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# Pathophysiology of autoimmune-associated congenital heart block

*Yongxia Qu[1,2], Mohamed Boutjdir[1,2,3]*

*[1]VA New York Harbor Healthcare System, Brooklyn, NY; [2]State University of New York Downstate Medical Center, Brooklyn, NY and [3]New York University School of Medicine, New York, NY, USA*

**Abstract**

Congenital heart block (CHB), detected at or before birth, in a structurally normal heart, is strongly associated with autoantibodies to SSA/Ro-SSB/La ribonucleoproteins. The historical hallmark of CHB is complete atrioventricular block (AVB) which is irreversible. CHB occurs in 2% of primigravid mothers with anti-Ro/La antibodies, and in 20% of women who had a previous CHB offspring. Although not consistent, emerging clinical research expanded the spectrum of CHB to include lesser degrees of AVB, sinus bradycardia, QT prolongation, and late onset cardiomyopathy. Despite over a century since the discovery of CHB, the wide spectrum of CHB manifestation has posed a challenge in the elucidation of the true underlying pathogenesis and thus effective therapy. This review focuses on the recent knowledge and some hypotheses proposed for CHB pathogenesis, mainly the apoptosis and the Ca channel hypotheses. Experimentally, the first challenge in establishing the association between anti-Ro/La antibodies and CHB was overcome by the development of *in vivo* animal models for CHB. The incidence of conduction abnormalities in these models was fairly similar to that clinically, indicating that anti-Ro/La antibodies are essential but not sufficient for the disease expression. Subsequent *in vitro* experiments demonstrated that maternal anti-Ro/La antibodies directly cross react and inhibit Ca channels. This inhibition of Ca channels provides a logical explanation for the AVB, sinus bradycardia and perhaps even the cardiomyopathy because Ca channels play a vital role in the action potential genesis and conduction at the atrioventricular node and sinus node; and in excitation-contraction coupling of the developing heart, respectively. Because Ro/La antigens are intracellularly located, apoptosis was proposed as a process that helps Ro/La antigens translocate to the fetal cell surface membrane where they will be accessible to circulating maternal antibodies. In this regard, extensive evidence showed that in experimentally induced apoptotis in human fetal ventricular myocytes, the intracellular Ro/La antigens translocate to the sarcolemma, interact with anti-Ro/La antibodies, divert normal clearance of apoptotic cardiac myocytes by healthy cardiac myocytes toward clearance by professional macrophages with the release of the inflammatory and/or fibrosing cytokines which leads to fibrosis and permanent CHB. Despite the significant progress made in the last decade in the understanding of the CHB pathogenesis, the wide spectrum of CHB expression point to the multifactorial nature of CHB pathogenesis and to the need for continuous and joint international efforts to dissect the complex pathways involved in CHB.

**Key words:** atrioventricular block, neonatal lupus, anti-Ro/SSA antibodies, Ca channels, apoptosis

## Abbreviations

| | |
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| 5-HT4: | Serotonin 4 receptors |
| AVB: | Atrioventricular block |
| CHB: | Congenital heart block |
| CHB-IgG: | Maternal IgG containing anti-Ro/La antibody from mothers with CHB children |
| CM: | Cardiomyopathy |
| ECG: | Electrocardiogram |
| EFE: | Endocardial fibroelastosis |
| ET: | Endothelin |
| Fc R: | Fc gamma receptor |
| GST: | Glutathione S-transferase |
| IC: | Immune complexes |
| IKs: | The delayed rectifier K current |
| INa: | The fast Na current |
| Ito : | Transient outward K current |
| LV: | Left ventricle |
| NLS: | Neonatal Lupus Syndromes |
| PRIDE: | PR Interval and Dexamethasone Evaluation |
| QTc: | Corrected QT interval |
| RV: | Right ventricle |
| SA : | Sinoatrial |
| siRNA: | Small interfering RNA |
| ssRNA: | Single strand RNA |
| TGFβ: | Transforming growth factor beta |
| TLR7: | Toll-like receptor 7 |
| uPA: | Urokinase plasminogen activator |
| uPAR: | Urokinase plasminogen activator receptor |

## Introduction

Autoimmune-associated congenital heart block (CHB) which occurs in the absence of structural abnormalities is a passively acquired autoimmunity consequent to placental transport of antibodies reactive with SSA/Ro-SSA/La ribonucleoprotein complex (1, 2). CHB is a manifestation of the so called neonatal lupus syndromes (NLS). Third degree atrioventricular block (AVB) is the hallmark of CHB and carries a significant mortality (20-30%) and morbidity (67% of surviving affected children require permanent pacemaker before adulthood (2-8). Other manifestations of NLS include cutaneous, liver and hematological disease, all of which generally disappear with the clearance of maternal antibodies from the neonatal circulation at about 6-8 months (2). The most common window period for CHB detection is between 20 and 24 weeks gestation, which is relevant for timing of fetal surveillance (2). Emerging clinical studies show that conduction abnormalities seen in CHB have expanded beyond AVB to include sinus bradycardia (9-11) although controversial (12), QT abnormalities (13), and late onset fatal cardiomyopathy (14-16) as late as 10 years of age. The incidence of CHB is low in the general population (1 of every 15,000) but increases to 5% in women with Lupus (2). However, the recurrence rate for CHB in a subsequent pregnancy is alarming and approaches 20% (2), affecting the decision of the mother to have another child. A network of research registries worldwide is needed to enable the acquisition of these rare disease cases and to facilitate further the investigation of epidemiology, clinical and basic research aspects of CHB. To date, and despite the significant progress made in the last decade at all fronts, CHB continues to pose a challenge to both basic scientists and clinicians in the understanding of the true pathogenesis underlying the disease.

