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Induction of Antibodies Reactive with SSA/Ro-SSB/La and Development of Congenital Heart Block in a Murine Model

M. Eugenia Miranda-Carús,* Mohamed Boutjdir,† Chung-E Tseng,* Francis DiDonato,* Edward K. L. Chan,‡ and Jill P. Buyon2*

To correlate the arrhythmogenic effects of maternal autoantibodies with the genesis of congenital heart block, female BALB/c mice were immunized with human recombinant 48-kDa SSB/La, 60-kDa SSA/Ro, 52-kDa SSA/Ro (52α), and 52β (amino acids 169–245 deleted) as well as with murine recombinant 52-kDa SSA/Ro. Control animals received β-galactosidase or a polypeptide encoded by pET-28 alone. Following primary immunization and two boosters, high titer responses to the respective Ags were established by ELISA, immunoblotting, and immunoprecipitation. Sera from mice immunized with either human 52α or 52β immunoprecipitated murine 52Ro. mRNA and protein expression of 52Ro was demonstrated in the newborn murine heart. A spectrum of atrioventricular nodal conduction abnormalities was identified by electrocardiogram. First-degree block was detected in 7% of 27 pups born to mothers immunized with 48La, 20% of 54 pups born to 60Ro-immunized mothers, 6% of 56 pups born to 52α-immunized mothers, 7% of 86 pups born to 52β-immunized mothers, and 9% of 22 pups born to mothers immunized with murine 52Ro. Advanced conduction abnormalities were only identified in offspring of 52α or 52β-immunized mice. In the 52α group, one pup had complete block and another had second-degree block (Wenckebach type); in the 52β group, five pups had complete block. Maternal Abs to the primary immunogens were detected in the pups. No control had any conduction abnormalities. This Ab-specific animal model provides strong evidence for a pathogenic role of anti-SSA/Ro-SSB/La Abs, particularly 52Ro, in the development of congenital heart block. The range and frequency of conduction defects suggest that additional factors promote disease expression. The Journal of Immunology, 1998, 161: 5886–5892.

Pregnant women with autoantibodies to 48-kDa SSB/La, 52-kDa SSA/Ro, or 60-kDa SSA/Ro ribonucleoproteins are at risk for having infants with isolated congenital heart block (CHB)1, which carries significant morbidity and mortality (1, 2). Autoimmune-associated CHB is currently considered a model of passively acquired autoimmunity, in which the mother (who may have systemic lupus erythematosus (SLE), Sjögren’s syndrome, or be entirely asymptomatic (3)) synthesizes anti-SSA/Ro-SSB/La Abs that cross the placenta and presumably injure the conduction system of the developing fetus. The atrioventricular (AV) block can be first, second, or third degree, with the former being quite rare (1, 2) and the latter two being most common. Despite identical circulating Abs, the maternal heart is never affected.

