

Maternal Antibody Responses to the 52-kd SSA/Ro p200 Peptide and the Development of Fetal Conduction Defects

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Objective. To identify a finer level of antibody specificity for risk of congenital heart block (CHB) than reactivity to 52-kd SSA/Ro (Ro 52).

Methods. Serum from mothers enrolled in the Research Registry for Neonatal Lupus and the observational PR Interval and Dexamethasone Evaluation (PRIDE) study was evaluated for reactivity against peptide aa200-239 of Ro 52 (p200), recently reported to be associated with a higher risk of CHB.

Results. The majority of 156 Ro 52–positive sera tested were reactive with p200 (>3 SD above control), irrespective of the clinical status of the child. Optical density (OD) values of p200 did not differ significantly among mothers of children with CHB (mean \pm SD 0.187 \pm 0.363), mothers of children with rash (mean \pm SD 0.176 \pm 0.356), and mothers of children without neonatal lupus (mean \pm SD 0.229 \pm 0.315). Reactivity against p200 was found in 80 of 104 mothers of children with CHB (77%), 24 of 30 mothers of children with rash (80%), and 21 of 22 mothers who delivered healthy children and had no children with neonatal lupus (95%) (*P* not significant for all comparisons). Sera from 4 mothers of children with CHB with varied p200 titers

(OD range 0.025–1.818) bound to the surface of nonpermeabilized apoptotic, but not proliferating, human fetal cardiocytes. In 32 Ro 52–positive women who completed the PRIDE study (22 with no child with neonatal lupus, 7 with a child with CHB, and 3 with a child with rash) in whom p200 levels were determined during pregnancy, the correlation between level of p200 (OD range 0.000–1.170) and maximal fetal PR interval (range 115–168 msec) was not significant ($\rho = 0.107$, *P* = 0.58).

Conclusion. Reactivity to p200 is a dominant but not uniform anti-Ro 52 response in women whose children have CHB. Since exposure to this antibody specificity was observed with a similar frequency in children without CHB born to mothers with anti-Ro 52, additional factors are necessary to convert risk to disease expression.

Accumulated data suggest that women with anti-SSA/Ro antibodies identified by commercial enzyme-linked immunosorbent assay (ELISA) face a 2% risk of having a child with neonatal lupus–congenital heart block (CHB), most often third-degree (1). While recent reports suggest that this risk may increase to 5% in the presence of anti-SSB/La antibodies (2), identification of more specific antibody reactivity would be a major advance in the translational approach to CHB. There is a clear need to identify an early marker of CHB, given that established third-degree block has not been reversed to date, despite treatment with maternal steroids known to be effective in the fetal circulation (3). Presently, weekly echocardiographic evaluation of all fetuses exposed to anti-SSA/Ro antibodies has been recommended to detect potentially reversible incomplete blocks (4). The discovery of an antibody specificity that induces injury to the developing human conduction system could lead to treatments directed toward removal

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Table 1. Clinical status of mothers of children with CHB, mothers of children with neonatal lupus rash but no CHB, and mothers of healthy children with no manifestations of neonatal lupus*

Children's diagnosis	Mothers' diagnosis, %				
	Asymptomatic	UAS	SS	SLE	SLE/SS
CHB	23	20	25	21	10
Neonatal lupus rash (no CHB)	10	10	40	30	10
Healthy (no neonatal lupus)	18	14	27	36	4

* CHB = congenital heart block; UAS = undifferentiated autoimmune syndrome; SS = Sjögren's syndrome; SLE = systemic lupus erythematosus.

of the putative autoantibody from the maternal circulation before placental transfer becomes effective.

Antibodies to the 52-kd SSA/Ro protein (Ro 52) are found in >80% of mothers whose children have CHB (2,5–7). Initial epitope mapping of this response revealed an immunodominant region spanning aa169–291 (which contains the leucine zipper) that was recognized by the majority of the CHB sera, frequently in the context of HLA-DRB1*0301, DQA1*0501, and DQB1*0201 (5). The finer specificity of the anti-Ro 52 response has been confirmed and extended, with the current focus on aa200–239 (p200) (7). In a limited study of 9 mothers of children with CHB and 26 anti-SSA/Ro-positive mothers of healthy children, antibodies to p200 predicted CHB with greater certainty than currently available testing for either 60-kd or 52-kd SSA/Ro (7). Recent studies integrating an in vivo rodent model and an in vitro culturing system suggest that anti-p200 antibodies bind neonatal rodent cardiocytes and alter calcium homeostasis (8).

To address both the clinical necessity and the sufficiency of this newly identified p200 reactivity in the development of CHB, as well as the reduced risk of CHB reportedly associated with aa176–196 (p176) and aa197–232 (p197) (7), maternal sera from the Research Registry for Neonatal Lupus (RRNL) (9) and the prospective, multicenter study, PR Interval and Dexamethasone Evaluation (PRIDE) in CHB (4) were evaluated. In addition, the PRIDE study provided the opportunity to address whether the level of anti-p200 antibodies correlated positively with the length of the Doppler mechanical PR interval.