## Spectrum of CHB

### *First degree AVB and progression of conduction abnormalities*

Historically, the signature lesion of CHB is complete AVB. Over the years, as the technology to diagnose CHB improved, a lesser degrees of AVB were readily detectable in utero. Existing data regarding the true incidence of first degree AVB and its potential to predict later progression to more advanced AVB is conflicting (17, 18, 15, 19, 20). A high incidence of first degree AVB was observed by Sonesson et al. (20), in a prospective study. Twenty-four Ro 52 seropositive

women were followed up weekly between 18 and 24 weeks of gestation, with two Doppler echocardiographic methods designed to estimate the time delay between hemodynamic events caused by atrial and ventricular depolarization. Two hundred eighty-four women with normal pregnancies served as controls. A PR interval >135 ms was considered abnormal. In Ro 52 seropositive women, eight of 24 fetuses had signs of first degree AVB, one of which progressed to complete AVB and six spontaneously reverted to normal conduction before or shortly after birth. The authors concluded that Ro 52 seropositive pregnant women frequently carry fetuses with first degree AVB and progression to a more severe degree of block may occur in some. This observation was not reproduced by Freidman et al., in the PRIDE study (19). Ninety five Ro 52 seropositive women had fetal echocardiograms performed weekly from 16 to 26 weeks gestation and biweekly from 26 to 34 week in 98 pregnancies. PR intervals of >150 ms was used as cutoff for first degree AVB. Of 98 fetuses, only two fetuses had first degree AVB detected at or before 22 weeks, and each reverted within 1 week with 4 mg dexamethasone. Ninety-two fetuses had normal PR intervals. Three fetuses had complete AVB, none of whom had a preceding abnormal PR interval. The conclusion was that prolongation of the PR interval was uncommon and did not precede more advanced block.

Re-evaluation of the PRIDE study using the cut off value of 135 ms as PR interval prolongation revealed consistency between the two studies, with about one-third of the fetuses in PRIDE having a prolonged PR interval. Interestingly, in the PRIDE study, all fetuses with PR interval of 135-150 ms spontaneously reversed by the next echocardiogram. In Sonesson's study (20), however, only two fetuses had PR prolongation as defined by PRIDE criteria. In summary, the discrepancy between the two studies was mostly related to the difference in the definition of PR prolongation. Mild PR prolongation might be common and can revert spontaneously. A universal cutoff for a pathogenic PR-interval to predict disease progression and standardization of the diagnostic approach for fetal AVB is warranted.

### *Sinus bradycardia*

Sinus bradycardia unrelated to AVB was first reported in animal models of CHB (21-23), and in the in vitro experiments using Langendorff-perfused isolated hearts (21, 22, 24). These observations were later confirmed in some clinical cases by Brucato et al. (11), and Hamilton's group (10). Brucato and colleagues (11) reported a significant transient sinus bradycardia in 4 infants among 24 otherwise healthy children from anti-Ro antibody positive mothers whose ECGs were obtained within the first 3 days of life. In all cases, sinus bradycardia disappeared within 10 days after birth, with no sequelae. An 11% of chronotropic incompetence of the sinus node was found in infants with AVB from mothers with anti-Ro antibodies by Menon et al. (10). However, Costedoat-Chalumeau did not find any significant difference in mean heart rate when comparing the ECGs of 58 anti-Ro-positive children with those of 85 anti-Ro-negative children of the same age (25). Although sinus bradycardia has been consistently demonstrated in animal models, the clinical sinus bradycardia may be a rare and seemingly reversible part of the spectrum of anti-Ro/La antibody related cardiac disease. Its prediction of further cardiac conduction abnormalities in CHB has not been fully illucidated.

### *Prolongation of QT interval*

QT prolongation has been reported in infants from mothers with anti-Ro/La antibodies (13, 26), but these observations have not been confirmed by others (19, 25). QT prolongation in adults positive for anti-Ro/La antibodies has also been reported (27-29). Cimaz and colleagues (13) reported a mean QTc

prolongation in the absence of CHB in 21 children born to anti-Ro-positive mothers when compared to 7 children born to anti-Ro-negative mothers. QTc prolongation was resolved during the first year of life. Costedoat-Chalumeau et al. (25), have addressed the same issue in a study that compared ECGs in 58 consecutive children aged 0 to 2 months and born to anti-Ro-positive mothers with a carefully defined control group of 85 infants aged 0 to 2 months born to anti-Ro-negative mothers with connective tissue disease. No difference was found for QTc between these two groups. Interestingly, the mean QTc interval recorded during the period from 2 to 4 months showed a significant lengthening in comparison with those obtained during the period from 0 to 2 months in both anti-Ro-positive and anti-Ro-negative groups. In agreement with this, Schwartz and colleagues (30) have shown that there was a physiological lengthening of the QTc interval at the second month of life in a prospective study involving 4205 healthy newborns. This could explain why Cimaz and colleagues (13) found a QTc prolongation in the anti-SSA/Ro-positive group in which ECGs were recorded at 90 days, compared with 7 days for the anti-SSA/Ro-negative controls.