A molecular definition for all three target autoantigens has been provided by the isolation of cDNA clones. 60-kDa SSA/Ro contains an RNA-binding protein consensus motif (5, 6), which could account for its direct interaction with small cytoplasmic hY RNAs (7). More recent studies demonstrate that the “zinc finger” in human 60-kDa SSA/Ro is not conserved across species (8). It has been suggested that 60-kDa SSA/Ro may function as part of a novel quality control or discard pathway for 5S ribosomal RNA production in Xenopus oocytes (9). Anti-SSB/La Abs recognize a 48-kDa polypeptide that does not share antigenic determinants with either 52-kDa or 60-kDa SSA/Ro (10, 11). SSB/La facilitates the maturation of RNA polymerase III transcripts, directly binds a spectrum of RNAs, and associates at least transiently with 60-kDa SSA/Ro (12, 13). In addition to the well-characterized 60-kDa SSA/Ro and 48-kDa SSB/La autoantigens, another target of the autoimmune response in mothers whose children have CHB is the 52-kDa SSA/Ro protein (14). The full-length protein, 52α, has three distinct domains: an N-terminal region rich in cysteine/histidine motifs containing two distinct zinc fingers known as RING finger and B-box, a central region containing two coiled coils with heptad periodicity (one being a leucine zipper with the potential for intramolecular dimerization), and a C-terminal “ret finger protein-like” domain (15, 16). An alternatively spliced transcript, 52β, has been identified recently (17) in which exon 4 encoding amino acids 169–245, inclusive of the leucine zipper and an immunodominant epitope (18, 19), has been deleted. In vitro-translated 52β is immunoprecipitated by antisera from mothers whose children have CHB (17), which is consistent with reports of an additional N-terminal epitope on 52α that is retained in 52β (18, 19). mRNA expression of 52β is maximal in the human fetal heart between 14 and 16 wk of gestation (17, 20). Clinical and experimental data support a pathogenic role of SSA/Ro and SSB/La Abs in the development of CHB. Maternal IgG anti-SSB/La Igs were identified on the surface of fetal myocardial fibers (21), and anti-SSA/Ro Abs have been eluted from an
affected fetal heart (22). Complete AV block is induced in the rabbit heart (23) as well as the human fetal heart (24) after perfusion of the aorta with sera containing anti-SSA/Ro Abs and affinity-purified anti-52-kDa SSA/Ro Abs. Furthermore, these same Ab preparations inhibit whole-cell and single-channel L-type calcium channels (23, 24). Despite these compelling observations, an animal model has yet to be established. Accordingly, this study was initiated to develop an Ab-specific murine model to correlate the arrhythmogenic effects of maternal autoantibodies with the in vivo genesis of CHB. This was approached by immunizing BALB/c mice with each of the components of the SSA/Ro-SSB/La complex. Mice were mated after the establishment of primary immune responses, and electrocardiograms (EKGs) were performed on all pups within 1 day of birth.

Materials and Methods

Preparation of recombinant Ags

Based on previously reported cDNA sequences, primers containing internal restriction sites to facilitate in-frame directional cloning were designed to amplify cDNA fragments encoding full-length human 48-kDa SSB/La, full-length 52a, 52b (an alternative form of 52-kDa SSA/Ro in which amino acids 1–245 are deleted (17)), 60-kDa SSA/Ro, murine 52-kDa SSA/Ro, and β-galactosidase (β-gal). cDNA fragments were subcloned into pET-28 (Novagen, Madison, WI) for expression of the 6× H isotagged proteins and subsequent Nε2107+ purification. DNA sequencing confirmed the identity of each subclone. *Escherichia coli* strains were transformed with each recombinant plasmid, and cultures were induced with isopropyl thiogalactose for expression of the recombinant proteins. Stored cells were thawed and lysed in 5 ml of 6 M GuHCl, 0.1 M sodium phosphate, and 0.01 M Tris/HCl (pH 8.0). Following sonication and centrifugation, supernatants were collected and added to 8 ml of a 50% slurry of Ni-nitrilotriacetic acid resin (Qiagen, Chatsworth, CA). Recombinant proteins were incubated for 45 min with the Ni-nitrilotriacetic acid resin, and the slurry was loaded onto a 1.5-cm diameter column. Columns were washed with 50 ml of 0.1 M sodium phosphate, and 0.01 M Tris/HCl (pH 8.0) until the A280 was <0.01. This washing was followed by an additional wash with 8 M urea, 0.1 M sodium phosphate, and 0.01 M Tris/HCl (pH 6.3) until the A280 was <0.01. The recombinant proteins were eluted with 20 ml of 8 M urea, 0.1 M sodium phosphate, and 0.01 M Tris/HCl (pH 5.9) followed by 20 ml of 8 M urea, 0.1 M sodium phosphate, and 0.01 M Tris/HCl (pH 4.5) and 20 ml of 6 M GuHCl plus 0.2 M acetic acid (each collected in 3-ml fractions). Elution fractions were analyzed by SDS-PAGE, and samples were dialyzed in 6 M urea and 0.01 M Tris (pH 7.0). Estimations of protein concentration were made using the bichinchonic acid protein assay (Pierce, Rockford, IL).