PATIENTS AND METHODS

Patients. The study included mothers enrolled in the RRNL (9) who had at least 1 child with CHB (and in many cases healthy children as well), and RRNL mothers who had at least 1 child with isolated neonatal lupus rash and no children with CHB. In addition, mothers were recruited from the

PRIDE in CHB study, which had 2 aims: to evaluate pregnant women with anti-SSA/Ro antibodies (regardless of pregnancy history) by fetal echocardiography weekly from 16 to 26 weeks and biweekly from 26 to 32 weeks in an attempt to find a reversible marker of fetal cardiac injury, and to evaluate the efficacy of maternal oral dexamethasone (open-label, nonrandomized) in the treatment of an established conduction abnormality (first-, second-, or third-degree block). The distribution of maternal disease was similar among the groups (Table 1). All mothers were known to have anti-SSA/Ro antibodies. Since the RRNL does not require that antibody testing be done during an affected pregnancy, serum from the RRNL mothers was not necessarily available from the time of an affected pregnancy. In contrast, sera obtained from women in the PRIDE study were evaluated during the time of pregnancy. Serum was also obtained from 13 healthy controls. Studies were approved by the Institutional Review Board prior to their initiation, and informed consent was obtained from subjects prior to their enrollment.

Recombinant proteins and synthetic peptides. Recombinant human SSA/Ro and SSB/La autoantigens were produced using the expression plasmid pET28 system in *Escherichia coli* BL21 (DE3; Novagen, Madison, WI), as previously described (5,10). Synthetic peptides p200, p197, and p176 were synthesized at the 25- or 100- μ mole scale using solid-phase 9-fluorenylmethoxycarbonyl chemistry with a peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA) operated by the Protein Chemistry Core Facility at the University of Florida Interdisciplinary Center for Biotechnology Research. Each peptide was analyzed by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. Crude peptides were >70% pure and were subjected to preparative HPLC to achieve >95% purity.

ELISA for detection of antibodies to Ro 52 and Ro 52 peptides. Standard protocol for ELISA using recombinant proteins was used, as previously described (10,11). Briefly, nickel column affinity-purified recombinant proteins were diluted in phosphate buffered saline (PBS) to a final concentration of 1 μ g/ml and then coated on Immulon 2 microtiter plates (Dynatech, Alexandria, VA). Human sera were diluted at a ratio of 1:1,000 and then incubated in the antigen-coated wells. Horseradish peroxidase–conjugated goat anti-human IgG (Caltag, San Francisco, CA) was used at a 1:5,000 dilution and the substrate ABTS was added as the detection reagent. Each sample was analyzed in duplicate, and the average optical

density (OD) at 405 nm with a substrate development time of 15–45 minutes was used for data analysis. The cutoff value designating a positive reaction was 3 SD above the mean OD in 13 normal sera.

The peptide ELISA protocol described by Salomonsen and colleagues (7) was followed closely, but with the following modifications. High-binding 96-well plates (Nunc, Rochester, NY) were coated with 100 μ l of 10 μ g/ml peptide per well in carbonate buffer (pH 9.6). Plates were coated overnight at room temperature and for at least 24 hours at 4°C before use. After this, all steps were performed at room temperature. Plates were washed 3 times with PBS–Tween (PBST) and blocked with 200 μ l PBST/5% milk powder per well for 30 minutes. After washing 3 times with PBST, 100 μ l of patient sera, diluted 1:1,000 in PBST/1% milk powder, was added and incubated for 2 hours. Plates were washed 3 times with PBST and peroxidase-conjugated goat anti-human IgG and IgM reagents, and developed as described above for recombinant protein ELISA.

Controls comprised 13 healthy donors with no known reactivity to any component of the SSA/Ro–SSB/La complex. The cutoff for positivity with each peptide was OD 0.002 (mean + 3 SD).

Echocardiographic recordings in human pregnancies.

The methodology of this technique, which allows noninvasive measurement of the atrioventricular (AV) cardiac time delay in the human fetus, has been previously described (12) and validated (13). Briefly, 2-dimensional fetal echocardiograms were recorded with commercially available ultrasound systems. All images were recorded on videotape and measurements were obtained using commercially available software packages. A gated–pulsed Doppler sample volume was placed in the left ventricle at the junction of the anterior leaflet of the mitral valve and the left ventricular outflow tract in an apical 5-chamber view, and simultaneous display of left ventricular filling and emptying was obtained. Time intervals were measured from the onset of the mitral A wave (atrial systole) to the onset of the aortic pulsed Doppler tracing (ventricular systole). This represented the mechanical PR interval. Three successive mechanical PR intervals were obtained and averaged. The mechanical PR interval was independent of gestational age (between 17 and 40 weeks) and heart rate in normal controls. The normal mechanical PR interval in the fetus is 120 ± 20 msec (mean \pm 2 SD; 95% confidence interval 100–140 msec). An interval of >140 msec is considered prolonged, and therefore consistent with first-degree AV block.

