbit Anti-Ro/SSA Adsorption with Human and Bovine IgG

Ligand[*]	Unadsorbed (10^{-3} dilution)	Control adsorbed[‡] (10^{-3} dilution)	IgG adsorbed at dilution of:[§]			NRS[ǁ]
			10^{-1}	10^{-2}	10^{-3}	
Ro/SSA	0.72	0.84	0.78	0.28	0.09	0.01
Human IgG	0.77	0.89	0.06	0.05	0.03	0.06
Bovine IgG	0.53	0.69	0.09	0.01	0.04	0.11

[*] Ligands were bound to the solid phase in ELISA for detection of antibody. Values expressed as OD at 405 nm (OD_{405}).

[‡] Rabbit anti-Ro/SSA adsorbed over an ethanolamine-blocked Sepharose 4B control column.

[§] Rabbit anti-Ro/SSA adsorbed over bovine and human IgG bound to Sepharose 4B.

[ǁ] Nonimmune rabbit serum.

formed adsorption of anti-IgG activity to enrich the anti-Ro/SSA specific activity of the antisera and to prevent artifacts that might arise from anti-IgG contaminating the anti-Ro/SSA serum. The rabbit antiserum was passed sequentially through bovine and human IgG affinity columns. Adsorption reduced the anti-bovine and anti–human IgG activity by almost 1,000-fold. There was, however, a concomitant loss of nearly 99% of the anti-Ro/SSA activity with IgG adsorption since a 100-fold greater concentration of adsorbed serum was required to achieve similar optical density measurements in this assay (Table II). Rabbit anti-Ro/SSA serum was also passed through an ethanolamine-blocked Sepharose 4B column with no loss of activity to Ro/SSA or IgG. Therefore, nonspecific binding of anti-Ro/SSA to Sepharose 4B was not responsible for the loss of anti-Ro/SSA activity. Nonimmune rabbit serum lacked any anti-Ro/SSA activity in this solid-phase assay and did not bind the IgG affinity columns.

IgG Inhibits Anti-Ro/SSA Activity in ELISA. The anti-Ro/SSA ELISA was used to determine if the loss of anti-Ro/SSA activity after IgG adsorption was due to crossreactive specificities of the Ro/SSA protein and IgG (Fig. 3). A 10^{-4} serum dilution of either rabbit or prototype human anti-Ro/SSA was preincubated with 10–5,000 µg/ml bovine IgG. The human anti-Ro/SSA activity was reduced by up to 55% by bovine IgG, and the rabbit anti-Ro/SSA by as much as 75%.

To assess the contribution of anti-IgG/anti-Ro/SSA complexes to these phenomena, the 7S anti-Ro/SSA from both human and rabbit serum was isolated
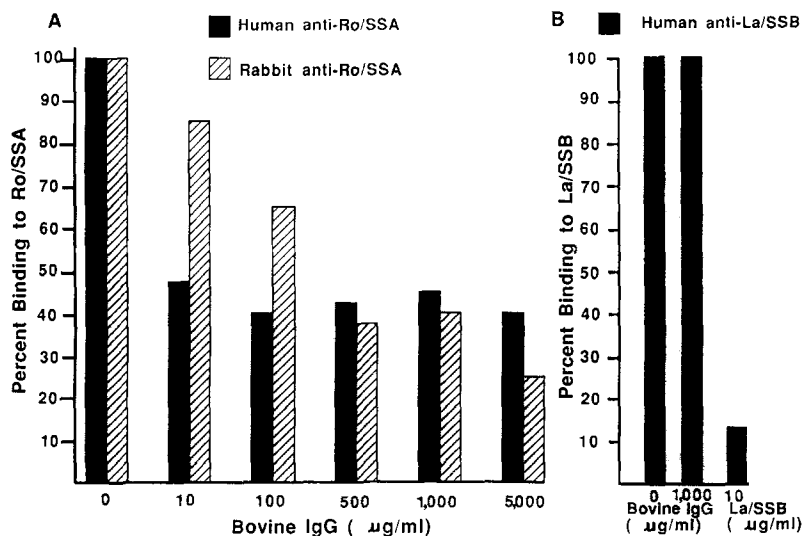
FIGURE 3. (A) Bovine IgG inhibition of anti-Ro/SSA activity. A 10^{-4} dilution of rabbit anti-Ro/SSA or 3×10^{-5} dilution of prototype human anti-Ro/SSA was preincubated with bovine IgG before addition to an anti-Ro/SSA-detecting ELISA. Percent binding to Ro/SSA is calculated as (inhibited sample OD_{405}/uninhibited sample OD_{405}) $\times$ 100. (B) Bovine IgG inhibition of prototype human anti-La/SSB activity. A 10^{-5} dilution of prototype human anti-La/SSB serum was preincubated with 1,000 μg/ml bovine IgG or 10 μg/ml affinity-purified La/SSB before determining the anti-La/SSB activity.