Immunizations of BALB/c mice

We immunized 6 to 8 wk-old female BALB/c mice (purchased from Charles River, Wilmington, MA) with either recombinant human 48-kDa SSB/La, 52a (full-length) SSA/Ro, 52b SSA/Ro, 60-kDa SSA/Ro, or murine 52-kDa SSA/Ro. For the initial immunization, 50 μg of recombinant protein in CFA was injected i.p. 25 μg of the same preparation in CFA was administered s.c. to the right and left scapular region. Control animals were given the same injections with recombinant β-gal or a Ni2+ affinity purification-purified polypeptide encoded by pET-28 alone in CFA. The first two boosters were tested in parallel against control polypeptides derived from 1/100, 1/500, and 1/1000) for 1 h at 22°C. Goat anti-mouse IgG (whole serum) (Cappel) was administered s.c. to the right and left scapular region. Control animals were given the same injections with recombinant β-gal or a Ni2+ affinity purification-purified polypeptide encoded by pET-28 alone. Results were considered positive if they were >2 SD greater than the mean obtained with sera from pET-28 control-immunized mice.

SDS immunoblots were performed as described previously (25). Recombinant proteins (1–2 μg/lane), human fetal heart extracts, or murine newborn heart extracts were separated by SDS-PAGE in 15% gels using a high rate of acrylamide and a crosslinker. The gels were stained as detailed previously (26). Separated proteins were electrotransferred to a nitrocellulose sheet for 3 h at a constant voltage of 60 V. The nitrocellulose strips were incubated for 1 h in PBS-Tween containing 3% nonfat milk followed by incubation with a 1:100 dilution of sera and were washed extensively in the PBS-Tween solution. Membranes were then incubated for 1 h with a blocking buffer containing a 1:5000 dilution of horseradish peroxidase-linked anti-mouse IgG (New England Bio-Labs, Beverly, MA). Filters were washed again in PBS-Tween, and detection was accomplished using the Phototope-horseradish peroxidase Western blot detection kit (New England Bio-Labs) according to the manufacturer’s instructions. Membranes were wrapped in Saran Wrap and exposed to x-ray film.

In vitro transcription/translation and immunoprecipitation

The Bluescript plasmids p52FL (containing the full-length cDNA for the 52-kDa SSA/Ro/Ro genetic constructs) (15), pATHSP (containing the full-length cDNA for 60-kDa SSA/Ro/Ro) (5), pM9 (containing the full-length cDNA for 48-kDa SSA/Ro/Ro) (10), and p52M (containing the full-length cDNA for murine 52-kDa SSA/Ro/Ro) were linearized and used as substrates for in vitro transcription using T3 RNA polymerase. The resultant RNA was translated in vitro using a rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of 50 mM methionine (Translabel, 70% methionine, and 15% cysteine; ICN Biochemicals, Costa Mesa, CA) as described previously (18).

For immunoprecipitation, 10 μl of murine antisera (1/1 dilution with PBS and 0.1% sodium azide) were mixed with 50 μl of 50% protein A-Sepharose, 100 μl of 10 mg/ml BSA, 200 μl of reaction buffer (150 mM NaCl, 4 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), and 1–10 μl of labeled in vitro translation product. The reaction mixtures were rotated at 4°C for 1–3 h in 1.5-ml Eppendorf tubes. The beads were washed four times with chilled reaction buffer, eluted and boiled with reducing SDS sample buffer, and run on 15% high-ratio monomer/cross-linker acrylamide gels overnight at 7.5 mAmperes. The gels were then stained with Coomassie blue and destained to visualize m.w. markers and ensure uniformity of Ig heavy and light chains precipitated in each reaction, dried with heat and vacuum, and placed with film at ~70°C for periods of time ranging from overnight to 3 days.