The measurement of the fetal mechanical Doppler PR interval was initially established in 56 normal pregnancies (12), and interobserver variability among 15 investigators was assessed in a subsequent validation study (13). Using the approach of Bland and Altman (14), as well as the paired *t*-test, the mean difference in measurements was found to be not significant. Intraobserver variability was estimated by comparing the normal fetal mechanical Doppler PR interval established in the initial study (12) with the mean \pm 2 SD results of the validation study (13): 120 ± 10 msec and 122 ± 11 msec, respectively.

Cardiocyte isolation and staining. Human fetal cardiocytes of gestational age 16–24 weeks were obtained aseptically

after elective termination of normal pregnancy in accordance with the guidelines of the Institutional Review Board and after obtaining consent from the mothers, and cultured as previously described (15). By day 4 in culture, spontaneous contraction (>30 beats/minute) was observed by phase-contrast microscopy. More than 75% of the cells were stained by a murine monoclonal anti-actinin (sarcomeric) antibody, which stains Z lines and dots in stress fibers of skeletal and cardiac muscle, but not in nonsarcomeric muscle elements, such as connective tissue, epithelium, nerves, or smooth muscle (16). Human fetal cardiocytes were treated with tumor necrosis factor α (5 ng/ml; Invitrogen, Carlsbad, CA) on tissue culture dishes coated with poly-(2-hydroxyethyl methacrylate) (P3932; Sigma, St. Louis, MO) (17) for 24 hours at 37°C. Apoptosis was assessed by anti-active caspase 3. Briefly, assessments of apoptosis in nonpermeabilized cells included immunofluorescence (Hoechst 33258 counterstain). Prior to analysis using FACScan (BD Biosciences, San Jose, CA), cells were treated with digitonin permeabilization (51-2090KZ; BD Biosciences) to permit evaluation of active caspase 3 (Apo active antibody caspase-3; Cell Technology, Mountain View, CA). Fluorescence-activated cell sorting assessment of maternal IgG, bound to intact or apoptotic cells, used maternal sera (1:100 dilution at 22°C for 30 minutes) and phycoerythrin-conjugated goat anti-human IgG (Sigma) (1:100 dilution at 22°C for 30 minutes).

Statistical analysis. Calculations were performed using GraphPad InStat software (GraphPad Software, San Diego, CA). The Kruskal-Wallis nonparametric test was used to compare mean values of reactivity to p200 among the 3 groups of mothers (those whose children had CHB, those whose children had rash, and those whose children had no manifestations of neonatal lupus, respectively). Fisher's exact test was used to compare the number of positive results in each group. Spearman's rank correlation was used to plot levels of p200 versus the Doppler mechanical PR interval. *P* values less than 0.05 were considered significant.

RESULTS

Reactivity to p200, p197, and p176 peptides among mothers whose children had CHB, neonatal lupus rash, or no manifestations of neonatal lupus. Of 148 sera available from mothers enrolled in the RRNL, 108 were from mothers who had at least 1 child with CHB (and, in many cases, healthy children as well), and 40 were from mothers who had at least 1 child with isolated neonatal lupus rash and no children with CHB. In addition, sera were available from 29 anti-SSA/Ro–positive women enrolled in the PRIDE study who have had only healthy children. Reactivity with full-length Ro 52 was found in 104 of 108 mothers of children with CHB (96%), 30 of 40 mothers of children with rash (75%), and 22 of 29 anti-SSA/Ro–positive women who had only healthy children (76%). Reactivity with p200, p197, and p176 was initially evaluated in all sera. While

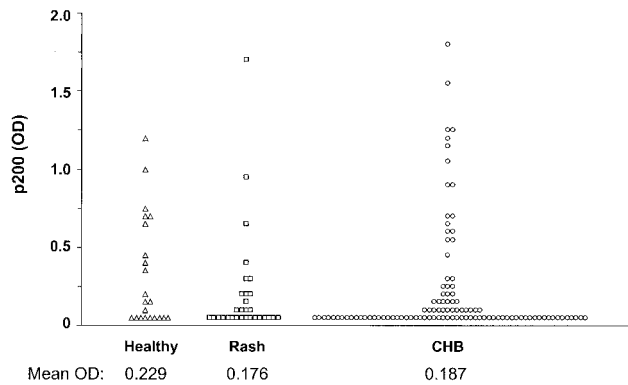


Figure 1. Serum levels of anti-p200 peptide. Distribution of anti-p200 (expressed as optical density [OD] units) in 22 anti-Ro 52-positive mothers who delivered healthy children and had previously not had any children with neonatal lupus, 30 mothers of children with an isolated neonatal lupus rash, and 104 mothers of children with congenital heart block (CHB).

there were sera that reacted with Ro 52 but not p200 or the other 2 peptides, there were no sera reactive with any of the 3 peptides and not Ro 52 (data not shown). Therefore, only sera reactive with Ro 52 were included in subsequent analyses to evaluate the contribution of p200 reactivity to the risk of CHB. Of the 104 mothers of children with CHB and 30 mothers of children with rash, 34 of the CHB sera (33%) and 5 of the rash sera (17%) were evaluated from the affected pregnancy.

