Anti-Ro Autoantibody with Cross-Reactive Binding to the Heavy Chain of Immunoglobulin G

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Autoantibodies directed at the intracellular Ro ribonucleoprotein complex are found in the serum of patients with systemic lupus erythematosus (SLE) and related autoimmune diseases. The antigenic stimulus for the induction of these autoantibodies is unknown, although we have previously demonstrated that the Ro protein and immunoglobulin G (IgG) share immunologic determinants bound by anti-Ro antibodies. The present study further defines the fine specificity of this cross-reactive binding. Using both patient autoanti-Ro antibodies and antigen-induced rabbit anti-Ro serum, the binding specificity for IgG was located to the heavy chains of IgG outside the Fc domain. F(αβ')2 fragments of IgG were observed to inhibit specific Ro binding by either human or antigen-induced rabbit sera, while Fc fragments of IgG failed to inhibit Ro binding. Anti-Ro sera were found to bind the heavy chains of IgG in immunoblots, and the antibodies eluted from these heavy chains were capable of immunoprecipitating the Ro particle from human cell extracts. Not all patient sera with anti-Ro antibodies possessed IgG binding antibodies. Studies of cyanogen bromide digestion fragments of IgG implicate the hinge region of IgG as the region cross-reactive with the Ro protein. The nature of this cross-reactivity may be important in understanding the induction and/or perpetuation of the anti-Ro response in patients with autoimmune disease.

INTRODUCTION

The presence of serum autoantibodies to a wide variety of cellular constituents is an underlying theme in patients with connective tissue disease. In particular, autoantibodies directed at nucleic acids and at proteins which bind both DNA and RNA are diagnostic markers for patients with systemic lupus erythematosus [1]. These autoantibodies commonly include specificities for the Sm, U1(nRNP), Ro, and La ribonucleoproteins [1]. Autoantibodies specific for the intracellular RNA-protein complex designated "Ro" are observed in over half of patients with systemic lupus erythematosus (SLE), most patients with Sjogren's syndrome, and a substantial percentage of normal individuals [2,3]. The exact biological function of the Ro ribonucleoprotein is unknown, though its role as an important target in autoimmune responses has been known since the first description of this autoantibody by Anderson et al. in 1961 [4].

The Ro protein is associated with two to four uridine-rich RNAs, depending both on the species of mammalian cell and the type of somatic cell examined [5,6].

Abbreviations: BSA: bovine serum albumin  CNBr: cyanogen bromide  ELISA: enzyme-linked immunosorbent assay  FPLC: fast protein liquid chromatography  SLE: systemic lupus erythematosus

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humans, the Ro RNAs are from 83 to 112 nucleotides in length and are a product of RNA polymerase III in the cell. The Ro protein is also transiently associated on RNAs with another ribonucleoprotein designated “La,” and autoantibodies to these two ribonucleoproteins are frequently co-expressed in patient sera [7]. The Ro protein and its RNAs are precipitable from several mammalian species by patient sera, suggesting that many of the autoantigenic epitopes of the protein are evolutionarily conserved [6].

In a previous study, we have shown that anti-Ro antibody from both autoimmune patient serum and antigen-induced animal serum bind not only the Ro protein but also have IgG binding activity [8]. Consistent with this observation, it has been reported that 70 percent of patients with Ro precipitating autoantibodies also have rheumatoid factors [9]. It may be postulated, therefore, that the anti-Ro component of sera may contribute to the detection of positive rheumatoid factors. In this study, we have analyzed the fine specificity of anti-Ro antibody for the IgG molecule. We conclude that the IgG cross-reactivity of anti-Ro does not resemble conventional rheumatoid factors, since Fc regions of IgG are not bound by anti-Ro. We have now demonstrated that the anti-Ro antibody has binding specificity for the F(ab')2 heavy chain region of IgG. The dual specificity of binding by anti-Ro autoantibodies may help to explain how the autoantibody response is first initiated or how the response is maintained in patients with systemic autoimmune disease.