RT-PCR analysis for murine 52-kDa SSA/Ro mRNA transcripts

First-strand synthesis was accomplished using 5 μl of 1-day-old mouse heart total RNA containing 40 U RNasin (Promega, Madison, WI) as described previously (17). PCR was performed using a TCA9600 Cycler (Perkin-Elmer, Foster City, CA). Briefly, the 50 μl reaction in 1× Taq buffer contained 2 U Taq polymerase (Life Technologies, Gaithersburg, MD), 2.5 μM MgCl2, 200 μM of deoxynucleoside triphosphates, ~10 ng of cDNA, and 20 pmol of the following primers: 5′-GAAGTACCGTCAG-3′ (an alternative form of 52-kDa SSA/Ro, 60-kDa SSA/Ro, or murine 52-kDa SSA/Ro/Ro). For the initial immunization, 50 μg of recombinant protein in CFA was injected i.p. 25 μg of the same preparation in CFA was administered s.c. to the right and left scapular region. Control animals were given the same injections with recombinant β-gal or a Ni2+ affinity purification-purified polypeptide encoded by pET-28 alone in CFA. The first two boosters were tested in parallel against control polypeptides derived from 1/100, 1/500, and 1/1000) for 1 h at 22°C. Goat anti-mouse IgG (whole serum) (Cappel) was administered s.c. to the right and left scapular region. Subsequent booster injections were continued with a similar regularity until primary responses were detected in all mice of a given group. Thereafter, booster injections were given approximately every 3 wk. Test bleeds were performed every 7–10 days after immunization and boosters.

ELISA and immunoblotting

These assays were performed as described previously (18), with some modifications. Briefly, wells were coated with overnight with 0.1–0.2 μg of recombinant protein in PBS, washed with PBS containing 0.05% Tween 20 (PBS-Tween), blocked with 3% BSA/PBS-Tween, washed with PBS-Tween, and incubated with serial dilutions of Ab in PBS-Tween (1/50, 1/100, 1/500, and 1/1000) for 1 h at 22°C. Goat anti-mouse IgG (whole serum) (Cappel) was administered s.c. to the right and left scapular region. Subsequent booster injections were continued with a similar regularity until primary responses were detected in all mice of a given group. Thereafter, booster injections were given approximately every 3 wk. Test bleeds were performed every 7–10 days after immunization and boosters.

Blood collection

Blood was obtained from the tails of adult mice by and cardiocentesis under anesthesia (inhaled ethyl ether) in pups within the first day of life.

Electrocardiographic recordings

On the day of delivery, standard EKGs were performed. The pups were placed in the supine position, and leads I, II, III, aVR, aVL, and aVF were recorded with limb leads attached using miniature electrodes and a portable
Primary immune responses of mice immunized with 48-kDa SSB/La, 52α, 52β, and 60-kDa SSA/Ro and confirmation of their placental transfer. Representative sera from mice in each immunization group (described above) were used to immunoprecipitate radiolabeled in vitro translation products (described below each lane). For example, sera obtained from either 52α- or 52β-immunized mice recognized both 52α and 52β SSA/Ro (lanes 10, 11, 14, and 15) but not 48-kDa SSB/La (lanes 9 and 13) or 60-kDa SSA/Ro (lanes 12 and 16). The serum obtained from a pup born to a mother immunized with 52β contained Abs reactive with both 52β and 52α (lanes 22 and 23) but not with 48-kDa SSB/La (lane 21) or 60-kDa SSA/Ro (lane 24).

EKG machine. Paper speed settings were adjusted to 25 and 50 mm/s; voltage amplification was 20 mV. Each lead was recorded for at least 15 s to precisely identify P waves and QRS complexes, and map PR intervals. No anesthesia was necessary. PR prolongation was defined as >92 ms, which corresponded to the mean ± 2 SD obtained in the pups born to β-gal-immunized mothers.