Figure 1 summarizes the overall results for each of the 3 groups of anti-Ro 52-positive maternal sera tested. There was no statistically significant difference in the mean OD values of p200 among the mothers of children with CHB (mean \pm SD 0.187 \pm 0.363), mothers of children with rash (0.176 \pm 0.356), or mothers who had only healthy children (0.229 \pm 0.315). Moreover, the frequency of reactivity to p200 was not statistically different among the groups. Specifically, anti-p200 antibodies were found in 80 of 104 mothers of children with CHB (77%), 24 of 30 mothers of children with rash (80%), and 21 of 22 mothers who had only healthy children (95%). Serial samples obtained from 1 mother demonstrated that, despite identical fetal exposure to anti-p200 antibodies during 2 consecutive pregnancies, 1 child had fatal CHB and the other was healthy (Figure 2). The mean OD value for reactivity to p197 and p176 was low in all 3 groups of sera tested: for p197, the mean was 0.030, 0.007, and 0.001 in the CHB, rash, and healthy children groups, respectively; for p176, the mean was 0.004, 0.002, and 0.005, respectively.

In vitro surface binding of cultured apoptotic human fetal cardiocytes by anti-p200 antibodies. Given the previously reported surface binding and intracellular calcium accumulation in the presence of anti-p200 antibodies (8), sera from 4 mothers of children with CHB with anti-p200 antibodies and 1 healthy anti-Ro/La-negative mother (control) were evaluated for surface binding in proliferating and apoptotic human fetal cardiocytes. As shown in Figure 3, sera from the 4 mothers of children with CHB with varied p200 titers (OD range 0.025–1.818) bound to the surface of nonpermeabilized apoptotic, but not proliferating, human fetal cardiocytes.

Maternal anti-p200 reactivity and fetal PR intervals during pregnancy. Although the mean level and frequency of p200 reactivity were not significantly increased in sera from women who had children with CHB compared with those who had never had a child with CHB, the possibility remained that reactivity with p200 might be useful in identifying women at high risk for having a fetus with a prolonged PR interval. This would be clinically important since first-degree block is a potential early marker of more advanced injury to the fetal cardiac conduction system. Accordingly, sera were evaluated from 32 pregnant anti-Ro 52-positive women who were followed prospectively with fetal echocardiographic examinations weekly from 16 to 26 weeks of gestation and biweekly from 26 to 32 weeks. Twenty-two of these women had never had a child with neonatal lupus, 7 had at least 1 child with CHB, and 3 had at least

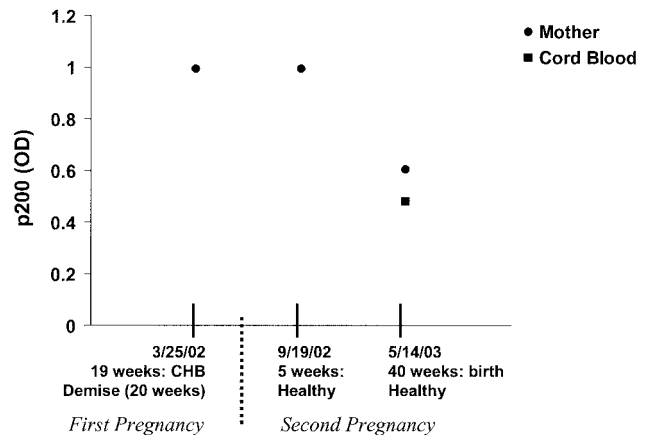


Figure 2. Serial evaluation of maternal anti-p200 antibody levels (expressed as optical density [OD] units) in maternal sera and cord blood of 1 mother during 2 consecutive pregnancies, 1 in which the child had congenital heart block (CHB) and died at 20 weeks gestation, and the other resulting in the birth of a healthy child.