METHODS

Purification of Ro Protein

Bovine Ro protein was purified according to a previously reported procedure [10]. Briefly, bovine spleen was cut into two cm³ pieces and mixed with an equal amount (weight/volume) of PBS with 2 mM dithiothreitol. The mixture was homogenized, centrifuged at 10,000 g, and the supernatant of this cell lysate was incubated with 40 percent (volume/volume) DE-52 (Whatman Co., Clifton, NJ) for four hours at 4°C with occasional stirring. The DE-52 was thoroughly washed with PBS and extracted with 1.0 M NaCl in 0.02 M phosphate buffer, pH 7.2. The extract was then run through an affinity column made from the IgG fraction of a patient serum with high titers of anti-Ro antibody. The column was washed and eluted with 3.0 M MgCl₂, and the eluate was dialyzed against 0.02 M Tris, 0.15 M NaCl, pH 7.2 (TBS). Ro protein purified by this method consists of a single characteristic 60 kD band by SDS-PAGE and is free of contaminating IgG or other related ribonucleoproteins [8].

Isolation of IgG Binding Fractions from Anti-Ro Sera

Rabbit anti-Ro antibodies were generated by immunization and subsequent boosts with affinity-purified 60 kDa Ro protein, as previously described [6]. The IgG binding components of either human or rabbit anti-Ro serum were purified by affinity chromatography. Rabbit or human anti-Ro and normal human and rabbit sera as controls were adsorbed to affinity columns consisting of Cohn fraction V of normal human IgG (Sigma Chemical Co., St. Louis, MO), attached to cyanogen bromide-activated Sepharose 4B. The columns were extensively washed with TBS and eluted with 3 M MgCl₂. The fractions were dialyzed against TBS, concentrated (Amicon Co., Danvers, MA), and studied for their Ro and IgG binding properties, as described below.
Western Immunoblots and Elution of Antibody from IgG Heavy and Light Chains

Blotting of human IgG substrates was performed by established techniques [11]. Briefly, human IgG (Sigma) was run under denaturing conditions in 7.5 percent (weight/volume) polyacrylamide gels and electrophoretically transferred to nitrocellulose (Bio-Rad, Richmond, CA). The nitrocellulose was blocked in 1 percent BSA and probed with a $10^{-2}$ dilution of rabbit anti-Ro or pre-immune rabbit serum. Goat anti-rabbit alkaline phosphatase conjugate was incubated with nitrocellulose and developed with β-naphthyl acid phosphate and o-diansidine, as previously described [12].

Alternatively, antibodies were eluted from the heavy or light chains of IgG [13]. Human IgG was electrophoresed in reducing PAGE gels and transferred to nitrocellulose. Human and rabbit anti-Ro ($10^{-2}$ dilutions) were incubated with the blot, and the heavy and light chains of IgG were each cut from the nitrocellulose. Antibodies were twice eluted from the nitrocellulose strips with 15 mM glycine, 0.5 M NaCl, 0.1 percent Tween, and 100 μg/ml BSA (pH 2.3) for two minutes. Eluted antibody was immediately neutralized to pH 7.2 with 1.0 M Tris, pH 8.0. Antibodies eluted in these fractions were then used in the RNA immunoprecipitation assay.

Immunoprecipitation of Ro RNAs

The RNAs associated with the Ro protein were immunoprecipitated as previously reported [14]. HeLa cell stocks were maintained at 37°C, 5 percent CO$_2$ in RPMI 1640 (Gibco Laboratories, Grand Island, NY), supplemented with 10 percent fetal calf serum, 60 μg/ml penicillin, and 100 μg/ml streptomycin. Log phase HeLa cells were centrifuged (400 g, five minutes), washed once with TBS, and resuspended to $2 \times 10^5$ cells/ml in phosphate-free minimal essential medium (Gibco), supplemented with 10 μCi/ml $^{32}$P orthophosphate (Amersham, Arlington Heights, IL). After 16 hours (37°C, 5 percent CO$_2$), the cells were washed with TBS, resuspended in NET-2 buffer (50 mM Tris, 150 mM NaCl, 0.05 percent NP-40, pH 7.4), and sonicated. The cell sonicate was centrifuged at 10,000 g for 30 minutes, and the lysate was mixed with protein A-Sepharose 4B (Pharmacia Co., Piscataway, NJ), bound to rabbit anti-Ro or affinity-purified IgG binding fractions of the rabbit sera. After 60 minutes, the samples were washed five times with NET-2 and deproteinized with phenol/chloroform/isoamyl alcohol (50:50:1). The aqueous layer was removed, and the nucleic acids were precipitated with 100 percent cold ethanol. The samples were denatured at 65°C for three minutes in sample buffer (10 M urea, 0.025 percent bromophenol blue, and 0.025 percent xylene cyanol FF in Tris-borate EDTA buffer [90 mM Tris, 90 mM borate, 1 mM EDTA, pH 8.6]) and electrophoresed in a 7 M urea/10 percent polyacrylamide gel. The gel was dried and exposed to photographic film (Eastman Kodak Co., Rochester, NY) against an intensifying screen for 20 hours at −70°C.