Two prospective studies of QTc prolongation have been recently published. Motta et al. (12) compared the ECGs obtained from 51 infants born to anti-Ro positive mothers with those from 50 control infants from mothers with connective tissue disease but were negative for anti-Ro antibodies. Mean QTc of infants born from anti-Ro positive mothers was slightly prolonged but did not reach the statistical significance when compared with controls. In another study by Gerosa et al. (31), 60 anti-Ro positive and 36 anti-Ro negative mothers were prospectively followed before/during pregnancy and underwent weekly fetal echocardiography from 18$^{th}$ to 26$^{th}$ week gestation. Infants' ECG and/or ECG-holter were performed at 1, 3, 6 and 12 months. ECGs of 200 consecutive neonates were used as a healthy control group. No differences in the prevalence of QTc interval prolongation (>440 ms) was observed between the anti-Ro-positive and negative groups. ECG-holter showed QTc prolongation >440ms in 59% infants of anti-Ro positive mothers, 60% in controls, QTc>470ms in four infants of anti-Ro positive group and two infants in controls. Genetic causes of QTc prolongation were excluded. These studies showed that QTc prolongation is frequent in infants with mothers with autoimmune disease, independent of maternal antibody profile. Thus, the current data do not show convincing evidence for the association of anti-Ro/La with QTc prolongation in the infants. Multinational studies that will systematically investigate the QTc prolongation as another potential spectrum of conduction abnormalities in infants with CHB are warranted.

### *Cardiomyopathy*

Approximately 15-20% of CHB affected fetuses develop more diffuse myocardial disease manifested as cardiomyopathy (CM) usually associated with endocardial fibroelastosis (EFE) (32-34), with or without clinical conduction abnormalities. The prognosis for fetuses and infants with diffuse CM/EFE is generally poor, with death or need for cardiac transplantation in 85% of the cases despite pacemaker therapy (14, 33, 34, 35). Nield et al. (34), described 13 children with CHB, associated with EFE predominantly involving the left ventricle. Severe ventricular dysfunction was present in all cases and led to death in nine and cardiac transplantation in two. The same year, those authors also reported 3 cases of severe EFE, mainly ventricular, in children without CHB born to mothers with anti-Ro antibodies (33). Guettrot-Imbert et al. (36), in contrast, recently reported 5 cases of mild EFE from 4 mothers with anti-Ro antibodies, detected at ages between 22-24 week gestation. The main EFE affected sites were the left atrium, aortic and tricuspid annuli. Only one case had left ventricular involvement. The appearance of EFE on fetal echocardiography remained stable, regard-

less of treatment (2 cases received betamethasone). One pregnancy was medially aborted at 30 weeks. During a 5 year follow up, one case had most severe EFE with significant right ventricular involvement, requiring surgery at age 6. The remaining 3 cases showed satisfactory development. The presentations in this case report contrast with previous reports about the severity of EFE (32-34). Two similar cases were reported by Raboisson et al. (37) and Pises et al. (38). Roboisson et al. (37) reported one case RV EFE associated with first degree AVB and a second case of isolated LV/RV EFE without any AV conduction prolongation from infants born to mothers with anti-Ro/La antibodies. In both cases, the fetal LV function was normal and EFE was resolved after dexamethasone treatment. Pises et al. (38) reported a prenatal diagnosis of right ventricular EFE without left ventricular involvement and without complete AVB in a fetus of a mother with anti-Ro/La antibodies. Improvement of EFE with maternal dexamethasone therapy was similar to three patients reported by Raboisson et al. (37). Of note is that none of the cases from Guettrot-Imbert's study were associated with CHB (36). The finding of EFE in the absence of AVB and the evolution of late CM/EFE despite adequate pacing suggest that CM/EFE and AVB may be two separate disease manifestations in NLS but a causal relationship cannot be ruled out. In summary, despite early institution of cardiac pacing, some infants with CHB will develop cardiomyopathy. Patients with CHB require close follow-up, not only for their cardiac rate and conduction abnormalities, but also for their ventricular function.

## Pathogenesis of CHB

### *Role of anti-Ro/La antibodies in the pathogenesis of CHB*

While almost all CHB diagnosed before the age of 26 weeks gestation were from mothers with anti-Ro/La antibodies, the risk of CHB is only 2% in mother known to have anti-Ro/La antibodies without prior children with CHB or rash (2). It is thus logical to question whether the anti-Ro/La antibody is just an innocent bystander or is there any causal relationship between anti-Ro/La antibodies and CHB. In this regard, a number of *in vivo* and *ex vivo* animal models were developed to investigate the potential role of anti-Ro/La antibodies in the pathogenesis of CHB.

### Passive mice model of CHB

To test a potential association between the presence of anti-Ro/La antibodies and the electrocardiographic abnormalities in CHB, a passive model of CHB was developed in mice (23). Timed pregnant mice were injected with anti-Ro/La antibodies purified from mothers with CHB children. ECG screening of the pups from the injected mothers showed sinus bradycardia and AVB when compared to controls. First-degree AVB was observed in 88% (14/16), 90% (9/10) and 47% (14/30), and sinus bradycardia was also present in 44% (7/16), 70% (7/10) and 33% (10/30) of pups from mothers injected at 8, 11 or 16 days gestation, respectively. Interestingly, no complete AVB was observed. The greater percentage and degree of sinus bradycardia and PR prolongation in the 11-day group correlates with the „window period" of susceptibility observed in humans. The high incidence of sinus bradycardia suggests possible sinoatrial (SA) node involvement. No follow-up ECGs were performed to examine the natural progression of the observed lesser degrees of AV block and sinus bradycardia in this study.

