Reactivity to the p305 Epitope of the α1G T-Type Calcium Channel and Autoimmune-Associated Congenital Heart Block

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Background—Only 2% of mothers positive for anti-SSA/Ro (Ro) antibodies have children with congenital heart block (CHB). This study aimed to determine whether reactivity with p305, an epitope within the α1G T-type calcium channel, confers added risk over anti-Ro antibodies.

Methods and Results—Using sera from anti-Ro-exposed pregnancies resulting in offspring with CHB, no disease but CHB-sibling, and no disease and no CHB-sibling, as well as disease (lupus without anti-Ro) and healthy controls, reactivities were determined for binding to Ro60, p305, and an epitope within Ro60, p133-Ro60, which shares structural properties with p305, including key amino acids and an α-helical structure. Candidate peptides were further evaluated in an in vitro model that assessed the binding of maternal antibodies to apoptotic cells. In anti-Ro-positive mothers, anti-p305 autoantibodies (>3 SD above healthy controls) were detected in 3/59 (5%) CHB pregnancies, 4/30 (13%) unaffected pregnancies with a CHB-sibling, and 0/42 (0%) of unaffected pregnancies with no CHB-sibling. For umbilical bloods (61 CHB, 41 healthy with CHB sibling), no association of anti-p305 with outcome was detected; however, overall levels of anti-p305 were elevated compared to mothers during pregnancy in all groups studied. For anti-p133-Ro60, reactivity paralleled that of anti-p305. In the screen employing apoptotic cells, p133-Ro60, but not p305, significantly attenuated the binding of immunoglobulin G isolated from a mother whose child had CHB (42.1% reduced to 13.9%, absence/presence of p133-Ro60, respectively, \(P<0.05\)).

Conclusions—These data suggest that anti-p305 is not a robust maternal marker for assessing increased risk of CHB during an anti-SSA/Ro pregnancy. (J Am Heart Assoc. 2015;4:e001836 doi: 10.1161/JAHA.115.001836)

Key Words: apoptosis • heart block • risk factors

Autoimmune-associated isolated congenital heart block (CHB) most often develops during 18 to 24 weeks of gestation and is histologically characterized by fibrotic replacement of the atrioventricular node, which can extend to the working myocardium and endocardium.1,2 The rapidity of clinically detectable injury is supported by the report of normal sinus rhythm progressing to irreversible third-degree block within 1 to 2 weeks.3 In fetuses identified with CHB, antibodies to components of the SSA/Ro-SSB/La ribonucleoprotein complex are nearly universally identified in the mother, regardless of her health status.1 This strong association was first noted more than 3 decades ago.4 However, only 2% of women with anti-SSA/Ro antibodies face the prospect of having a child with CHB5 thus propelling the search for additional reactivities.

The most viable antibody candidates should be those that recognize cognate ligands within pathways that might account for a putative disease mechanism. There are 2 non-mutually exclusive hypotheses that have been proposed to explain the way in which anti-SSA/Ro autoantibodies, whose antigens are normally sequestered intracellularly, initiate injury in the fetal heart. The apoptosis hypothesis states that during development, physiologic apoptosis results in the surface translocation of intracellular SSA/Ro-SSB/La antigens, which then become accessible to circulating maternal antibodies, resulting in immune complexes phagocytosed by macrophages and secretion of proinflammatory and profibrosing cytokines.6,7 The second hypothesis is based on cross-reactivity, wherein autoantibodies cross-react with myocyte surface proteins. Several candidates involving calcium channels have been advanced in accordance with the latter hypothesis.8 It has been recently reported that sera from a small cohort of

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anti-SSA/Ro-positive mothers of children with CHB recognize an extracellular epitope of the α1G T-type calcium channel. Reactivity was mapped to a peptide designated as p305 (corresponding to aa 305 to 319 of the extracellular loop linking transmembrane segments S5-S6 in α1G repeat I).

To address the cross-reactivity hypothesis and further evaluate the utility of p305 antibodies as a specific biomarker for assessing risk of CHB in an offspring, maternal sera were collected during pregnancies from anti-SSA/Ro-positive mothers whose offspring were identified with CHB or were unaffected as well as cord blood from anti-SSA/Ro exposed CHB and unaffected neonates. In addition, potential homology between p305 and a peptide segment in Ro60 (p133-Ro60) was investigated. The functional effects of both peptides, p305 and p133-Ro60, were assessed by screening for inhibition of anti-Ro60 antibody binding to the surface of apoptotic cells.