Enzyme-Linked Immunosorbent Assays (ELISA)

Twenty SLE patient sera positive for anti-Ro in immunodiffusion studies and negative for rheumatoid factor by latex fixation were examined for binding to purified Ro, F(ab')$_2$, and Fc fragments of IgG by ELISA, according to established methods [10,15]. Purified human F(ab')$_2$ and Fc fragments (Cappel Laboratories, Malvern, PA) were adsorbed to microtiter plates at 10 μg/ml in carbonate buffer, pH
9.6. Purified Ro was similarly plated at 5 μg/ml. Plates were blocked with 1 percent bovine serum albumin (BSA), and 10⁻² serum dilutions of patient sera were applied to duplicate wells for two hours at room temperature. The plates were washed, and the F(ab’)₂-coated plates were incubated with anti-human Fc conjugated to alkaline phosphatase (Sigma). Conversely, the Ro- and Fc-coated plates were incubated with anti-human F(ab’)₂ conjugate. After thorough washing and the addition of p-nitrophenylphosphate substrate, the optical density was measured at 405 nm (MR 580 ELISA reader, Dynatec Laboratories, Alexandria, VA).

Fragments of human IgG and purified Ro protein were investigated for their ability to inhibit Ro binding activity of human and rabbit anti-Ro serum. The IgG fraction of a prototype human anti-Ro serum was biotinylated by the method of Wofsy [16]. Rabbit anti-Ro and biotinylated human anti-Ro were pre-incubated with 100 μg/ml of human F(ab’)₂, Fc, whole IgG, or 10 μg/ml purified Ro protein. Samples were applied to Ro-coated plates, and binding was detected with goat anti-rabbit IgG-alkaline phosphatase (Sigma) or avidin-alkaline phosphatase, as described above (Sigma). All data represent the mean of duplicate wells from representative experiments.

Cyanogen Bromide Cleavage of IgG

Specific fragments of the IgG molecule were obtained by cyanogen bromide (CNBr) digestion, according to previously described methods [17,18]. Pooled human IgG was dissolved in 70 percent formic acid to a concentration of 20 mg/ml. Solid CNBr was added 5:1 (weight/weight), and the reaction mixture was incubated 24 hours at room temperature. The contents were brought up in ten volumes of water and lyophilized to stop the reaction. Fragments were isolated by fast protein liquid chromatography (FPLC) (Pharmacia) over a Superose 12 gel filtration column (Pharmacia) with 0.1 M acetic acid as running buffer. Sample peaks collected from the FPLC were analyzed for molecular weight by comparison of retention times with standard molecular weight markers (Bio-Rad), run under identical conditions. Peak fractions were then adsorbed to microtiter plates in PBS for ELISA binding analysis of rabbit anti-Ro sera, as described above.

RESULTS

Antibody Eluted from IgG Binds the Ro Ribonucleoprotein

IgG binding antibodies were obtained first by immunoaffinity purification on human IgG-Sepharose 4B. Both whole rabbit serum (Fig. 1, lanes 1 and 2) and the rabbit serum fraction eluted from the IgG affinity column immunoblotted the 60 kDa purified Ro protein (lanes 3 and 4) as well as the 50 kDa heavy chain of human IgG. The sera failed to bind the light chains of IgG in immunoblots, and pre-immune
rabbit serum failed to bind either purified Ro protein or the heavy or light chains of IgG (lanes 5 and 6). These results support our previous study that demonstrated binding to Ro protein in solid phase immunoassays by antibodies eluted from IgG-Sepharose affinity columns [8].