### Active mice model of CHB

Unlike the passive model of CHB where mice were directly injected with anti-Ro/La antibodies, the active model of CHB was developed by immunizing female mice and rabbit with human or murine Ro 52, Ro 60 or La 48

antigens (21, 39-41). The average incidence of complete AVB was 2.5% which is similar to the incidence reported in humans. Specifically, in the first study by Boutjdir et al. (21), female mice were immunized with Ro 52 recombinant protein and ECG was recorded from pups at birth. Nine out of 20 pups from the immunized mothers had conduction system abnormalities but none of the 22 control pups exhibited conduction abnormalities. Four pups had sinus bradycardia, three pups had PR prolongation and two pups had complete AV dissociation. Subsequently, Miranda-Carus et al. (40), also identified a spectrum of AV nodal conduction abnormalities on the ECG tracings obtained from pups born to mice immunized with Ro/La complex but not in the control groups. PR prolongation was observed in 7% of pups born to mothers immunized with 48 kDa La, in 6–7% of pups born to 52α Ro and 52β Ro-immunized mothers, 20% of pups born to 60 kDa Ro-immunized mothers, and 9% of pups born to murine 52 kDa Ro-immunized mothers. Second degree AVB was observed in 2% of offspring from 52α Ro-immunized mothers. Complete AVB was observed in 2–6% of the pups from 52α Ro and 52β Ro-immunized mothers. Suzuki et al. (41) demonstrated that maternal immunization with 60kDa Ro, 48 kDa La or recombinant calreticulin in mice resulted in 9-18% second degree AVB and 0% in controls.

Salomonsson et al. (42) were using a peptide from the Ro 52 spanning amino-acids 200-238 referred to as p200 to immunize female rats. PR prolongation was induced in 19% (10/52) of rat pups. No second- or third degree AVB was reported in this rat model (42). Xiao et al. (39) immunized seven female rabbits with human Ro 52 antigen. Of 152 pups, 31 pups (20.4%) were born dead, 1 pup (0.7%) had second degree AVB, 7 pups (4.6%) had sinus bradycardia, 8 pups (5.3%) had PR prolongation, and 5 pups (3.3%) had both sinus bradycardia and AVB. The remaining 100 pups had normal sinus rhythm and normal PR interval.

Altogether, the results from both the passive and active animal models of CHB provide evidence for a necessary pathogenic role of maternal anti-Ro/La antibodies in the development of CHB.

## Maternal anti-Ro/La antibodies induce sinus bradycardia and AVB in isolated Langendorff-perfused hearts

While the consequence of *chronic* exposure of fetal heart to anti-Ro/La antibodies were examined in animal models as described above, the acute effects of anti-Ro/La antibodies on the isolated completely denervated hearts were tested on Langendorf-perfused human fetal (21) and rat hearts (22). Perfusion of these hearts with IgG containing anti-Ro/La antibody from mothers with CHB children (CHB-IgG) resulted first in sinus bradycardia, followed by first and second degree AVB that subsequently degenerated into complete AVB within 15-20 min of CHB-IgG perfusion. In contrast, perfusion of the heart with normal IgG devoid of anti-Ro/La antibodies from healthy mothers with healthy children did not alter ECG parameters (21, 22). The sinus bradycardia and/or AVB were similarly demonstrated in Langendorff-perfused hearts by others (9, 24, 43, 44) indicating that the electrocardiographic abnormalities seen in CHB can be induced by an *acute* initial effect which may be distinct mechanistically from the *chronic* effect associated with long term exposure of the heart to maternal antibodies.

Collectively, animal models and in vitro experiments provide strong evidence for a pathogenic role of anti-Ro/La antibodies in the development of CHB. The spectrum of conduction abnormalities was varied and included a greater incidence of first degree AVB than that reported in humans. The rate of advanced degree of AVB approximated the 1–5% risk for a mother with anti-Ro/La antibodies to have a child with CHB and suggests that additional factors are required to promote disease expression.

### *Apoptosis-inflammation-fibrosis hypothesis*

Maternal anti-La antibodies were identified on the surface of fetal myocardial fibers (45), and anti-Ro antibodies have been eluted from an affected fetal heart (46). Because the Ro/La antigens are localized inside the cell and maternal antibodies (IgG) cannot cross the sarcolemma of a healthy cardiac myocyte, apoptosis was proposed as a mechanism by which the intracellular Ro/La antigens are translocated to the cell surface to be accessible to maternal antibodies.

### Translocation of intracellular autoantigens to the cell membrane

Substantial experimental evidence has been proposed to account for the translocation of Ro/La antigen to the cell surface, including viral infection, UV light and IFNg treatment (47, 48). The limitation of the above studies is the use of non-cardiac myocytes. Miranda et al. (49), using human fetal ventricular myocytes in culture from 16- to 24-week hearts demonstrated surface expression of Ro/La protein in early and late apoptotic cells induced by staurosporine but not in non-apoptotic cardiac myocytes. It was suggested that experimental induction of apoptosis led to the translocation of Ro/La antigens to cell surface membrane to subsequently be accessible to binding by circulating anti-Ro/La antibodies, triggering the inflammation cascade and eventually fibrosis. Perhaps the strongest evidence supporting the role of apoptosis in provoking cardiac injury is the immunohistochemical analysis of hearts from several fetuses dying with CHB (50, 51). Remarkably, not only is apoptosis detectable, but exaggerated 30-fold in septal tissue of CHB affected hearts compared to age-matched controls. These histologic clues suggest either an unchecked exuberance of apoptosis or a potential defect in clearance.