Statistical analysis

The Student t test for unpaired data was used to compare heart rates between the different groups. Fisher’s exact test was used to compare the frequency of first-degree block between control and experimental groups. p values of <0.05 were considered significant.

Results

Establishment of murine autoantibody responses

To establish a murine model of autoimmune-associated CHB, female BALB/c mice were immunized with the following human recombinant proteins: full-length 52α SSA/Ro (n = 8 mice), 52β SSA/Ro (n = 5), 60-kDa SSA/Ro (n = 7), 48-kDa SSB/La (n = 10), or murine 52-kDa SSA/Ro (n = 5). Mice were bled every 7–10 days, and sera from each mouse were separately analyzed for reactivity by ELISA, immunoblotting, and immunoprecipitation. For each recombinant immunogen, high-titer IgG responses were generated, which may indicate limited B cell tolerance to these proteins in normal mice. No anti-52-kDa SSA/Ro, -60-kDa SSA/Ro, or anti-48-kDa SSB/La responses were detected in mice immunized with pET-28 alone (n = 7) or with β-gal recombinant protein constructed in pET-28 (n = 3).

As described previously (27), primary immune responses to each of the human recombinant proteins were readily demonstrated by ELISA and immunoblotting (data not shown). To unambiguously demonstrate the specificity of each Ab and evaluate reactivity to conformationally dependent epitopes that might not be detected by ELISA or immunoblotting, murine antisera were tested by immunoprecipitation of [35S]methionine-labeled in vitro translation products. This approach provided an independent assay of reactivity, because the translation products were derived from different vectors (pBluescript) than those used for the preparation of the immunizing recombinant proteins, thus eliminating reactivity with pET-28 vector-derived polypeptides or E. coli proteins. Representative immunoprecipitations are shown in Fig. 1. Mice immunized with recombinant 48-kDa SSB/La recognized in vitro-translated 48-kDa SSB/La (lane 5). Mice immunized with recombinant full-length 52α SSA/Ro or the 52β isoform recognized both 52α and 52β (lanes 10, 11, 14, and 15). Mice immunized with 60-kDa SSA/Ro recognized 60-kDa SSA/Ro (lane 20). Immunoprecipitations were also performed using antisera obtained from the F1 generations as a proxy to determine whether the maternal Abs reactive with each of the recombinant proteins were effectively transferred across the murine placenta. All sera (collected in the first day of life) from several pups born to mothers in each immunized group confirmed the presence of the appropriate Ab. As exemplified in Fig. 1 (lanes 22 and 23), the serum obtained from a pup born to a mother immunized with 52β contained Abs that were reactive with 52α and 52β.

There is a 91% sequence similarity between murine and human 48-kDa SSB/La (28) and a 95% similarity between murine and human 60-kDa SSA/Ro (8). However, the similarity between murine and human 52α SSA/Ro is only 81% (29). Because the primary Ab responses generated to human 52α SSA/Ro may represent alloseactivity rather than true autoreactivity, murine sera were also tested against murine 52-kDa SSA/Ro. Mice immunized with either human 52α or 52β immunoprecipitated in vitro-translated mouse 52-kDa SSA/Ro (Fig. 2, lanes 2 and 4). Mice immunized with murine 52-kDa SSA/Ro recognized both human and murine 52-kDa SSA/Ro (data not shown).

Demonstration of 52-kDa SSA/Ro in murine heart

To confirm that the murine Ab responses were directed against the Ags present in the target tissue, mRNA and protein expression of 52-kDa SSA/Ro was evaluated in murine hearts isolated from 1-day-old pups. Using primers based on murine sequences, mRNA
transcripts for murine 52-kDa SSA/Ro were readily amplified from mouse heart cDNA (Fig. 3A, lane 1). Mice immunized with human 52-kDa SSA/Ro recognized the respective Ags in MOLT-4 and mouse heart lysates (Fig. 3B, lanes 3 and 4).