The IgG eluates were then used to immunoprecipitate the Ro particle from 32P-labeled Hela cells in order to determine if the shared antibody binding site between Ro and IgG is present on the native Ro particle (Fig. 2). Total ethanol-precipitated RNA from Hela cell extracts is shown in lane 1. The Ro RNAs are not detectable in total RNA extracts from cell lysates because of their low copy number in the cell. Pre-immune serum failed to precipitate Ro RNAs (lane 2), while rabbit anti-Ro whole serum immunoprecipitated the typical Ro RNAs designated hY1 to hY5 (lane 3). Rabbit anti-Ro antibody eluted from the human IgG affinity column also precipitated the Ro RNAs (lane 4).

Rabbit anti-Ro was eluted from the heavy and light chains of IgG in Western immunoblots, as described above. Elution steps from the light chains of IgG failed to precipitate the Ro RNAs (Fig. 2, lane 5), while antibody eluted from heavy chains weakly precipitated the typical Ro RNAs (lane 6). Antibody eluted from the heavy chains is therefore capable of binding the native Ro particle from crude cell extracts. As further controls, anti-Sm human sera were adsorbed to IgG blots and eluted as above. All elutions failed to immunoprecipitate the typical Sm RNAs from HeLa cell extracts (data not shown).

**Anti-Ro Preferentially Binds F(ab')2 and Heavy Chain Fragments of IgG**

In attempts to localize the IgG binding site of anti-Ro antibodies, 20 patient sera with anti-Ro were screened in solid phase assays for Fc or F(ab')2 fragment binding
activity (Fig. 3). All sera examined were negative for rheumatoid factors by conventional latex agglutination assays. When purified human Fc fragments were adsorbed to microtiter plates, anti-human F(ab′)2-specific enzyme conjugate was used to detect patient IgG binding. Conversely, when human F(ab′)2 fragments were the source of antigen, anti-human Fc-specific conjugates were used to detect patient antibody. Purified human Fc, F(ab′)2, and bovine Ro were all plated at 5 μg/ml. Patient sera were tested at 10−2 dilutions for binding to fractions of IgG and at 10−3 serum dilutions for Ro protein binding. For all sera examined, relative Ro binding O.D. 405 was greatest when compared to their respective Fc or F(ab′)2 binding activity. All sera displayed weak Fc binding relative to normal serum controls. Twelve of 20 sera showed significant binding (>2 S.D. above the mean of normal serum controls) to the F(ab′)2 fragments of human IgG. Twenty sera specific for the Sm and/or U1 autoantigens by immunodiffusion failed to bind significantly the Fc, F(ab′)2, or Ro protein antigens in this solid phase assay by the above criterion (data not shown).

Inhibition of Ro Binding by Fragments of IgG

Purified Ro protein, purified IgG, and fragments thereof were examined in solid phase assays for their ability to inhibit Ro binding activity (Table 1). Prototype human anti-Ro was first biotinylated so that human IgG used in inhibition assays would not interfere in the interpretation of the results. Purified Ro protein inhibited
83 percent of the Ro binding activity. Fc fragments only weakly inhibited Ro binding (7 percent) relative to inhibition by whole human IgG (34 percent) or F(ab')$_2$ fragments (48 percent). Likewise, the rabbit anti-Ro serum was best inhibited by purified Ro protein (94 percent). Fc fragments failed to inhibit Ro binding (18 percent) as well as whole IgG (61 percent) or F(ab')$_2$ fragments (41 percent).

**Anti-Ro Antibody Binding to Cyanogen Bromide-Digested Fragments of IgG**

In order to characterize further the binding site on IgG of the Ro binding antibodies, CNBr-cleaved fragments of human IgG were assessed for antibody binding in ELISA assays. Pooled human IgG was first completely digested with cyanogen bromide in 70 percent formic acid and lyophilized. Fragments were resuspended and separated by FPLC over a gel filtration column. Five separate peak fractions were obtained (Fig. 4), as previously observed by other investigators [18]. Fractions were collected and adsorbed to the solid phase of microtiter plates. Rabbit anti-Ro whole serum and biotinylated human anti-Ro were assayed for binding to the CNBr digestion fragments of human IgG (Table 2). Both human and rabbit anti-Ro bound fraction 41 best among the fractions collected. Binding to fraction 41 by human or rabbit anti-Ro serum was significantly inhibited by pre-incubation with purified Ro protein (82 percent and 68 percent, respectively; data not shown). The molecular weight of fraction 41 is approximately 10 kDa when compared to standard molecular weight markers for column calibration. The CNBr profiles obtained in this study correspond to those obtained by gel filtration chromatography in previous studies [17,18,20]. Although this peak was not identified by amino acid sequence analysis, it does correspond identically to a CNBr digestion product of the hinge region of the gamma chain of IgG [18].