### Phygocytosis of cardiac myocytes

The above observations point to an exaggerated apoptosis as the initial link between maternal anti-Ro/La antibodies and tissue injury (52, 53). Under physiologic conditions, apoptosis is a distinct form of cell death in which the cell commits to a suicide program, leading to rapid elimination without inflammation (54, 55). Thus, the pathologic cascade from apoptosis to inflammation and fibrosis initiated by autoantibodies in CHB has been an active area of research. Clancy et al. (56) used phase-contrast and confocal microscopy on co-cultured healthy and apoptotic human fetal ventricular myocytes to demonstrate that the healthy cardiac myocytes are capable of phagocytosing apoptotic cardiac myocytes. Experiments were then performed with apoptotic cardiac myocytes preincubated with CHB-IgG. These treated cells were co-cultured with healthy human fetal cardiac myocytes. Pretreatment of apoptotic cells with CHB-IgG inhibits the normal phagocytosis of the apoptotic cells. In contrast, preincubation of apoptotic cells with control IgG had no effect on engulfment. Given the role of the cardiac myocyte in the physiologic removal of apoptotic cells, perturbation of this developmental function by maternal anti-Ro/La antibodies could account for the exaggerated apoptosis observed in the autopsy sections from fetuses dying with CHB. Blocking physiologic apoptotic cell removal by inadvertent antibody binding to apoptotic cells would be expected to direct the pool of IgG-apoptotic cell complexes toward proinflammatory clearance by infiltrating professional scavengers, the macrophages which may lead to fibrosis and permanent heart block. Support for this model are data demonstrating that supernatants from macrophages co-cultured with opsonized apoptotic human fetal cardiac myocytes transdifferentiate cardiac fibroblasts to myofibroblasts, a scarring phenotype (57). Prolonged secretion of cytokines such as TGFβ may contribute to the exuberant scarring seen in CHB (58).

## Mechanism of decreased clearance of apoptotic cells

Given recent evidence implicating the urokinase plasminogen activator receptor (uPAR) as a "don't eat me" signal during efferocytosis, experiments were performed to address whether surface bound anti-Ro antibody inhibits apoptotic cell removal via an effect on the expression/function of the urokinase-type plasminogen activator protease uPA/uPAR system. As assessed by flow cytometry and confocal microscopy, uPAR colocalizes and interacts with Ro 60 on the surface of apoptotic human fetal ventricular cardiac myocytes. Blocking of uPAR enhances phagocytosis of apoptotic cardiac myocytes by healthy cardiac myocytes and reverses the anti-Ro 60 antibody dependent impaired clearance of apoptotic cardiac myocytes. Binding of anti-Ro 60 antibody to apoptotic cardiac myocytes results in increased uPAR expression, as well as enhanced uPA activity. It was suggested that increased uPAR expression and uPA activity induced by anti-Ro antibody 60 binding to the apoptotic fetal cardiac myocyte provides a molecular basis by which these antibodies inhibit efferocytosis, resulting in exacerbated apoptosis and ultimately to scaring of the fetal conduction system and the working myocardium (59). Interestingly, only Ro 60, but not Ro 52 or La 48 interacts with uPAR. This finding further illustrates the complexity of the pathogenesis of CHB as anti-Ro 52 antibody is known to be a major player in the development of CHB (13, 21, 42, 60-62).

## Cascade linking inflammation to fibrosis

Alvarez et al. (63) continued the efforts in investigating downstream crosstalk between inflammatory and fibrosis pathways initiated by binding anti-Ro/La antibodies to their cognitive antigens on the apoptotic cells. Incubation of macrophages with immune complexes (IC) comprised of Ro 60, hY3 ssRNA and anti-Ro 60 antibody induces the Toll-like receptor 7 (TLR7)-dependent generation of supernatants which provoked a fibrosing phenotype in human fetal cardiac fibroblasts. Supernatants from macrophages incubated with IC induced the fibroblast secretion of TGF, which was inhibited by an antagonist of TLR7. Under the same conditions, the induced fibroblast secretion of TGFβ was decreased by inhibitors of the endothelin (ET)-1 receptors ETa or ETb, or by an anti-ET-1 antibody, but not control antibody. Exogenous ET-1 induced a profibrosing phenotype while fibroblasts transfected with either ETa or ETb siRNA were unresponsive to the profibrosing effects of the IC-generated macrophage supernatants. Immunohistochemistry of the hearts from two fetuses dying with CHB revealed the presence of ET-1-producing mononuclear cells in the septal region in areas of calcification and fibrosis. The authors concluded that these cells are a major source of TGFβ, and that ET-1 is one of the key components responsible for the profibrosing effects generated by stimulated macrophages. A novel role of ET-1 in linking TLR7 inflammatory signaling to subsequent fibrosis was suggested.

