

# Antibody Reactivity to $\alpha$ -Enolase in Mothers of Children with Congenital Heart Block

CAROLINA LLANOS, EDWARD K.L. CHAN, SONGQING LI, GRANT X. ABADAL, PETER IZMIRLY, CAROLINE BYRNE, ROBERT M. CLANCY, and JILL P. BUYON

**ABSTRACT.** *Objective.* To evaluate the frequency of anti- $\alpha$ -enolase antibodies in the sera of mothers whose children have congenital heart block (CHB), given provocative results in which  $\alpha$ -enolase, a membrane protein, was recognized by monoclonal antibodies reactive with the peptide p200 of 52 kDa Ro/SSA in a neonatal rat heart library.

*Methods.* An ELISA using a recombinant  $\alpha$ -enolase protein was developed. Sera from 100 anti-Ro52+ CHB mothers in the Research Registry for Neonatal Lupus, 50 patients with systemic lupus erythematosus (SLE; 7 anti-Ro52+), and 48 healthy controls were tested for anti- $\alpha$ -enolase reactivity.

*Results.* There were no significant differences in the median values obtained from CHB mothers, patients with SLE, or controls at each of the dilutions tested. Only 7 (7%) at 1:100 dilution and 2 (2%) at 1:1000 dilution of 100 CHB sera were 3 standard deviations above the mean value obtained for controls. Preincubation with recombinant Ro52 did not inhibit anti- $\alpha$ -enolase reactivity.

*Conclusion.* The low frequency of anti- $\alpha$ -enolase antibodies in the sera of CHB mothers and the absence of apparent cross-reactivity with Ro52 suggest that antibodies to Ro52 are not likely to mediate CHB via binding to  $\alpha$ -enolase. (J Rheumatol First Release Feb 1 2009; doi:10.3899/jrheum.080860)

*Key Indexing Terms:*

CONGENITAL HEART BLOCK  
 $\alpha$ -ENOLASE

SYSTEMIC LUPUS ERYTHEMATOSUS  
CHILDREN

More than 85% of mothers whose fetuses are identified with conduction abnormalities in structurally normal hearts have circulating autoantibodies to the Ro/SSA-La/SSB complex<sup>1</sup>. This association is independent of whether the mother is asymptomatic or has a defined rheumatologic condition such as systemic lupus erythematosus (SLE) or Sjögren's syndrome (SS). In addition to the well characterized 60 kDa Ro/SSA and 48 kDa La/SSB ribonucleoproteins, another target of the immune response in these mothers is 52 kDa Ro/SSA (Ro52)<sup>2-4</sup>. The fetal heart appears to be specifically vulnerable to these antibodies, because there is only a single

case in the literature in which the maternal heart had a conduction abnormality<sup>5</sup>.

One of the greatest challenges in defining the pathogenic role of anti-Ro/SSA-La/SSB antibodies is the intracellular location of the cognate antigens. It has been reported that a subset of anti-Ro52 antibodies, which recognize amino acids 200–239 (p200) of the Ro52 protein, bind neonatal rat cardiocytes and cause congenital heart block (CHB) by disturbing calcium homeostasis<sup>6</sup>. Screening a neonatal rat heart expression library with monoclonal anti-p200 Ro52 antibodies identified  $\alpha$ -enolase as a potentially cross-reactive antigen<sup>7</sup>. Enolase is a key glycolytic dimeric enzyme formed from 3 subunits:  $\alpha$ ,  $\beta$ , and  $\gamma$ , all encoded by 3 different genes<sup>8,9</sup>.  $\alpha$ -enolase has been recently observed on the surface of several hematopoietic, epithelial, and endothelial cells. It is a multifunctional protein that may participate in fibrinolytic processes as a plasminogen receptor<sup>8</sup>. Potentially pertinent to CHB,  $\alpha$ -enolase (composed by 2  $\alpha$  subunits) is the isoform predominantly expressed in the rat cardiocyte during fetal development. Although the embryonic  $\alpha$ -enolase form remains present during postnatal development, a transition toward  $\alpha$ -enolase with an increase in the expression of  $\beta$  subunits occurs<sup>9</sup>. Also, decrease of  $\alpha$ -enolase activity has been observed in rodents at the adult stage<sup>10</sup>. Reactivity to  $\alpha$ -enolase has been detected in patients with SLE, particularly those with renal disease<sup>11</sup>, and in patients with other autoimmune disorders<sup>12,13</sup>.