**DISCUSSION**

Our previous study had demonstrated that a subset of Ro binding autoantibodies also bound human and bovine immunoglobulin G [8]. Initially, upon adsorption of anti-Ro serum over IgG affinity columns, the relative specific Ro binding activity of the effluent was dramatically decreased when compared to unadsorbed serum. Furthermore, the eluates from these IgG columns retained Ro binding activity in ELISA assays. The present study extends our previous work [8] in order to characterize further the specificity of the IgG binding site by both human autoimmune serum and heteroimmune rabbit anti-Ro antibodies. We have also surveyed the IgG

### TABLE 1

| Human F(ab')$_2$ and Fc Inhibition of Ro Binding Activity |
|----------------------------------------------------------|
| Ro Binding | % Inhibition$^a$ |
|------------|------------------|
| O.D. 405   | F(ab')$_2$ | Fc | Whole IgG | Ro |
| Human anti-Ro | 0.34 | 48 | 7 | 34 | 83 |
| Rabbit anti-Ro | 0.69 | 41 | 18 | 61 | 94 |

$^a$Rabbit and biotinylated human anti-Ro were pre-incubated with 100 μg/ml each of human F(ab')$_2$, Fc, whole IgG, or 10 μg/ml of purified Ro protein prior to their addition to Ro-coated plates. Percentage of inhibition was calculated as: 1 - (inhibited O.D. 405/untreated O.D. 405) × 100. Normal human serum and pre-immune rabbit serum background was < 0.09 O.D. 405.
binding activity from a series of human anti-Ro sera in order to understand the prevalence of these cross-reactive antibodies.

Rabbit anti-Ro antibodies have been used in this and other studies of the 60 kDa Ro ribonucleoprotein [6,8]. The use of antigen-induced animal serum in these studies lends insight into the origins of patient autoantibodies, which arise from undetermined origins. Our rabbit antibodies resemble human autoantibodies in a number of respects. Both sera show identity for Ro in Ouchterlony immunodiffusion, bind the 60 kDa Ro protein in immunoblots, and compete for Ro epitopes in solid phase assays [6,8]. Both human and rabbit anti-Ro bind the native Ro particle, as

FIG. 4. Fragments of cyanogen bromide-digested human IgG. Pooled human IgG was digested with CNBr, and polypeptides were separated by FPLC over a gel filtration column. Fractions were collected for the peaks as indicated. Retention of molecular weight standard markers (66 kDa, 45 kDa, 21 kDa, and 14 kDa) are indicated at the top of the figure.

| Fraction | 7 | 16 | 20 | 24 | 30 | 41 |
|----------|---|----|----|----|----|----|
| Human anti-Ro | .07 | .18 | .20 | .28 | .15 | .64 |
| Rabbit anti-Ro | .09 | .08 | .11 | .06 | .12 | .22 |

*aCyanogen bromide fragments of human IgG were isolated by FPLC (Fig. 4) and adsorbed to the solid phase of microtiter wells. Rabbit and prototype human anti-Ro sera were then tested for their ability to bind the isolated fragments (O.D. 405). Non-immune sera had background binding of <0.11 for all fractions.
shown by the ability to immunoprecipitate the Ro RNAs from human cell lysates (Fig. 2). While it has been demonstrated recently that some human sera bind a distinct 52 kDa Ro protein [19], the rabbit anti-Ro serum only precipitates the 60 kDa Ro protein. The rabbit serum serves as an excellent model of anti-Ro specificities, since it is a true autoantibody, as measured by its ability to bind autologous rabbit Ro protein [8].