In summary, the apoptosis hypothesis stipulates that in apoptotic human fetal ventricular myocytes, the intracellular autoantigens to maternal anti-Ro/La antibodies translocate to the cardiac myocyte sarcolemma where they are accessible to circulating anti-Ro/La antibodies which might inadvertently divert normal clearance of apoptotic cardiac myocytes by healthy cardiac myocytes thus increasing uPAR expression and uPA activity, toward clearance by professional macrophages (via FcγR) with the release of the inflammatory and/or fibrosing cytokines. ET-1 is one of the key components responsible for the profibrosing effects generated by stimulated macrophages. Components of the proposed cascade include macrophages (representing the inflammatory component) and fibroblasts (representing the scarring component).

### *Calcium channel hypothesis*

As mentioned above, maternal antibodies can also interact and recognize proteins other than their own autoantigens. Anti-Ro and/or anti-La antibodies were reported to cross-react with laminin B-1 chain, with human cardiac myosin heavy chain (64), calreticulin (41, 65), 5-$HT_4$ serotoninergic receptor (62), and alpha-enolase (66) and Ca channels (39, 67, 68).

### Calcium channel

The formulation of the Ca channel hypothesis is driven by the fact that AV node electrogenesis and action potential propagation to the ventricle is under the control of L-type Ca channel. L-type Ca channel is a protein complex which consists of an α1 pore forming subunit, β, and α2δ accessory subunits. The 1 subunit is composed of four homologous (I-IV) domains containing six transmembrane segments. The Ca channel hypothesis proposes that circulating maternal antibodies directly bind to specific epitopes of the Ca channel pore-forming subunit and inhibit Ca entry to the cell. This inhibition of the Ca channel function by anti-Ro/La antibodies is per se sufficient to cause abnormal electrocardiographic abnormalities similar to those seen in CHB.

### Acute effect of anti-Ro/La antibodies

We have previously established that CHB-IgG stained the sarcolemma of non-premeabilized fetal cardiac myocytes using confocal immunostaining, directly cross-reacts with L-type Ca channel α subunit by Western blot, functionally block the channel activity by the patch-clamp techniques and cause conduction abnormalities including sinus bradycardia and AVB by surface ECG (21, 22, 67-69).

*At the whole heart level,* perfusion of isolated beating hearts with CHB-IgG resulted in the development of AVB as documented by ECG recordings (21,22). Using optical mapping technique which has the unique ability to simultaneously record action potentials from multiple sites, perfusion of CHB-IgG on isolated young rabbit hearts resulted in sinus bradycardia and various degrees of AVB including complete AV dissociation (24). Activation maps revealed marked conduction delay at the sino-atrial junction but only minor changes in overall atrial and ventricular activation patterns. No conduction disturbances were seen in the presence of control IgG from mothers with healthy children (24). The AVB and sinus bradycardia were similarly demonstrated in Langendorff-perfused human fetal (21) and animal hearts (9, 24, 43, 44).

*At the tissue level,* using the standard double microelectrode technique, CHB-IgG but not control IgG superfusion of dissected hearts to specifically isolate and expose the sino-atrial and AV nodal areas resulted in action potential conduction block between the atria and AV node followed by complete inhibition of AV nodal action potential and consequently conduction to the ventricle (21).

*At the single cell level,* CHB-IgG, but not control IgG, selectively inhibits L- and T-type Ca currents in freshly isolated cardiac myocytes from SA node, AV node, and the ventricle (21, 67-69). CHB-IgG had no effect on the transient outward K current ($I_{to}$) (22), the delayed rectifier K current ($I_{Ks}$) (22) and the fast Na current ($I_{Na}$) (22) indicating specificity for Ca channels. Furthermore, CHB-IgG also inhibited 1C L-type Ca channel expressed solely in Xenopus oocytes and in mammalian tsA201 cells to eliminate contamination by other ion channels present in the native cardiac cells (69). Altogether, the data demonstrate that *acute* exposure of cardiac myocytes to CHB-IgG functionally inhibits L-type Ca current resulting in conduction abnormalities including sinus bradycardia, PR prolongation and complete AVB.

## Anti-Ro/La antibodies and the newly discovered α1D L-type Ca channel in the heart

Because AV block has been the hallmark phenotype for CHB, the AV node, rather than the sinoatrial node, has been the main focus of previous publications (2-4), and during clinical diagnosis of CHB (2-4, 6, 70). Sinus bradycardia unrelated to AV block was first reported in animal models of CHB (21-23, 39), which was later confirmed clinically in patients by Brucato et al. (11) and Menon et al. (10). These observations indicate that the spectrum of conduction abnormalities in CHB extends beyond the AV node to also affect the SA node. Up until recently, the ionic and molecular basis of this sinus bradycardia was not known. Several currents have been implicated in the sinus node function (71-74). The major currents involved in the diastolic depolarization and pacemaker activity include the hyperpolarization-activated funny current, If; the delayed rectifier, $I_K$ and the T-type Ca current. While there is a consensus that the α1C L-type Ca current plays only a minimal role in the diastolic depolarization, the α1D isoform of L-type Ca channel emerged as a key player in the sinus node pacemaker. The expression of α1D L-type Ca channel has always been thought to be restricted to the neuroendocrine tissue until the publication of the knockout mouse model for congenital deafness (75). Interestingly, the pups born to mice with this α1D L-type Ca channel knockout also exhibited a cardiac phenotype, sinus bradycardia and AV block reminiscent of CHB (75). This finding triggered further research aimed at first investigating the potential presence and later the function of α1D L-type Ca channel in the heart (75-78). Indeed, studies performed in the mouse revealed that α1D L-type Ca channels is expressed in the heart with (75-77) unique expression in the supraventricular tissue, especially the SA node (75-77) where it was confirmed that α1D L-type Ca current plays a major role in phase 4 diastolic slope which controls sinus rate. Our group was first to characterize the expression of this channel in the human fetal heart (67) and across other species (67,79). We found that this channel has differential developmental expression profile, including a more abundant expression level, and universal localization in fetal and neonatal stages compared to adult heart (79). To test the potential role of α1D L-type Ca channel in the sinus bradycardia observed in animal models of CHB, the effects of CHB-IgG on this channel were investigated. CHB-IgG, but not control IgG, did in fact inhibit α1D L-type Ca current (67). This inhibition may explain the sinus bradycardia seen in CHB given that α1D L-type Ca current contributes to phase 4 diastolic depolarization (10, 11, 21-23, 39).