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From the Department of Medicine, Division of Rheumatology, New York University School of Medicine, New York, New York; and the Department of Oral Biology, University of Florida, Gainesville, Florida, USA.

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C. Llanos, MD, Department of Medicine, Division of Rheumatology, New York University School of Medicine; E.K.L. Chan, PhD; S. Li, BMed; G.X. Abadal, Department of Oral Biology, University of Florida; P. Izmirly, MD; C. Byrne, MD; R.M. Clancy, PhD; J.P. Buyon, MD, Department of Medicine, Division of Rheumatology, New York University School of Medicine.

Address reprint requests to Dr. C. Llanos, Th-407, 560 First Avenue, New York University School of Medicine, New York, NY 10016, USA.

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Accordingly, the goal of our study was to evaluate the frequency of anti- $\alpha$ -enolase reactivity in the sera of mothers whose children have autoimmune CHB. An ELISA using recombinant human  $\alpha$ -enolase was developed for this purpose. One hundred anti-Ro52-positive mothers of children with CHB from the Research Registry for Neonatal Lupus (RRNL) were evaluated.

## MATERIALS AND METHODS

**Patients and controls.** Our study included mothers enrolled in the RRNL, established in September 1994 by the National Institute for Arthritis and Musculoskeletal and Skin Diseases<sup>14</sup>, who had at least 1 child with a cardiac conduction abnormality (94 = third degree, 9 = second degree, 4 = first degree). All mothers had been previously tested and were positive for anti-Ro52 antibodies. Since the RRNL does not require that antibody testing be done during an affected pregnancy, sera from the RRNL mothers were not necessarily available from the time of an affected pregnancy. In addition to these 100 sera, sera from 50 patients with SLE were also tested. Sera from 48 healthy controls that did not contain reactivity to any component of the Ro/SSA-La/SSB complex were evaluated to establish normal ranges for the ELISA (see below).

**Recombinant proteins and synthetic peptides.** Recombinant human Ro52 and  $\alpha$ -enolase autoantigens were generated using the expression plasmid pET28 system in *Escherichia coli* BL21 (DE3; Novagen, Madison, WI, USA), for expression of the 6XHis tagged proteins and subsequent Ni<sup>2+</sup> resin purification, as described<sup>15</sup>.

**Construction of 6XHis-enolase plasmid.** Full-length human  $\alpha$ -enolase cDNA was polymerase chain reaction (PCR) amplified from the Image clone 6063437 using PfuUltra<sup>®</sup> High-Fidelity DNA polymerase (Stratagene, La Jolla, CA, USA) and primer attB1 primer enolase1 5'-AAA AAG CAG GCT CGA TGT CCA TTC TAA AGA TCC ATG CCA GGG AGA-3' (start codon of enolase underlined) and attB2 primer enolase2 5'-AGA AAG CTG GGT ACT TGG CCA AGG GGT TTC TG-3'. The 1.3 kb PCR product was purified and then subcloned into pDONR207 vector (Invitrogen, Carlsbad, CA, USA) using Gateway<sup>®</sup> BP clonase<sup>®</sup> enzyme mix (Invitrogen). For the 6XHis-enolase expression construct, a recombination reaction was performed between pDONR207-enolase and pDEST17 Gateway<sup>®</sup> destination vector using the LR Clonase<sup>®</sup> II (Invitrogen) following the manufacturer's protocol. All constructs were sequenced to confirm that they were in-frame and consistent with reference sequences.