The rabbit anti-Ro serum binds both the 60 kDa Ro protein and 50 kDa heavy chains of IgG in immunoblots (Fig. 1). Light chain binding is not apparent in these studies. In addition, a prototype human anti-Ro serum binds the heavy chain of bovine IgG in immunoblots (data not shown). Antibody eluted from the heavy chains of IgG in these blots immunoprecipitates the Ro RNAs (Fig. 2), suggesting that the cross-reactive epitope is present on both the native Ro particle and the reduced heavy chain of IgG. The 52 kDa Ro protein does not possess the cross-reactive epitope, since our sera do not bind this Ro protein. Our data support the initial observation that the 52 kDa protein has epitopes different from those on the 60 kDa Ro protein [19].

Binding to human Fc, F(ab')2, and purified Ro was assayed in a series of patient sera with precipitin-positive anti-Ro antibodies. All sera bound Ro with at least tenfold greater relative titers than either fragment of IgG (Fig. 3). No sera were observed to bind Fc fragments significantly over normal human serum controls, while 12 of 20 sera bound F(ab')2 fragments in the ELISA assay. The Ro binding antibodies in human and rabbit sera were also significantly inhibited by purified Ro and human F(ab')2 fragments but not by Fc fragments (Table 1). This observation suggests that the cross-reactive epitope lies on the heavy chain outside the Fc domain of IgG.

The IgG fragments derived from cyanogen bromide digestion have now been well characterized [17,18,20]. We utilized these digests to confirm further the location of the IgG epitope bound by anti-Ro sera. Cyanogen bromide digestion of IgG yielded four major peaks (Fig. 4). Polypeptides from fraction 41, the smallest polypeptides in molecular weight among the peaks collected, were bound best by both human and rabbit anti-Ro serum. The peak from fraction 41 corresponds to a molecular weight of approximately 10 kDa that is consistent with a fragment of IgG, located at the hinge region of the gamma heavy chain [20]. The region is rich in hydrophilic amino acids, which may lend itself to expression as an immunogenic region of the surface of the IgG molecule.

Previous studies have demonstrated the presence of autoantibodies specific for the Fab and F(ab')2 fragments of IgG [21,22]. These autoantibodies are typically found in patients with rheumatoid arthritis and systemic lupus erythematosus (SLE). The co-expression of anti-Ro with anti-F(ab')2 in these patients may be related to the expression of cross-reactive epitopes between the Ro particle and IgG, as described in this study. Alternatively, these cross-reactive epitopes may be responsible for the initiation and/or perpetuation of the autoantibody responses.

In evaluating the series of patient sera with precipitin-positive anti-Ro, the sera with highest anti-Ro titers by ELISA also had the highest anti-F(ab')2 antibody responses. Only one exception among this group was notable for relatively low anti-Ro ELISA titers with measurable anti-F(ab')2 activity. Not surprisingly, it has previously been reported that high titers of antinuclear antibodies and rheumatoid factors contribute to anti-Ro precipitin formation in immunodiffusion studies [9].
Our observations suggest that a subset of anti-Ro antibodies bind a cross-reactive epitope of IgG. Not all precipitin-positive sera possessed IgG binding properties. These cross-reactive antibodies are most likely to appear as the anti-Ro responses mature and become high-titered in the patient.

Based on end point dilutions in ELISA, the antigen-induced rabbit serum had relative anti-Ro titers as high as our prototype patient serum and also possessed the cross-reactive idiootype. The animal model of anti-Ro antibody induction thus resembles the human autoantibody and suggests that the anti-Ro response in humans is induced by the 60 kDa Ro antigen itself.

Other antibodies with cross-reactive properties to IgG have been previously described in patients with autoimmune disease [23–25]. Autoantibodies with specificity for histones and native DNA have been found to possess rheumatoid factor-like activity. Binding to these multiple epitopes was removed by adsorption to IgG, just as anti-Ro binding is removed by IgG adsorption in our study. Amino acid sequence studies of histone proteins reveal no similarities with IgG that would suggest a structural analog for the existence of cross-reactive epitopes, just as the available sequence of the human 60 kDa Ro protein does not demonstrate homology with IgG [26]. Indeed, amino acid sequence homology is not necessarily a requisite of immunologic cross-reactivity. The basis of the cross-reactivity between Ro and IgG will become more obvious as specific structural properties and studies of immunologic determinants of the Ro protein become available.

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