by sucrose density gradient centrifugation. The 7S anti-Ro/SSA fraction of the rabbit and human serum was tested in competitive inhibition assays. IgG was seen to inhibit 7S anti-Ro/SSA activity as effectively as it inhibited unfractionated anti-Ro/SSA serum (data not shown). These data suggest that rheumatoid factor bound to anti-Ro/SSA does not contribute to the apparent crossreactivity of Ro/SSA and IgG.

To determine whether IgG inhibition was unique to the Ro/SSA–anti-Ro/SSA interaction, we used a similar solid-phase immunoassay for the detection of a related autoantibody, anti-La/SSB. Preincubation of a prototype human autoimmune anti-La/SSB serum with up to 1,000 μg/ml bovine IgG failed to inhibit binding to La/SSB in ELISA (Fig. 3B), whereas preincubation of the anti-La/SSB serum with 10 μg/ml of purified La/SSB antigen reduced binding by 85%. Preincubation of human anti-histone antibody from a SLE patient with up to 5,000 μg/ml bovine IgG also failed to inhibit binding to histones by ELISA (data not shown).

Heteroimmune rabbit anti-Ro/SSA was also preincubated with human IgG at concentrations from 10 to 5,000 μg/ml. We saw similar IgG concentration–dependent inhibition with human IgG as was shown with bovine IgG in Fig. 3. Human IgG inhibited up to 45% of the rabbit anti-Ro/SSA activity. The residual anti-Ro/SSA activity of the rabbit and human anti-Ro/SSA sera that had been adsorbed with IgG-Sepharose was not inhibited by preincubation with IgG (data not shown). This phenomenon, as well as the previous data showing partial inhibition of anti-Ro/SSA with IgG, suggests that some epitopes on the Ro/SSA
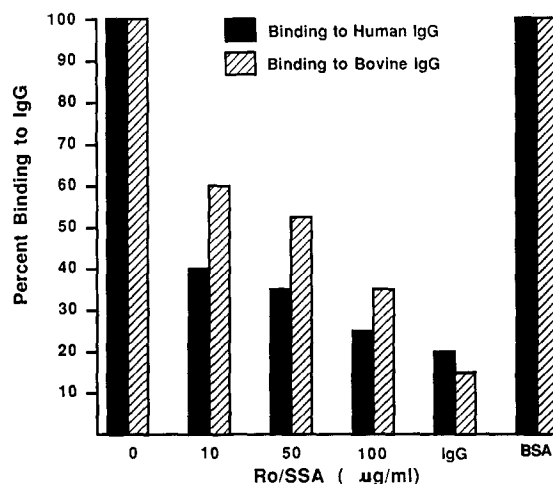
FIGURE 4.   Purified Ro/SSA inhibition of anti-IgG activity. A 10^{-4} dilution of rabbit anti-Ro/SSA was preincubated with purified Ro/SSA, 10 μg/ml bovine or human IgG, or 100 μg/ml BSA and was assayed for anti-IgG activity by ELISA.

protein are identified by the polyclonal sera that are crossreactive with IgG while other epitopes are not.

Ro/SSA Inhibition of Anti-IgG Activity.   Since we observed that IgG is an inhibitor of anti-Ro/SSA activity, we performed reciprocal assays using purified Ro/SSA to inhibit anti-IgG activity (Fig. 4). A 10^{-4} serum dilution of heteroimmune rabbit anti-Ro/SSA was incubated with 10–100 μg/ml of affinity-purified bovine Ro/SSA before its addition to an anti-IgG-detecting ELISA. Maximal inhibition of anti-IgG activity by Ro/SSA was achieved by preincubation with 100 μg/ml purified Ro/SSA. The anti–bovine IgG and anti–human IgG activities of these anti-Ro/SSA sera were inhibited 75 and 63%, respectively. As a control, 100 μg/ml BSA did not inhibit rabbit anti-Ro/SSA-binding to either bovine or human IgG. Preincubation of either the rabbit or the human anti-Ro/SSA with up to 20 μg/ml purified La/SSB or total histones also failed to inhibit the anti-IgG activity of either sera (data not shown). These data again suggest that the apparent crossreactivity found between Ro/SSA and IgG is not found with other autoantigens.