**ELISA for detection of antibodies to Ro52, p200, and  $\alpha$ -enolase antigens.** A standard protocol for ELISA using recombinant proteins was developed. Briefly, nickel column affinity-purified recombinant proteins were diluted in phosphate buffered saline (PBS) to a final concentration of 10  $\mu$ g/ml and 100  $\mu$ l were coated on microton microtiter plates (Greiner bio-one, Monroe, NC, USA). Human sera were diluted 1:1000 and 1:100 in PBS-0.01% gelatin. Antigen coated plates were blocked for 1 h using PBS-0.01% gelatin. After washing 3 times with PBS-Tween, diluted sera were added and incubated for 1 h at room temperature. After 3 washes using PBS-Tween, alkaline phosphatase-conjugated goat anti-human IgG (Sigma Aldrich, St. Louis, MO, USA) was diluted 1:2000 in PBS-0.01% gelatin. After washing 3 times, the substrate p-nitrophenyl phosphate (Sigma Aldrich) was added as the detection reagent and incubated at 37°C. Each sample was analyzed in duplicate, and the average optical density (OD) was determined at 405 nm when the OD of a rabbit anti- $\alpha$ -enolase antibody (Abcam, Cambridge, MA, USA) for  $\alpha$ -enolase was 1.0. The cutoff value designating a positive reaction was 3 standard deviations (SD) above the mean OD obtained for sera from the 48 controls. Cutoffs for positivity for  $\alpha$ -enolase (mean + 3 SD) were 0.289 OD and 0.126 OD for 1:100 and 1:1000 dilutions, respectively. For blocking experiments, diluted serum samples were incubated for 2 h at room temperature with each antigen at a concentration of 50  $\mu$ g/ml and then added to the plate and incubated according to the ELISA protocol described.

In our study all samples were evaluated for both anti-Ro52 and anti- $\alpha$ -enolase on the same day to assure viability of the stored samples. These same samples had been previously evaluated for reactivity against the p200 peptide of Ro52 as reported<sup>16</sup>. This latter assay was not repeated.

**Statistical analysis.** Calculations were done using GraphPad InStat software (GraphPad Software, San Diego, CA, USA). The Mann-Whitney nonparametric test was used to compare median values of  $\alpha$ -enolase reactivities among the 3 groups. A paired Student's t test was used to compare reactivity for  $\alpha$ -enolase between inhibited and uninhibited samples in the blocking experiments using  $\alpha$ -enolase.

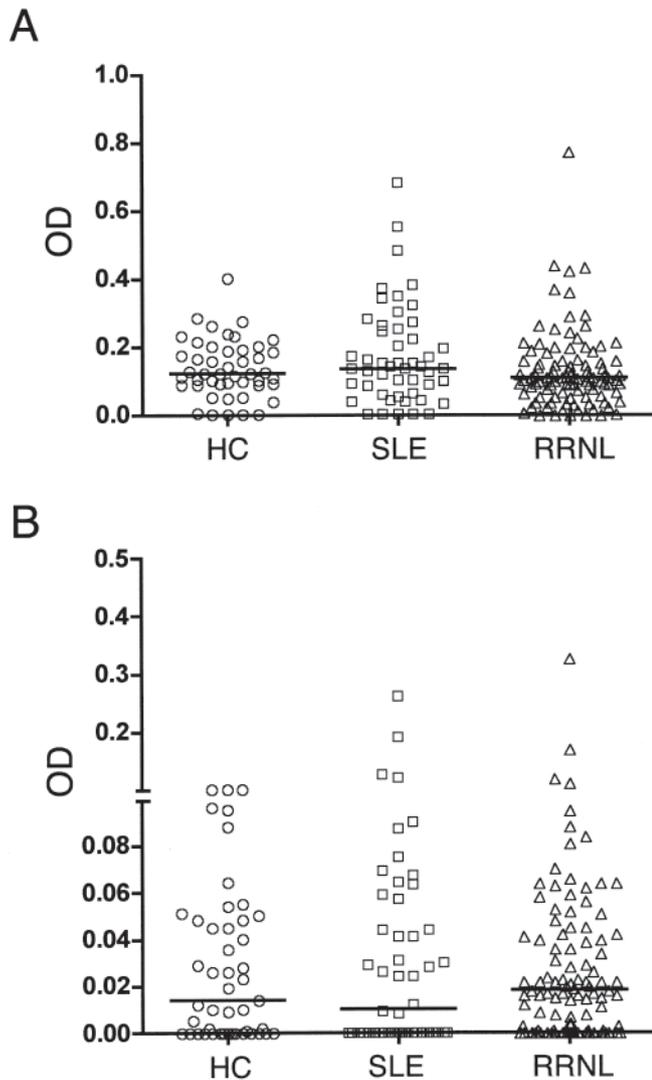
## RESULTS

**Reactivity to  $\alpha$ -enolase in sera from mothers of children with CHB.** One hundred sera from previously known anti-Ro52-positive mothers enrolled in the RRNL with at least 1 child with CHB were tested for reactivity against recombinant human  $\alpha$ -enolase. The majority of the mothers included in the study were Caucasian (85%) and were asymptomatic or characterized as having an undifferentiated autoimmune syndrome (58%) or diagnosed with either SLE (12%) or SS (22%), or both diseases (8%). The SLE cohort included a majority of women (94%), predominantly Caucasian (46%), and none had children with CHB.