## Cross-reactivity and binding site of anti-Ro/La antibodies on L-type Ca channel

Because CHB-IgG functionally inhibits both α1C and α1D L-type Ca channels, we sought to determine whether CHB-IgG cross-react and physically bind to the Ca channel. Using immunoprecipitation and Western blot, our previous experiments showed that CHB-IgG recognizes and cross-reacts with both the α1C and α1D pore forming protein subunit (39, 67, 68). Next, we generated GST fusion proteins corresponding to the extracellular loop S5–S6 of each of the four domains that form the pore of the Ca channel α1D subunit and tested the reactivity to CHB sera from mothers with CHB infants. A fraction (14.4%) of these sera reacted with the extracellular loop of S5–S6 of the first but not the second, third or fourth domains of the α1D subunit as demonstrated by both ELISA and Western blots (80).

The consequences of the inhibition of both α1C and α1D L-type Ca channels by CHB-IgG in the fetal ventricular myocytes could also account for the observation that around 20% of children with CHB develop late onset cardiomyopathy despite appropriate pacing (14, 33, 34, 35). Because both

α1C and α1D L-type Ca channels are expressed in the fetal heart ventricles and may participate in the excitation-contraction coupling especially during fetal stages where the myocyte relies heavily on Ca entry from the sarcolemma. Inhibition of these channels may exert negative inotropic effect and/or electro-mechanical uncoupling leading to contractile dysfunction. To date, experimental data supporting this proposition are still lacking.

## Consequence of chronic exposure of Ca channels to anti-Ro/La antibodies

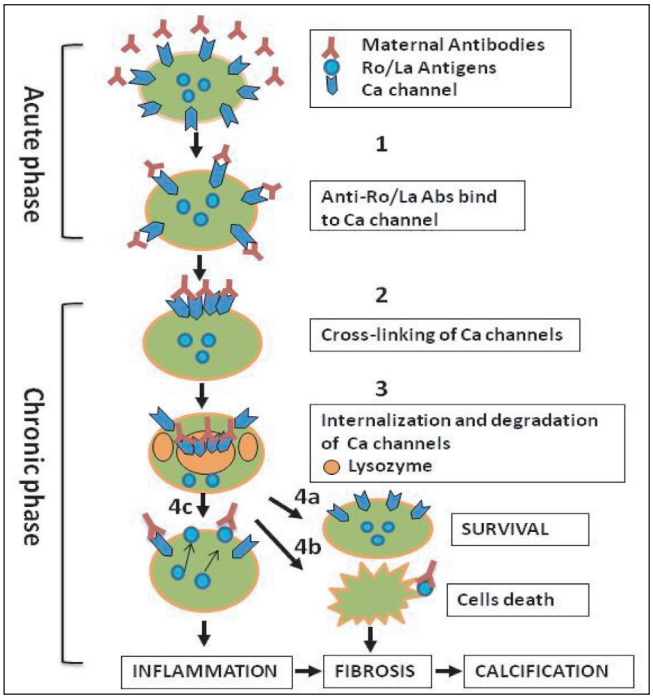
The above electrophysiological and biochemical findings clearly indicate that maternal anti-Ro/La antibodies interact directly with L-type Ca channels to acutely inhibit their function. To investigate the consequence of *chronic* exposure of pups to maternal autoantibodies as it occurs during pregnancy, we used our previously established murine model for CHB (21, 68) where mice were immunized with recombinant Ro 52 protein and ECG measured in pups at birth. The hypothesis tested was that chronic prolonged exposure of pups heart to maternal antibodies during pregnancy could lead to reduced Ca channels density. Patch-clamp techniques were used to record whole cell L-type Ca currents in pups born to immunized mothers and controls. L-type Ca current density in cardiac myocytes from pups in Ro 52-immunized group was reduced by 38.6 ± 4.5% ($P < 0.02$, $n = 17$) (68). Similarly, in the rabbit CHB model immunized with Ro 52 protein (39), a 31 ± 3.4% ($P = 0.02$, $n = 24$) reduction of L-type Ca current density and a 19 ± 1.6% ($P = 0.031$ ) decrease in Ca channel protein as well were observed in the pups born to immunized mothers compared to controls. These observations indicate that during *chronic* exposure *in utero* to maternal antibodies, the reduced L-type Ca current density could be attributed to a decrease in the number of functional Ca channels probably due to internalization and degradation, setting the stage for irreversible AVB at birth where proliferation of cardiac myocytes is minimal.