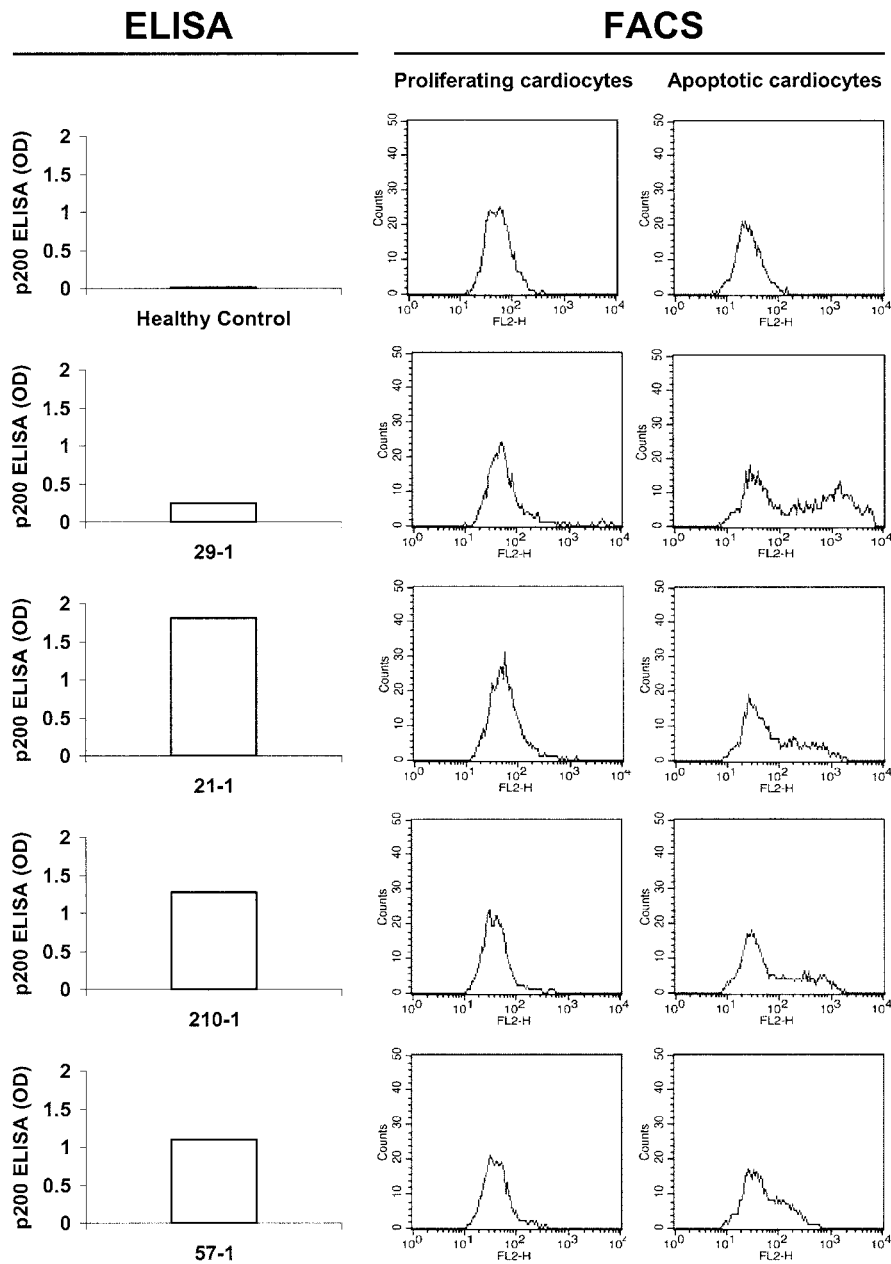


Figure 3. Single-staining of nonpermeabilized apoptotic and proliferating cardiocytes using maternal sera to evaluate accessibility of p200 epitope. Human fetal cardiocytes (proliferating cardiocytes) were obtained as monolayer cultures. Apoptotic cardiocytes were prepared by plating fetal human cardiocytes on poly-(2-hydroxyethyl methacrylate) + tumor necrosis factor α (10 ng/ml for 18 hours at 37°C). A suspension of each preparation was stained with maternal serum, which was obtained from 4 mothers of children with CHB (patients 29-1, 21-1, 210-1, and 57-1) whose p200 immunoreactivity varied over a broad range, as measured by enzyme-linked immunosorbent assay (ELISA) (OD 0.025–1.8), and with a control serum from an anti-Ro/La–negative mother of healthy children. Each row includes the ELISA result and fluorescence-activated cell sorting (FACS) profile for staining of proliferating and apoptotic cardiocytes. Intact and apoptotic cardiocytes were single-stained using phycoerythrin-conjugated goat anti-human IgG to assess binding of maternal antibody. FL2-H = fluorescence channel 2 (see Figure 1 for other definitions).