Methods
Patients
The study included maternal blood from 4 groups of pregnant women in which the outcome of the offspring was known and 55 nonpregnant healthy volunteers without anti-SSA/Ro antibodies. Maternal Group 1 comprised 59 anti-SSA/Ro positive sera from pregnancies complicated by CHB. Maternal Group 2 comprised 30 anti-SSA/Ro positive sera from unaffected pregnancies but in whom a child with CHB was born prior or subsequent. These 2 groups were obtained from enrollees in the Research Registry for Neonatal Lupus. Maternal Groups 3 and 4 comprised sera from mothers with systemic lupus erythematosus enrolled in the Predictors of Pregnancy Outcome: Biomarkers in Antiphospholipid Antibody Syndrome and Systemic Lupus Erythematosus (PROMISSE) study—42 positive for anti-SSA/Ro and 41 negative for anti-SSA/Ro, respectively, who never had a child with CHB (described in ref. 11). Plasma was obtained from the umbilical cord blood of 102 neonates exposed to maternal antibodies. Maternal Group 1 comprised 61 anti-SSA/Ro positive sera from pregnancies complicated by CHB and unaffected neonates but in whom a child with CHB was born prior or subsequent. These 2 groups were obtained from enrollees in the Research Registry for Neonatal Lupus and/or PROMISSE and evaluation of blood samples.

Protein and Synthetic Peptides
Ro60 purified from bovine spleen and thymus was purchased from ArOtec. Synthetic peptide p305 (YNSSSNTTCVNWNOY), biotinylated p305 (b-YNSSNTTCVNWNOY), and synthetic Ro60 peptides, p133-Ro60 (KKDLKESMKCGMWGRAKKK) and p197-Ro60 (GSGVTKYTKGWEVHELKYEKALSV) were >90% pure (purchased from Mimotopes).

Ro60, p305, and p133-Ro60 Enzyme-Linked Immunosorbent Assays (ELISAs)
Full-length Ro60 (1 μg/well) and candidate peptides (0.1 to 2 μg/well, p305 or 0.1 μg/well, p133-Ro60) in phosphate-buffered saline were coated onto a 96-well microtiter plate overnight at 4°C. After washing (phosphate-buffered saline/0.1% Tween 20), nonspecific sites were blocked with 0.1% gelatin/phosphate-buffered saline. Human sera (adults) and plasma (cord bloods) were applied (1:1000 for full-length Ro60; 1:500 for peptides in blocking buffer) for 1 hour at room temperature. Alkaline phosphatase–conjugated rabbit anti-human immunoglobulin G (IgG) (g-chain specific; Sigma) was used (1:20000) with phosphatase substrate. To ensure uniformity of each experiment, a patient with high-titer anti-p305 antibodies was included in each ELISA. Sera were considered positive if the binding units were 3 SDs above the mean optical density of healthy controls.

Induction of Apoptosis in Murine Embryonic Fibroblasts (MEFs)
Apoptosis was induced by treating fibroblasts with 0.1 mg/mL murine interferon-γ for 24 hours as previously described. Cells were then seeded onto tissue culture dishes coated with poly (2-hydroxyethyl methacrylate), which disrupts adhesion to the surface, in the presence of interferon-γ (0.1 mg/mL), tumor necrosis factor-α (5 ng/mL), and cycloheximide (100 mg/mL) for 4 to 18 hours.

Flow Cytometry for Evaluation of Ro60 Expression
Surface expression of Ro60 was assessed on nonfixed, nonpermeabilized, early apoptotic fibroblasts gated as annexin V+, PI−. Cells (apoptotic) were incubated with 300 μg/mL CHB IgG (isolated from an anti-SSA/Ro60 and Ro52-SSB/La-positive mother of a child with CHB [no antibodies to dsDNA]) or CHB IgG preincubated with peptide (30 minutes prior to addition to cells) for 45 minutes at room temperature. Control IgG obtained from a pool of healthy donors (300 μg/mL) that was negative for anti-SSA/Ro and SSB/La was also used. After incubation, cells were washed twice (phosphate-buffered saline/1% bovine serum albumin/0.02% sodium azide) and stained with anti-human IgG–fluorescein isothiocyanate (1:200) for 30 minutes. For apoptotic cells, 5 mL annexin V-allophycocyanin (BD Biosciences) and 5 mg/mL propidium iodide (Invitrogen) in annexin V binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl2 [pH 7.4]) were added for 15 minutes at room temperature. Binding was assessed on a LSRII flow cytometer (BD Biosciences).
binding of CHB IgG to apoptotic cells is reflected by the percent of positive cells (after staining with anti-IgG fluorescein isothiocyanate and gating events versus control IgG).