## Possible reversal of electrocardiographic abnormalities by up-regulation of Ca channels

Now that reduced Ca channel density by exposure to maternal antibodies is demonstrated, we hypothesized that if inhibition of the Ca current is critical in cardiac conduction disorders seen in CHB, then up-regulation of the Ca channels should rescue or reverse the electrocardiographic abnormalities seen in CHB. Transgenic (TG) mice overexpressing the α1C subunit of the L-type Ca channel were used. The cardiac-specific overexpression of the α1C subunit of the L-type Ca channel and the detailed molecular, hemodynamic and electrophysiological characteristics of these TG mice are reported elsewhere (81). Briefly, the α1C transcript was increased by 2.8 fold. Similarly, the density of L-type Ca current was increased by 44% to 52% in cardiac myocytes from the TG mice. This percent increase is ideal because CHB-IgG inhibits Ca current within the same range, 40–60% (21, 22, 67). Therefore, we postulated that immunization of the TG mice overexpressing α1C Ca channel protein should give birth to pups with no or fewer electrocardiographic abnormalities. Indeed, a lesser degree of sinus bradycardia and fewer AV conduction abnormalities were observed in TG pups overexpression α1C Ca channel from immunized mothers compared to non-TG pups (82). These findings demonstrate that up-regulation of L-type Ca channels can be used as potential therapeutic approach to CHB by the development of cardiac specific Ca channel agonists.

All together, the various available data clearly and unambiguously indicate that anti-Ro/La antibodies from mothers with CHB children directly cross-react with both α1C and α1D Ca channel protein on the cell surface membrane and inhibit the channel activ-

ity in both the SA and AV nodes which could account for sinus bradycardia and AV block. The inhibition of L-type Ca channels in the ventricles might account for the ventricular dysfunction seen in CHB. A proposed molecular basis of the Ca channel hypothesis is depicted in the Figure: Two distinct consequences of Ca channel blockade by maternal antibodies can be identified: *Acute* (minutes, Step 1) and *chronic* (weeks-months, Steps 2-4) effects. In the *acute* phase (Step 1), circulating maternal anti-Ro/La antibodies bind to Ca channels at the cell membrane, and inhibit Ca current which *per se* is sufficient to cause sinus bradycardia and AVB as demonstrated on the ECG of ex-vivo hearts perfused with CHB-IgG (21,22). *Chronic* exposure of Ca channels to circulating maternal antibodies, as it is the case during pregnancy, could lead to a few scenarios: Binding of anti-Ro/La antibodies to Ca channels, leads to cross-linking (Step 2) followed by internalization and degradation of the channel protein (Step 3) as demonstrated by a decrease of Ca channel current densities and proteins from the hearts of pup chronically exposed to maternal anti-Ro/La antibodies (68,69). Cross-linking of adjacent ion channels by the two $F_{ab}$




***Proposed Ca Channel Hypothesis for the Development of Autoimmune Associated Congenital Heart Block.***

***Acute*** *(minutes, Step 1) and* ***chronic*** *(weeks to months, Steps 2-4) phases of CHB development. In the* ***acute*** *phase (Step 1), circulating maternal anti-Ro/La antibodies bind to Ca channels at the cell membrane, and inhibit Ca current. In the* ***chronic*** *phase, long term exposure of Ca channels to circulating maternal antibodies during pregnancy, leads to binding of anti-Ro/La antibodies to Ca channels, cross-linking (Step 2) followed by internalization and degradation of the channel protein (Step 3). AV nodal cells with sufficient amount of the Ca channel reserve will survive (Step 4a). Cells with lesser Ca channel density reserves (step 4b) will eventually die leading to fibrosis and ultimately calcification. Alternatively, internalization and degradation of Ca channels affects the dynamic intracellular Ca homeostasis which may lead to apoptosis (Step 4c), the translocation of intracellular Ro/La antigens to cell surface, the binding of the circulating anti-Ro/La antibodies and initiation of the inflammation, fibrosis and calcification.*

arms of IgG increases the rate of normal internalization of the target protein/antibody complex and thereby decreases the channel density on the cell surface as has been reported previously (83). Depending on the Ca channel density of the individual fetal cardiac myocyte, AV nodal cells with sufficient amount of the remaining Ca channel reserve will survive (Step 4a) and continue to conduct the impulse to the ventricle. Cells with lesser Ca channel density reserves (step 4b) will eventually die leading to fibrosis and ultimately calcification. Alternatively, internalization and degradation of Ca channels could also affect the dynamic intracellular Ca homeostasis which may lead to apoptosis (Step 4c), the translocation of intracellular Ro/La antigens to cell surface, the binding of the circulating anti-Ro/La antibodies and initiation of the inflammation and fibrosis. Collectively, ample experimental evidence show that anti-Ro/La antibodies and Ca channels are causally related to the development of CHB but the range and frequency of conduction defects suggest that additional factors must be necessary to explain the full spectrum of CHB.

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*Correspondence address*
Mohamed Boutjdir, PhD, FAHA
Research and Development (151)
VA New York Harbor Healthcare System
800 Poly Place
Brooklyn, NY 11209
USA
mohamed.boutjdir@va.gov