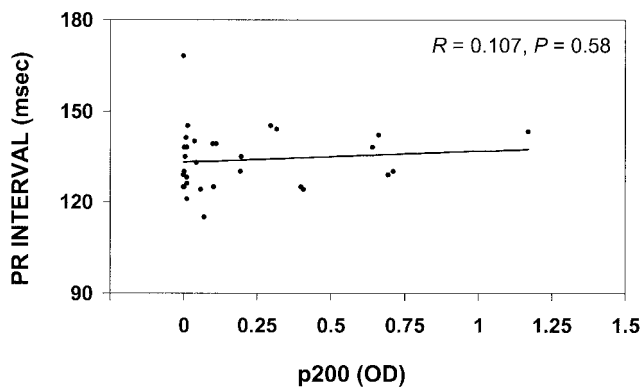


Figure 4. Fetal PR intervals in relation to levels of maternal anti-p200 antibodies. For each of 32 anti-Ro 52-positive women whose pregnancies were followed prospectively, anti-p200 levels were determined during pregnancy, and serial echocardiograms were obtained as described in Patients and Methods. Each point represents an assessment in which the fetus's maximal mechanical PR interval is plotted in relation to the mother's anti-p200 titer. OD = optical density.

1 child with isolated neonatal lupus rash and no children with CHB. There was no significant correlation observed when p200 levels were plotted against the highest AV time intervals measured in each fetus (Figure 4).

Seven mothers had fetuses with a PR interval of sufficient prolongation to represent first-degree block (>140 msec), 1 of which was >150 msec. The mother of this fetus had no detectable antibodies to p200. This mother began treatment with 4 mg/day of dexamethasone; the fetal PR interval measured 1 week later was 135 msec. Findings of an electrocardiogram (EKG) obtained from the child at birth were entirely normal and have remained so at followup to 2 years. Another fetus, whose highest recorded PR interval was 140 msec at 19 weeks, developed third-degree block at 20 weeks; the maternal p200 reactivity was low-positive at OD 0.038. Based on continual deterioration and hydropic changes despite treatment of the mother with 4 mg/day of dexamethasone, the pregnancy was terminated at 24 weeks.

DISCUSSION

The identification of a fetus at risk for developing CHB would be invaluable, given the substantial morbidity ($\sim 65\%$ require lifelong pacing) and mortality ($\sim 20\%$) associated with this disease (5,9,18–20). Moreover, current echocardiographic technologies are being evaluated for early in utero detection at a time when injury might still be reversible (12,13,21–23). Among putative factors likely to reveal the fetus at highest risk is

a maternal autoantibody specificity more precise than reactivity with full-length components of the SSA/Ro–SSB/La system that would segregate with affected but not unaffected pregnancies.

In this study, neither the mean level of p200 reactivity nor frequency was significantly different among anti-Ro 52-positive mothers who had children with CHB, mothers who had children with isolated neonatal lupus rash but not CHB, or mothers who had only healthy children with no neonatal lupus. Approximately 25% of the sera from the mothers of children with CHB did not display reactivity to p200. This contrasts with findings of a more limited study in which 9 of 9 anti-Ro 52-positive mothers of children with CHB demonstrated reactivity with p200, compared with lower titer and frequency in 26 mothers (not all anti-Ro 52-positive) of healthy children (7). Since our data show that antibody reactivity against p200 is not observed in the absence of reactivity to full-length Ro 52, evaluation of reactivity to p200 should be restricted to individuals positive for anti-Ro 52 to ascertain whether this finer specificity truly exaggerates the risk of CHB in a fetus. It is readily acknowledged that many of the sera tested were not obtained at the time of a given pregnancy. However, if titers of anti-p200 antibodies parallel levels of reactivity with full-length Ro 52, then steady-state levels of anti-p200 would be expected, since anti-Ro 52 antibody levels only rarely fluctuate over time (24). This would further validate the findings reported herein. In fact, many of the sera assigned to the group of mothers of children with CHB were actually obtained during the time of a healthy pregnancy, which suggests that pathogenicity of anti-p200 antibodies requires additional factors.

One difficulty in identifying a pathogenic effect of an autoantibody is accounting for the heterogeneity of that effect. CHB is a paradigmatic example in that not only is the injury seemingly rare, but the degree of injury varies along a spectrum from clinically inconsequential first-degree block through third-degree (complete) block and even, in some cases, an associated cardiomyopathy that is often fatal (25,26). Identification of a necessary or essential factor is only part of the challenge in defining the pathology of CHB, since recurrence rates from one pregnancy to the next are 18%, not 100%, and identical twins are with rare exception discordant for disease (27).

Even if anti-p200 or an even finer epitope specificity were truly pathogenic and cross-reactive with a structure on the cardiocyte surface, subsequent events are required to convert risk of disease to full expression. Many healthy fetuses were exposed to maternal anti-

p200 antibodies, as demonstrated by results observed in sera obtained during pregnancies followed prospectively in the PRIDE study. Salomonsson and colleagues (8) reported that 19% of rat pups born to mothers actively immunized with p200 had first-degree block. These same investigators concluded that an epitope within the predicted leucine zipper structure is recognized by similar antibodies from children with CHB and from rat pups with first-degree block, and by selected human anti-p200 monoclonal antibodies, although there was not complete uniformity among the groups when a series of peptides was evaluated (8). While the antibody testing herein did not evaluate the finer specificity of reactivity within the α -helical p200 region, the fact that the majority of anti-p200-exposed rat pups had normal AV conduction suggests that finer mapping is not likely to change the conclusions presented herein. Thus, data both from rodents and from humans support the notion of a partnership between maternal and fetal contributions in the pathogenesis of CHB. Other candidate factors include fetal genes relating to the pathologic cascade, which might amplify inflammatory and fibrosing responses (28,29), and environmental influences.