**Molecular Modeling and Bioinformatics**

ZEGA sequence alignment of the minimal reactive epitope of the α₁G p305 peptide (NTTCVNWNQY) reported by Strandberg et al with full-length human Ro60 (accession no. M25077) was performed with the Internal Coordinate Mechanics software (ICM-Pro Molsoft LLC, La Jolla, CA) as previously described, which localized the highest ungapped homology with this minimal p305 determinant to amino acid positions 138 to 147 in human Ro60, suggesting that the full putative homologous segment with p305 in Ro60 is amino acid positions 133 to 147. ICM ab initio folding of α₁G p305 and p133-Ro60 peptides was performed as previously described. Molecular graphical analysis was also performed with ICM-Pro.

**Statistical Analysis**

The prevalence of autoantibodies was compared by Fisher’s exact test. Differences between antibody binding to apoptotic cells in the absence and presence of peptides were determined using ANOVA, followed by the Tukey-Kramer test when findings with the ANOVA model were significant. Analyses were performed using GraphPad InStat version 3.10.

**Results**

**Structural Analysis and Ro60/TROVE2 Homology Evaluation of p305**

Published data suggest that p305 reacts with maternal CHB-associated serum IgG as an isolated 15-mer peptide such that all of the three-dimensional structural information relevant to this interaction is contained within the isolated peptide. Accordingly, we predicted the dynamic three-dimensional structure of p305 using in silico ab initio folding (see

![Figure 1](https://example.com/image1.png)

**Figure 1.** Predicted three-dimensional structures of p305 and p133-Ro60. A, Lowest-energy ab initio folding conformation of p305 peptide backbone: Left: The backbone is shown in purple ribbon depiction, and the side chains on 1 surface of the helix that exhibits some conservation between p305 and p133-Ro are shown in stick depiction. Right: the side chains are depicted with an additional volume depiction, with those conserved between p305 and p133-Ro colored green and others colored red. B, Best ZEGA (see Methods) alignment between minimal p305 active sequence and residues 138 to 147 of human Ro60. C, Superposition of folded p305 structure from (A) (purple ribbon; circled) onto a segment identified in (B) by sequence alignment within homology model of human Ro60 (orange ribbon). D, Lowest-energy ab initio folding conformation of p133-Ro60, colored and illustrated as in (A).
Methods) and found that p305 strongly prefers an α-helical conformation (Figure 1A). Since NTTCVNWNOY was found to be the sequence of the minimal reactive determinant of p305, the sequence of this p305 fragment was aligned with the sequence of full-length human Ro60. The best alignment was found between this segment and the Ro60 segment at amino acids 138 to 147 (Figure 1B). This segment is exposed on the surface of Ro60, is largely α-helical, and abuts a ssRNA hairpin binding site as shown in the superimposition of the human Ro60 homology model with the p305 folded free peptide (Figure 1C). These alignment data suggested that this segment in Ro60 might exhibit α-helical-based epitopes similar to those in p305. In order to determine whether the equivalent segment to p305 in Ro60 (the full-length equivalence would be from aa 133 to 147, termed p133-Ro60) continued to exhibit α-helical properties as an isolated 15-mer peptide for the purposes of ELISA testing, we predicted the dynamic three-dimensional structure of p133-Ro60 ab initio and found that p133-Ro60 also strongly prefers an α-helical conformation, mirroring p305 (Figure 1D). Thus, we hypothesized that p305 and p133-Ro60 mimic each other as autoantibody targets at the molecular level and that both could be tested using isolated 15-mer p305 and p133-Ro60 peptides in ELISA-based assays.

Maternal Reactivity to Full Length TROVE2 (Ro60) and p305 CACNA1G (p305)

As expected, positive reactivity with full-length Ro60 was found in 100% of Group 1 (59 CHB mothers), 87% of Group 2 (30 mothers of unaffected siblings), 100% of Group 3 (42 anti-Ro-positive, SLE, CHB-negative mothers), none of the Group 4 (41 anti-Ro-negative, SLE, CHB-negative mothers), and none of the healthy controls (Table 1).