Anti-Ro/SSA Activity of IgG Affinity Column Eluates.   Rabbit anti-Ro/SSA was adsorbed separately through human or bovine IgG affinity columns. Eluates from both human and bovine IgG adsorbents had anti-Ro/SSA-binding activity by the solid-phase assay, which was more than 70% inhibited with Ro/SSA, bovine IgG, or human IgG. Antibody eluted from the IgG columns also bound the 60-kD Ro/SSA protein in Western blot, while nonimmune rabbit serum did not bind.

A similar analysis was performed with prototype-human anti-Ro/SSA serum. The antibody eluted from the human IgG-Sepharose column contained anti-Ro/SSA activity, which could be inhibited by >90% with Ro/SSA. Western blot analysis demonstrated both the human anti-Ro/SSA serum and the human IgG-Sepharose eluate fraction of this serum-bound Ro/SSA.

Specific Anti-Ro/SSA Activity.   Heteroimmune rabbit anti-Ro/SSA was charac-

TABLE III

Relative Specific Activity of Rabbit Anti-Ro/SSA Compared with its Affinity-Isolated Anti–Ro/SSA and Anti–IgG Fractions

Fraction	Anti-Ro/SSA	Anti-Human IgG
Rabbit anti-Ro/SSA serum activity	1.0	1.0
Human IgG column eluate	1.8	3.4
Ro/SSA column eluate	6.8	1.5

Rabbit anti-Ro/SSA sera were tested at 10^{-4} and 3×10^{-5} dilutions, respectively. The rabbit anti-Ro/SSA was adsorbed to human IgG– or Ro/SSA–Sepharose 4B, washed, eluted and concentrated. The relative specific anti-Ro/SSA and anti-IgG activities were determined by ELISA and the normalized values for $OD_{405}/\mu g$ IgG were compared.

TABLE IV

Detection of Contaminating IgG in Ro/SSA by ELISA

Grams of IgG per ml	Human IgG	Bovine IgG
10^{-6}	0.720	0.755
10^{-7}	0.124	0.111
10^{-8}	0.041	0.180
10^{-9}	0.022	0.017
10^{-10}	0.002	0.004
Ro/SSA (10 μg/ml)*	0.016	0.008

Titrations of human or bovine IgG were added to purified Ro/SSA and adsorbed to microtiter wells. The sensitivity of IgG detection was measured by the addition of anti–human or anti–bovine alkaline phosphatase and the addition of paranitrophenylphosphate $(PNPP)^1$ as compared with uncontaminated Ro/SSA as the negative control. Values expressed as OD_{405}.

* Purified Ro/SSA at 5 μg/ml was adsorbed to microtiter wells and incubated with anti–human or anti–bovine IgG alkaline phosphatase followed by the substrate PNPP. OD_{405} values are relative to 0.1% BSA in PBS as the negative control.

terized for its relative specific anti-Ro/SSA and anti-IgG activity (Table III). The fraction of the rabbit anti-Ro/SSA serum that bound to human IgG had increased relative specific anti-IgG as well as anti-Ro/SSA activities. Likewise, both the anti-Ro/SSA and anti-IgG activities were concentrated in the fraction of this serum-binding Ro/SSA. Normal rabbit serum failed to bind either the Ro/SSA or human IgG columns in quantities sufficient for analysis.

Affinity-purified Ro/SSA Is Not Contaminated by IgG. Since interpretation of the crossreactivity data would be complicated if the Ro/SSA contained IgG, two approaches were used to assess possible Ig contamination of the purified Ro/SSA. First, by adding bovine and human IgG to the Ro/SSA preparation followed by separation on 7.5% SDS-PAGE, silver staining was shown to detect ≥2.0% (wt/wt) IgG in the purified Ro/SSA. In the purified Ro/SSA preparations, we saw no bands other than the 60-kD Ro/SSA in SDS-PAGE. Second, we used anti-IgG conjugated to alkaline phosphatase to detect IgG directly in the ELISA plates coated with Ro/SSA (Table IV). By intentionally contaminating a Ro/SSA