This study did not confirm results recently reported by Sonesson and colleagues (21), in which prolongation of the AV time interval and levels of anti-p200 antibodies were positively correlated in 8 of 24 anti-Ro 52-positive mothers (21). The fetal Doppler mechanical PR interval measurement is a putative surrogate marker for the earliest sign of CHB, i.e., first-degree AV block. It was initially reported (12) as a tool for surveillance of mothers at risk for the development of fetal CHB and has been validated in a multicenter study of a normal population (13) with proof of concept in an affected fetus (30). Differences between the findings in the present study and those of Sonesson and coworkers (21) may have arisen from ethnic differences between the 2 cohorts, differing gestational age range and a smaller number of anti-Ro 52-positive patients in the Swedish study (21), and interindividual variation of measurement, particularly in the onset of the mitral A wave. However, it should be acknowledged that, at least postnatally, PR intervals are highly variable and change over time, with alterations in heart rate and sympathetic and parasympathetic tone. For example, the length of the PR interval does not even correlate with level of digoxin, or with the degree of carditis in acute rheumatic fever (31).

In conclusion, fetuses can develop CHB in the absence of detectable maternal anti-p200 antibodies, and exposure to antibodies of this fine specificity does not invariably result in CHB. Accordingly, this reactivity

is neither necessary nor sufficient to account for all cases of isolated CHB detected in utero. Injury to the fetal conduction system and surrounding myocardium is rare and likely the consequence of several factors, both maternal and fetal. However, continued search for an essential antibody specificity, perhaps related to an epitope within the region of p200, remains important. Removal of this putative single maternal factor could prevent initiation of injury/inflammation and the subsequent scarring amplified by the fetus as well as the in utero environment.

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REFERENCES

1. Brucato A, Frassi M, Franceschini F, Cimaz R, Faden D, Pisoni MP, et al. Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. *Arthritis Rheum* 2001;44:1832-5.
2. Gordon P, Rosenthal E, Simpson JM, Sharland G, Brucato A, Franceschini F, et al. Anti-52 kDa Ro, anti-60 kDa Ro, and anti-La antibody profiles in neonatal lupus. *J Rheumatol* 2004;31:2480-7.
3. Saleeb S, Copel J, Friedman D, Buyon JP. Comparison of treatment with fluorinated glucocorticoids to the natural history of autoantibody-associated congenital heart block: retrospective review of the Research Registry for Neonatal Lupus. *Arthritis Rheum* 1999;42:2335-45.
4. Buyon JP, Clancy RM. Neonatal lupus: basic research and clinical perspectives. *Rheum Dis Clin North Am* 2005;31:299-313.
5. Buyon JP, Slade SG, Reveille JD, Hamel JC, Chan EK. Autoantibody responses to the "native" 52-kDa SS-A/Ro protein in neonatal lupus syndromes, systemic lupus erythematosus, and Sjögren's syndrome. *J Immunol* 1994;152:3675-84.
6. Julkunen H, Kurki P, Kaaja R, Heikkilä R, Immonen I, Chan EK, et al. Isolated congenital heart block: long-term outcome of mothers and characterization of the immune response to SS-A/Ro and to SS-B/La. *Arthritis Rheum* 1993;36:1588-98.
7. Salomonsson S, Dorner T, Theander E, Bremme K, Larsson P, Wahren-Herlenius M. A serologic marker for fetal risk of congenital heart block. *Arthritis Rheum* 2002;46:1233-41.
8. Salomonsson S, Sonesson SE, Ottosson L, Muhallab S, Olsson T, Sunnerhagen M, et al. Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeostasis, and mediate congenital heart block. *J Exp Med* 2005;201:11-7.
9. Buyon JP, Hiebert R, Copel J, Craft J, Friedman D, Katholi M, et al. Autoimmune-associated congenital heart block: demographics, mortality, morbidity and recurrence rates obtained from a national neonatal lupus registry. *J Am Coll Cardiol* 1998;31:1658-66.
10. Tseng CE, Caldwell K, Feit S, Chan EK, Buyon JP. Subclass distribution of maternal and neonatal anti-Ro(SSA) and La(SSB) antibodies in congenital heart block. *J Rheumatol* 1996;23:925-32.
11. Tseng CE, Chan EK, Miranda E, Gross M, Di Donato F, Buyon