Identification of conduction abnormalities in the F₁ generation

Offspring were obtained by breeding female mice with syngeneic males. The data on the number of fertile females and pups born in each group are summarized in Table I. The mean heart rate (in beats per minute (bpm)) was not significantly different between the experimental groups (48La, 312 bpm ± 91 SD; 52αRo, 363 bpm ± 131 SD; 52βRo, 329 bpm ± 101 SD; 60Ro, 285 bpm ± 120 SD; and murine 52Ro, 286 bpm ± 88 SD) and the β-gal group (321 bpm ± 124 SD). Pups born to mothers immunized with vector control had a higher mean heart rate when compared with the other groups (390 bpm ± 96 SD; p < 0.01). PR intervals could be reliably measured in all offspring of mothers immunized with 48-kDa SSB/La, murine 52-kDa SSA/Ro, and vector control as well as in 11 of 21, 47 of 56, 74 of 86, and 50 of 54 pups born to mice immunized with β-gal, human 52α, 52β, or 60-kDa SSA/Ro, respectively. The mean PR interval was similar in the experimental groups (48La, 59 ± 19 ms; 52αRo, 55 ± 24 ms; 52βRo, 59 ± 22 ms; 60Ro, 69 ± 27 ms; and murine 52Ro, 60 ± 21 ms) compared with β-gal group (58 ± 17 ms), whereas pups in the vector control group had a lower mean PR interval (45 ± 12 ms; p < 0.01).

First-degree block was defined as a PR interval of >92 ms (2 SD above the mean PR in offspring of β-gal-immunized mothers). As seen in Fig. 4, no pups in either control group had a PR interval that was >90 ms. In contrast, first-degree block was found in 2 pups born to 48-kDa SSB/La-immunized mothers, 3 born to 52α-immunized mothers, 5 born to 52β-immunized mothers, 10 born to 60-kDa SSA/Ro-immunized mothers, and 2 born to murine 52-kDa SSA/Ro-immunized mothers. In total, 22 (10%) of 220 pups in the experimental groups (in which PR intervals could be measured) demonstrated first-degree block compared with none of 43 pups whose mothers were immunized with vector alone (two-tailed p value = 0.03, Fisher’s exact test).

In addition to first-degree block, more extensive AV nodal conduction abnormalities were detected in several pups from the experimental groups: 2 of the 56 pups born to 52α-immunized mothers had advanced heart block (one complete and another with second-degree block (Wenckebach type)). Each of these pups was born to a different 52α-immunized mother. Complete block was also detected in 5 of 86 pups born to 52β-immunized mice. Three of these pups were from the same litter. Representative EKGs are shown in Figs. 5 and 6. No second- or third-degree block was found in the offspring of mothers immunized with 48-kDa SSB/La, 60-kDa SSA/Ro, or murine 52-kDa SSA/Ro. There was no difference in the titer (as measured by ELISA) of the immune responses to the respective Ags in mothers of affected pups compared with mothers of unaffected pups.

Discussion

To develop a murine model of autoimmune-associated CHB, three requisite conditions must be met: 1) establishment of Ab responses to the targeted human Ags, 2) confirmation of autoreactivity with murine homologues in cardiac tissue, and 3) demonstration of placental transfer of these murine Abs into the circulation of the progeny. All immunized mice developed high-titer responses to each of the human recombinant proteins as well as to murine 52-kDa SSA/ Ro. mRNA and protein expression of 52-kDa SSA/Ro was demonstrated in newborn murine hearts; the latter was recognized by

Table I. Murine Model of CHB

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Immunized Mothers (n)</th>
<th>Fertile Mothers (n)</th>
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* One 52β mother had three pups with CHB in the same litter; two other mothers each had one CHB pup.
antisera from the immunized mice. Sera obtained from the offspring of the experimental mice contained Ab responses that appropriately reflected the maternal immunogen.