In contrast to the reactivity with Ro60, reactivity with p305, as defined by 3 SD above the mean value of the healthy controls, was seen in only 3/59 (5%) sera of the Group 1 mothers during pregnancies with CHB, 4/30 (13%) of the Group 2 anti-Ro-positive mothers carrying unaffected siblings, 0/42 (0%) of the Group 3 anti-Ro-positive SLE mothers with no CHB children, 0/41 (0%) of the Group 4 anti-Ro-negative SLE patients with no CHB children, and 1/55 (2%) healthy subjects. There were no differences in mean serum levels between any of the groups (Figure 2).

Cord Blood Reactivity to p305

The mean plasma levels of anti-p305 from cord blood of CHB neonates, unaffected siblings, and healthy non-anti-Ro-exposed neonates were 0.203±0.225, 0.216±0.276, and 0.120±0.072, respectively (P not significant for all comparisons, Figure 3). Positive reactivity was identified in

### Table 1. Reactivity of Anti-p305 Autoantibody in Peripheral Blood of Healthy Controls, Research Registry for Neonatal Lupus Mothers, Lupus Patients, and Cord Blood of Healthy and Neonatal Lupus Children

| Group                        | N   | Ro60% Positive | p305% Positive | p133-Ro60% Positive |
|------------------------------|-----|----------------|----------------|---------------------|
| Healthy controls             | 55  | 0              | 2              | 0                   |
| CHB mothers                  | 59  | 100            | 5              | 11                  |
| Unaffected sibling mothers   | 30  | 87             | 13             | 17                  |
| anti-Ro+ SLE mothers         | 42  | 100            | 0              | ND                  |
| anti-Ro- SLE mothers         | 41  | 0              | 0              | ND                  |
| Healthy cord bloods          | 5   | 0              | 0              | 0                   |
| CHB neonates                 | 61  | 94             | 20             | 19                  |
| Unaffected sibling neonates  | 41  | 100            | 17             | 25                  |

CHB indicates congenital heart block; ND, not determined; SLE, systemic lupus erythematosus.

12/61 (20%) CHB cord bloods compared to 7/41 (17%) for the unaffected siblings (P=0.80). In instances of an assessment within the same family comparing discordant siblings, there was no association between the presence or titer of the...
anti-p305 autoantibody and outcome (Table 2). However, overall levels of p305 were higher in cord blood compared to levels in the mothers during pregnancy in all groups studied (19/102 [18.6%] positive CHB cord bloods versus 7/89 [7.8%] positive CHB mothers’ serum, \( P < 0.001 \), Table 1). Given the hypothesized homology between p305 and p133-Ro60, cord bloods were evaluated for reactivities to p133-Ro60. While a positive blood test for p133-Ro60 was found in 19% of cord bloods of CHB children, it did not associate with outcome (positive in 25% in cord bloods of anti-SSA/Ro-exposed healthy children). Higher reactivities of anti-p133-Ro60 in cord than maternal blood were also observed (Table 1).

**Divergence of Biological Effects of p133-Ro60 and p305 as Assessed by Blocking In Vitro Surface Binding of Cultured Apoptotic MEFs by CHB IgG**

In accord with the apoptotic hypothesis, the binding of anti-SSA/Ro to surface Ro60 on apoptotic cells was considered a proxy for the disease-provoking pathogenic scenario. MEFs were rendered apoptotic after an overnight incubation using a polyHEMA coated culture dish. Cells were incubated with and without CHB IgG in the absence and presence of prior incubation with the candidate peptide. As expected, the binding of CHB IgG to apoptotic murine fibroblasts was significantly increased (positive cells, 42.1% versus 5.9%, CHB IgG versus control IgG, respectively, Figure 4). The binding of CHB IgG to apoptotic MEFs was attenuated in the presence of p133-Ro60, but not p305. p197-Ro60, another peptide derived from the backbone of Ro60, also significantly lowered the binding of anti-Ro60 autoantibody to apoptotic MEFs.

**Discussion**

In this study, the association of CHB with a novel autoantibody against \( \alpha_{1G} \) was assessed leveraging sera from anti-SSA/Ro-positive mothers during pregnancy and plasma from umbilical cord blood. Reactivity in the latter would unambiguously substantiate exposure to maternal antibody. Based on ELISA using an epitope designated as p305 (aa305 to 319 of the T-type calcium channel, \( \alpha_{1G} \)), <10% of anti-Ro60 pregnant mothers whose fetuses either were identified to have CHB or were healthy but had previously affected fetuses demonstrated reactivity. While anti-p305 antibodies were detected in 20% of cord bloods from CHB children, reactivity did not associate with outcome since 18% of cord bloods from anti-SSA/Ro-exposed healthy children were also positive.