TABLE V

F(ab')₂-binding Activity of Autoanti-Ro/SSA Patient Sera

Specific binding activity	Anti-Ro/SSA patient sera									
	1	2	3	4	5	6	7	8	9	10
Ro/SSA binding										
Percent inhibition with Ro/SSA*	91	92	97	92	92	93	90	88	80	97
Percent inhibition with IgG*	65	28	71	44	0	22	0	0	0	56
F(ab')₂ binding‡	1.10	1.27	0.64	0.41	0.19	0.65	0.05	0.50	0.54	0.56

* Anti-Ro/SSA activity of Ro/SSA precipitation–positive sera was determined with and without incubation with 10 μg/ml Ro/SSA and 100 μg/ml bovine IgG, respectively.
‡ The OD_{450} of the solid-phase binding of patient sera to F(ab')₂ fragments of bovine IgG is presented. Minimal background binding ($\leq$0.05 OD_{450}) was found in normal controls.

preparation with IgG, it was shown that 0.2% (wt/wt) IgG contamination of the Ro/SSA could reliably be detected over background. However, no IgG was detected in the Ro/SSA preparations used for solid-phase assays, Western blot, or the Ro/SSA–Sepharose 4B affinity columns.

Survey of Autoanti-Ro/SSA Binding Activity. We observed that both the rabbit and prototype human anti-Ro/SSA sera bound F(ab')₂ fragments to a much greater extent than isolated Fc fragments. A group of 10 patients with precipitating Ro/SSA autoantibody were tested in the solid-phase assay for anti-F(ab')₂ and anti-Ro/SSA binding activity (Table V). In all samples, Ro/SSA binding was inhibited by >80% with purified Ro/SSA. In 6 of the 10 patients, Ro/SSA binding was inhibitable to various degrees by IgG, and the same 6 patients also had F(ab')₂-binding activity in their sera. Significant anti-F(ab')₂ antibody was additionally found in two patients (numbers 8 and 9) whose Ro/SSA-binding activity were not inhibited by IgG. These two sera may possess antibodies that bind epitopes exposed only after pepsin digestion of IgG (35). Consequently, these two sera do not have evidence for antibodies binding both Ro/SSA and intact IgG. Normal human sera had neither Ro/SSA nor F(ab')₂-binding activity.

Discussion

In this study we have demonstrated that the small ribonucleoprotein Ro/SSA is immunogenic and that both autoimmune and heteroimmune anti-Ro/SSA antibodies also bind IgG. Prior studies of this RNA-bound protein have exclusively used patient sera that precipitated the Ro/SSA protein in Ouchterlony immunodiffusion. We have found that immunization of rabbits with purified Ro/SSA induced antibody with precipitin identity to a prototype patient serum (Fig. 1), and that the rabbit and human sera had comparable Ro/SSA-binding titers in solid-phase assays.

Initial studies showed that the heteroimmune sera possessed anti-IgG activity in solid-phase assays. The sera were adsorbed to remove IgG-binding activity in an attempt to develop a monospecific reagent and enrich for the anti-Ro/SSA specific activity. Unexpectedly, adsorption of the sera with Sepharose-IgG reduced the anti-Ro/SSA activity by nearly 100-fold (Table II). The immunologic specificity between Ro/SSA and IgG was then characterized by competitive inhibition in the solid-phase assay. We found that IgG could partially inhibit the anti-Ro/SSA activity of both the heteroimmune and autoimmune anti-Ro/SSA

sera (Fig. 3), and conversely, that Ro/SSA inhibits the anti-IgG activity of the sera (Fig. 4). Histones, albumin, or a related ribonucleoprotein, La/SSB, failed to inhibit the binding of anti-Ro/SSA to Ro/SSA (Table I). We have also observed that antibody eluted from the specific Ro/SSA band in Western blotting (by the method of Smith et al. [36]) binds IgG in ELISA (data not shown), as does affinity-purified specific anti-Ro/SSA (Table III). Finally, the IgG-binding fraction of the rabbit serum had increased relative anti-Ro/SSA specific activity as compared with unadsorbed serum (Table III).

We performed additional experiments to rule out potential artifacts due to IgG contamination of Ro/SSA (Table IV) or to immune complexes (Ro/SSA-IgG and IgG-IgG). No IgG contamination of Ro/SSA preparations could be detected nor was evidence found for immune complexes within either the heteroimmune or autoimmune sera. Gel diffusion experiments were also performed, but failed to define the immunologic relationship of Ro/SSA and IgG. Other investigators (Herrera-Esparza, R., and L. A. Diaz, unpublished observations), however, have also immunized rabbits with Ro/SSA and have demonstrated partial immunologic identity between Ro/SSA and IgG in gel diffusion.