A spectrum of AV nodal conduction abnormalities was identified on the EKG tracings obtained from the F1 generation of mice immunized with components of the SSA/Ro-SSB/La complex but was not identified in control groups. First-degree AV block was observed in 7% of pups born to mothers immunized with 52-kDa SSB/La, in 6–7% of pups born to 52α- and 52β-immunized mothers; in 20% of pups born to 60-kDa SSA/Ro-immunized mothers, and in 9% of pups born to murine 52-kDa SSA/Ro-immunized mothers. Second-degree block was observed in 2% of offspring from 52α-immunized mothers. Third-degree block was observed in 2–6% of the pups from 52α- and 52β-immunized mothers.

A comparison of the frequency of second- and third-degree AV block observed in this inbred murine model with the disease in humans is difficult. Genetics notwithstanding, the frequency depends upon the denominator (i.e., the number of mothers at risk and/or the number of children born to these mothers). In one retrospective study of CHB, the rate was 7 of 259 live births in mothers with anti-SSA/Ro Abs, the rate was 6 of 79 (30). In another prospective study, none of 26 gravid lupus patients with anti-SSA/Ro and/or -SSB/La Abs gave birth to a child with CHB (31). Most recently, Simmons-O’Brien et al. (32) detected one case of CHB in 113 pregnancies of 43 anti-SSA/Ro Ab-positive women between 20 and 40 yr of age. Further confounding the estimation of risk is the fact that many mothers are asymptomatic; therefore, the identification of anti-SSA/Ro-SSB/La Abs is first made at the time heart block is detected in utero. Lee estimates the risk of CHB in a mother with the candidate Abs to be 1% (2) as calculated from the given rate of CHB in 1 in 15,000–20,000 live births (33) and the 0.5% frequency of anti-SSA/Ro Abs in asymptomatic pregnant women (34). Although data on the frequency of anti-52-kDa (vs. 60-kDa) SSA/Ro/Abs in healthy pregnant women are unknown, the advanced conduction defects in the murine offspring approximated those reported for most series in humans. Expression of disease did not correlate with titer of immune response, which is also consistent with human disease (35). The heterogeneity of the fetal response remains unexplained, but it is hoped that the availability of this murine model will facilitate such studies.

In humans, the characteristic cardiac conduction abnormality associated with maternal anti-SSA/Ro-SSB/La is complete AV block, rather than the first-degree block noted in the mice. This may be accounted for by differences in the pathogenetic properties of human compared with murine Abs or by differences in the response and repair of the fetal heart between the two species. Alternatively, it may simply be that first-degree block is underestimated in humans because it is not clinically detected as a bradycardia. In support of the latter possibility, routine EKGs are not performed on otherwise healthy infants of asymptomatic mothers and often not on infants whose mothers are known to have a rheumatic disorder, even if the candidate autoantibodies are established. However, there have been rare reports of autoimmune-associated first-degree AV block (4, 36). One illustrative case identified in the Research Registry for Neonatal Lupus supports the applicability of the mouse model to the disease in humans. A mother with an undifferentiated rheumatic disorder, even if the candidate autoantibodies are established. However, there have been rare reports of autoimmune-associated first-degree AV block (4, 36). One illustrative case identified in the Research Registry for Neonatal Lupus supports the applicability of the mouse model to the disease in humans. A mother with an undifferentiated autoimmune syndrome and documented anti-SSA/Ro Abs gave birth to a child whose EKG demonstrated first-degree block, as noted by a prolonged PR interval for age, at 11 h of life. By 18 mo, the block had progressed to second-degree, and the child required a pacemaker (36).