The paradoxical observation reported involving higher reactivities of anti-p305 in cord than maternal blood is intriguing, but cannot be definitively explained at this time. Higher reactivities of anti-p133-Ro60 (an amino acid segment that is exposed on the surface of Ro60 sharing key properties with p305, including key amino acids and an \( \alpha \)-helical structure) in cord than maternal blood were also observed. Possible explanations are maternal anti-idiotype IgM antibodies’ masking of epitopes, absence of equilibration of

**Table 2. Evaluation of Titers of Anti-p305 Antibody in Plasma From Cord Blood in Siblings Using an ELISA Approach as Described in Figure 2**

| Sibling Pair | p305 Optical Density | Date of Birth (Month, Year) | Outcome Sibling 1 | Sex | p305 Optical Density | Date of Birth (Month, Year) | Outcome Sibling 2 | Sex |
|--------------|----------------------|-----------------------------|-------------------|-----|----------------------|-----------------------------|-------------------|-----|
| 1            | 0.29                 | 4/10                        | Healthy           | F   | 0.05                 | 4/10*                       | CHB               | F   |
| 2            | 0.01                 | 10/09                       | CHB               | M   | 0.15                 | 10/09*                     | Healthy           | M   |
| 3            | 0.10                 | 3/08                        | Healthy           | M   | 0                    | 3/08*                      | CHB               | M   |
| 4            | 0                    | 11/06                       | CHB               | M   | 0                    | 12/08                      | CHB               | M   |
| 5            | 0.48                 | 5/05                        | CHB               | M   | 0.71                 | 5/07                       | Healthy           | M   |

CHB indicates congenital heart block.

* Twin.
Maternal Antibody-Specificity and Fetal CHB

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antibodies between maternal and fetal compartments, hemo
dilution, avidity effects, or as yet unidentified factors that exist only in the fetal or maternal compartment exclusively. The reactivity of blood specimens for p133-Ro60, which were within a low range (11% to 25%), represent values that are consistent with the findings by Scofield and coworkers who also reported weak reactivity of peptide versus native antigen and a peptide in the context of multimers.17,18

Because in vivo binding of anti-SSA/Ro to surface Ro60 on apoptotic cells is hypothesized to initiate the cascade to cardiac injury19 and this in vitro assay has robust perfor-
mance to evaluate surface accessibility to maternal extra-
cellular anti-SSA/Ro antibody,7,20 a finding that was replicated by Reed and coworkers,21 inhibition of anti-SSA/Ro antibody binding to apoptotic MEFs by p133-Ro60 but not p305 represents a divergence of biological properties for these 2 peptides. While observations evaluating the relationships between p305 and p133-Ro60 as targets of CHB antiseras are largely exploratory, they are, to our knowledge, the first reported link between the large body of literature on observed effects of CHB-associated autoantibodies on calcium channels and on Ro60. A limitation of our approach is that reactivity to the p305 peptide was not evaluated in the context of the whole molecule (α1G T-type calcium channel). Accordingly, these data do not rule out the possibility that there may be maternal reactivity against the α1G T-type calcium channel. While the anti-p305 antibody could confer pathogenicity, unlike anti-Ro52 or Ro60 it appears to be neither necessary (since the frequency is extremely low) nor sufficient (since it is present in mothers during unaffected pregnancies). These data cannot rule out whether maternal anti-p305 antibodies confer an added risk (presumably by affecting calcium electrogenesis) in certain fetuses.

In consideration of anti-p305 as a clinical biomarker for risk of CHB, an association of positivity and titer with outcome in different pregnancies from the same mother would be highly informative, given a recurrence rate that is ≈18%.22 However, the frequency and titer of reactivity to p305 in cord blood did not differentiate CHB from unaffected siblings. Also, while α1G is important to conduction, it should be emphasized that the clinical spectrum of injury is not restricted to the α1G expressing conduction tissue. In a recent study that interrogated autopsies, Llanos23 reported that in addition to atrioventricular nodal fibrosis, injury extended in some cases to nonconductive tissues.

While the clues to pathogenesis are related to the importance of understanding which targets are bound by antibodies, reactivities against p305 and p133-Ro60 did not perform as robust biomarkers in this study. In contrast, autoantibodies against Ro60 have a high sensitivity for CHB but fall short because of too many false-positive results, suggesting that other fetal or environmental factors are required for full disease expression and/or that the elusive target still awaits identification.

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**Disclosures**

None.

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