- JP. The 52-kd protein as a target of intermolecular spreading of the immune response to components of the SS-A/Ro-SS-B/La complex. *Arthritis Rheum* 1997;40:936-44.
12. Glickstein JS, Buyon J, Friedman D. Pulsed Doppler echocardiographic assessment of the fetal PR interval. *Am J Cardiol* 2000;86:236-9.
 13. Glickstein J, Buyon J, Kim M, Friedman D, and the PRIDE investigators. The fetal Doppler mechanical PR interval: a validation study. *Fetal Diagn Ther* 2004;19:31-4.
 14. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307-10.
 15. Tseng CE, Miranda E, Di Donato F, Boutjdir M, Rashbaum W, Chan EK, et al. mRNA and protein expression of SSA/Ro and SSB/La in human fetal cardiac myocytes cultured using a novel application of the Langendorff procedure. *Pediatr Res* 1999;45:260-9.
 16. Miranda ME, Tseng CE, Rashbaum W, Ochs RL, Casiano CA, Di Donato F, et al. Accessibility of SSA/Ro and SSB/La antigens to maternal autoantibodies in apoptotic human fetal cardiac myocytes. *J Immunol* 1998;161:5061-9.
 17. Clancy RM, Askanase AD, Kapur RP, Chiopelas E, Azar N, Miranda-Carus ME, et al. Transdifferentiation of cardiac fibroblasts, a fetal factor in anti-SSA/Ro-SSB/La antibody-mediated congenital heart block. *J Immunol* 2002;169:2156-63.
 18. Waltuck J, Buyon JP. Autoantibody-associated congenital heart block: outcome in mothers and children. *Ann Intern Med* 1994;120:544-51.
 19. Silverman ED. Congenital heart block and neonatal lupus erythematosus: prevention is the goal. *J Rheumatol* 1993;20:1101-4.
 20. Julkunen H, Kaaja R, Wallgren E, Teramo K. Isolated congenital heart block: fetal and infant outcome and familial incidence of heart block. *Obstet Gynecol* 1993;82:11-6.
 21. Sonesson SE, Salomonsson S, Jacobsson LA, Bremme K, Wahren-Herlenius M. Signs of first-degree heart block occur in one-third of fetuses of pregnant women with anti-SSA/Ro 52-kd antibodies. *Arthritis Rheum* 2004;50:1253-61.
 22. Rein AJ, Mevorach D, Perles Z, Shovali A, Elchalal U. Fetal first-degree heart block, or where to set the confidence limit: comment on the article by Sonesson et al [letter]. *Arthritis Rheum* 2005;52:366.
 23. Theander E, Brucato A, Gudmundsson S, Salomonsson S, Wahren-Herlenius M, Manthorpe R. Primary Sjögren's syndrome: treatment of fetal incomplete atrioventricular block with dexamethasone. *J Rheumatol* 2001;28:373-6.
 24. Hassan AB, Lundberg IE, Isenberg D, Wahren-Herlenius M. Serial analysis of Ro/SSA and La/SSB antibody levels and correlation with clinical disease activity in patients with systemic lupus erythematosus. *Scand J Rheumatol* 2002;31:133-9.
 25. Moak JP, Barron KS, Hougen TJ, Wiles HB, Balaji S, Sreeram N, et al. Congenital heart block: development of late-onset cardiomyopathy, a previously underappreciated sequela. *J Am Coll Cardiol* 2001;37:238-42.
 26. Askanase AD, Friedman DM, Copel J, Dische MR, Dubin A, Starc TJ, et al. Spectrum and progression of conduction abnormalities in infants born to mothers with anti-SSA/Ro-SSB/La antibodies. *Lupus* 2002;11:145-51.
 27. Buyon JP, Rupel A, Clancy RM. Congenital heart block: do fetal factors fuel the fire from inflammation to fibrosis? [review]. *Lupus* 2003;12:731-4.
 28. Clancy RM, Backer CB, Yin X, Kapur RP, Molad Y, Buyon JP. Cytokine polymorphisms and histologic expression in autopsy studies: contribution of TNF- α and TGF- β 1 to the pathogenesis of autoimmune-associated congenital heart block. *J Immunol* 2003;171:3253-61.
 29. Clancy RM, Kapur RP, Molad Y, Askanase AD, Buyon JP. Immunohistologic evidence supports apoptosis, IgG deposition, and novel macrophage/fibroblast crosstalk in the pathologic cascade leading to congenital heart block. *Arthritis Rheum* 2004;50:173-82.
 30. Rosenthal D, Friedman DM, Buyon J, Dubin A. Validation of the Doppler PR interval in the fetus. *J Am Soc Echocardiogr* 2002;15:1029-30.
 31. Allen HD, Clark EB, Gutgesell HP, Driscoll DJ, editors. *Moss and Adams heart disease in infants, children and adolescents*. 6th ed. Philadelphia: Lippincott Williams and Wilkins; 2001.