Two previous publications have addressed the development of a murine model of CHB; one used a completely different strategy. Kalush et al. reported that offspring of BALB/c mice immunized with the monoclonal anti-DNA Id 16/6 had conduction abnormalities (37). Of 31 pups born to mothers with experimental SLE, 8 had first-degree heart block, 2 had second-degree heart block, 2 had complete block, 10 had bradycardia, and 8 demonstrated widening of the QRS complex. None of these disorders could be detected in the 20 offspring of healthy control mice. One of the difficulties in interpreting these findings is that the immunized mothers synthesized a variety of autoantibodies, including those reactive with 16/6 Id, ss/dsDNA, Sm, RNP, cardiolipin, SSA/Ro, and SSB/La. In an earlier pilot study by our group in which female BALB/c mice were immunized with human 52-kDa SSA/Ro, 2 of 20 pups had CHB (24). The results reported herein confirm these findings and provide extensive characterization of the Ab reactivities as well as novel data regarding immunization with human 52β SSA/Ro, 48-kDa SSB/La, and 60-kDa SSA/Ro and with murine 52α SSA/Ro.

**FIGURE 4.** Increased frequency of first-degree block in pups born to mothers immunized with the SSA/Ro-SSB/La proteins. The PR interval was measured in milliseconds in all pups in which an unambiguous P wave could be seen. The number of pups whose PR interval falls within a specified range is given above each bar. Open bars represent PR intervals that fall within 2 SD of the β-gal control group; hatched bars represent prolonged PR intervals (>92 ms). The recombinant protein used for immunization is shown above each graph.
While pups from each of the experimentally immunized groups developed some degree of AV block, only those born to mothers immunized with human 52-kDa SSA/Ro, particularly the β isoform, demonstrated complete AV dissociation. Although the number of pups is too small to draw definitive conclusions, several explanations may contribute to this apparent selectivity. First, it has been demonstrated previously that affinity-purified anti-52-kDa SSA/Ro Abs induce AV block in a working heart model and inhibit whole-cell and single-channel slow, inward calcium currents (24). Second, 52-kDa SSA/Ro is the most prevalent Ag recognized on immunoblots by sera from mothers whose children have CHB, although in vitro-translated 60-kDa SSA/Ro is immunoprecipitated by all of these sera (25, 38, 39). Third, the mRNA expression of 52β relative to 52α in the human heart is greatest between 14 and 16 wk of fetal life (20), albeit not yet established in the mouse. A particular difficulty in attributing pathogenicity to anti-52-kDa SSA/Ro Abs is that the majority of mice demonstrate spreading of the immune response over time (most readily observed from 48-kDa SSB/La to 52-kDa SSA/Ro) (27). The absence of serious conduction abnormalities in the 48-kDa SSB/La and murine 52-kDa SSA/Ro groups of mice is unexplained but may relate to the limited number of pups. However, this absence may also reflect differences in Ab titer, affinity, or epitope specificity generated in response to a primary exogenous immunogen compared with a “secondary” self Ag. Further work with increased numbers of immunized mothers and early matings before detectable spreading should more precisely define Ab specificity and pathogenicity.

In conclusion, this murine model provides strong evidence for a pathogenic role of Abs reactive with SSA/Ro and SSB/La in the development of CHB. The spectrum of conduction abnormalities was varied and included a greater incidence of first-degree AV block than that reported for humans. The rate of second- and third-degree block approximates the 1–5% risk for a mother with anti-Ro/La Abs to have a child with CHB and suggests that additional factors are required to promote disease expression. Histologic evaluation of the affected hearts and more precise identification of the fine specificity of the arrhythmogenic Abs should provide invaluable clues to the pathophysiology of autoimmune-associated CHB.

**FIGURE 5.** Representative EKGs performed on pups born to 52β- or control-immunized mice. The tracing in the upper panel demonstrates complete heart block in a pup born to a 52β-immunized mother. The top arrows point to P waves, and the bottom arrows point to QRS complexes. The tracing in the bottom panel demonstrates normal sinus rhythm in a pup born to a control mother.

**FIGURE 6.** EKGs obtained from a litter born to a 52α-immunized mother. One pup had first-degree block, another had second-degree block, and two were in normal sinus rhythm.