In a study of patients with Ro/SSA-precipitating antibody, we found that a majority of patients possessed Ro/SSA-binding activity that was inhibitable with IgG in the solid-phase assay (Table V). A relationship between Ro/SSA and IgG binding antibody has been shown in clinical studies that may explain this observation. In patients with SLE and Sjögren's syndrome, rheumatoid factor is found in ~70% of those with Ro/SSA-precipitating antibody (17). Additionally, the titers of rheumatoid factor and anti-Ro/SSA antibody are directly associated in primary Sjögren's syndrome (18). In a study (19) designed to compare the solid-phase ELISA results with Ro/SSA precipitin data, it was found that higher levels of rheumatoid factor were found in the sera that formed Ro/SSA precipitins when compared with sera with the same level of anti-Ro/SSA by solid phase–binding, but that failed to form a precipitin. The results suggest that rheumatoid factor augments Ro/SSA precipitation. If some anti-Ro/SSA possess rheumatoid factor–like activity, enhanced precipitation would result by increasing the complexity of lattice formation between the autoantibody and Ro/SSA.

Two ligands that react with the same antibody will often share a common structural component. The structural analog that leads anti-Ro/SSA to bind IgG is not known, although other preliminary data (Mamula M. J., and J. B. Harley, unpublished observations) suggest that anti-Ro/SSA binds the H chain and the $F(ab')_2$ fragment of IgG. The binding to these sites on IgG raises the possibility that allotypes or particular idiotypes may be structurally important for this interaction. On the other hand, the effectiveness of both bovine and human IgG to inhibit binding to the Ro/SSA particle argues against contributions from IgG structural properties that are not similar between species.

In the context of autoimmune disease, our findings may bear similarities to the crossreactivity between IgG and histones that is found in some patients with rheumatoid arthritis who have positive antinuclear antibody (20). In this regard, Hannestad et al. (37) were successful in removing both antinuclear antibody and rheumatoid factor activity by adsorption of serum with human IgG, just as anti-Ro/SSA and anti-IgG activity were lost with IgG adsorption. Rubin et al. (24)

have demonstrated monoclonal rheumatoid factors that also bound histones and single-stranded DNA. While IgG-binding activity is a common theme of several studies of antibody multispecificity (20–26), its importance has yet to be exploited in defining crossreactive epitopes of different ligands. Rheumatoid factor and antihistone antibodies appear to have similar idiotypes in some patients, though the basis for the crossreactivity is perplexing since no obvious structural similar-ities exist between IgG and histones. Since no structural features of Ro/SSA are known, the physical basis for crossreactivity of Ro/SSA and IgG cannot, as yet, be understood. Models of crossreactivity should not, however, be limited to those of similar structure since antibody can bind multiple ligands at different regions within the antibody-combining site (38). Mechanisms of antigen crossreactivity appear to reach beyond explicit amino acid similarities and may rely on other physical properties, such as charge or spatial configuration. Nevertheless, multi-specificity of an antibody-binding site is one mechanism of generating diversity within the antibody repetoire.

This study adds the Ro/SSA ribonucleoprotein to the lengthening list of substances that possess functional immunologic resemblance to IgG (20–26). The association of Ro/SSA with IgG may be an important feature in the induction and perpetuation of the autoimmune response.

Summary

The rheumatic disease autoantigen, Ro/SSA, was immunogenic to a rabbit host. The heteroimmune rabbit serum bound the Ro/SSA particle in immuno-blots and in an ELISA. Both the rabbit anti-Ro/SSA and a human prototype anti-Ro/SSA serum also bound IgG; and moreover, IgG inhibited both rabbit and human anti-Ro/SSA activity. Anti-IgG activity of the rabbit and human anti-Ro/SSA sera bound Ro/SSA by Western blot and solid-phase assays. In addition, purified Ro/SSA inhibited the anti-IgG activity of the anti-Ro/SSA sera from rabbit and man. Affinity purification of the IgG- and Ro/SSA-binding fractions of the rabbit anti-Ro/SSA demonstrated that both the anti-Ro/SSA and anti-IgG activities were concentrated in these fractions. These data show that Ro/SSA and IgG share epitopes that are bound by anti-Ro/SSA antibody. Inhibition experiments suggest that this antibody is found in most human anti-Ro/SSA autoimmune sera and that the epitope(s) are found in the $F(ab')_2$ fragment of IgG.

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