In parallel with the anti- $\alpha$ -enolase ELISA, each serum was also tested for confirmation of anti-Ro52 reactivity. As previously identified, all CHB sera contained antibodies to Ro52, as did 7 of the SLE sera and none of the sera from the controls. The ELISA results for reactivity with  $\alpha$ -enolase are summarized in Figure 1. The median OD was 0.108 for the 100 CHB sera, 0.137 for the 50 patients with SLE, and 0.121 for the 48 controls for the 1:100 dilution [p = not significant (NS) for all comparisons, Mann-Whitney test]. In addition, each sample was also tested using a 1:1000 dilution. Similarly, there were no differences between the groups. Specifically, the median OD for the CHB mothers was 0.018, 0.010 for the patients with SLE, and 0.014 for controls for the 1:1000 dilution. (p = NS for all comparisons, Mann-Whitney test.)

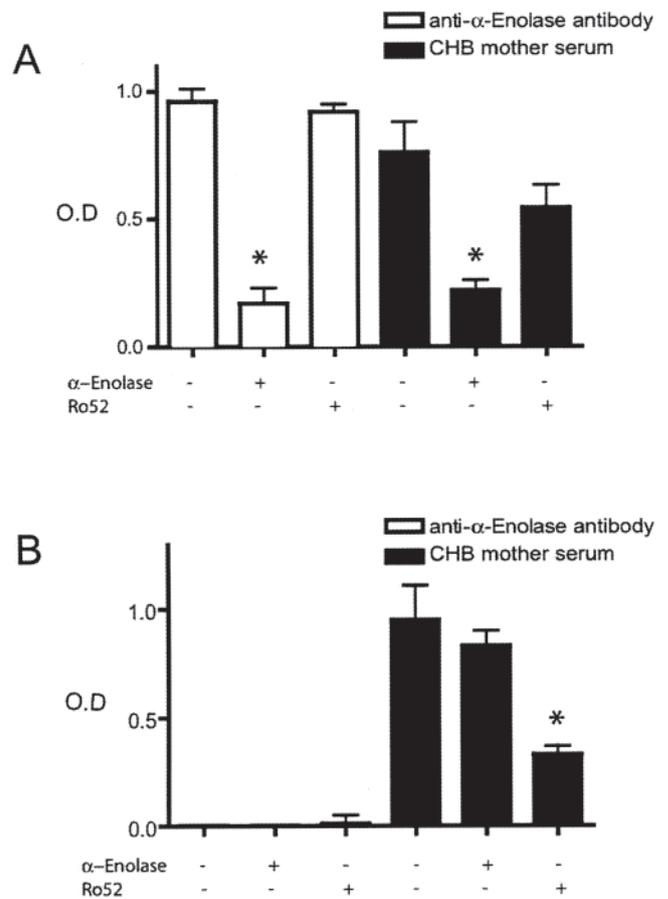
Using a cutoff of 3 SD above the mean for the control group, only 7 (7%) CHB sera were positive using a 1:100 dilution and 2 (2%) at 1:1000 dilution. Of the 7 CHB mothers who were anti- $\alpha$ -enolase-positive, 2 had SLE, 2 had SS, and 3 either were asymptomatic or were classified as having an undifferentiated autoimmune syndrome. Of the 2 patients who were also positive at the 1:100 dilution, one was asymptomatic and the other one was diagnosed with an undifferentiated autoimmune syndrome. In the patients with SLE (non-CHB), 8 (16%) sera at 1:100 dilution and 3 (6%) at 1:1000 dilution were reactive with  $\alpha$ -enolase.

Given the initial suggestion of cross-reactivity between  $\alpha$ -enolase and the p200 region of Ro52<sup>7</sup>, we compared our ELISA results with those previously tested for the p200 region<sup>15</sup>. In total, 4 of 34 CHB sera previously tested<sup>15</sup> that were reactive with p200 also demonstrated anti- $\alpha$ -enolase reactivity, including the 2 sera that were anti- $\alpha$ -enolase positive at 1:1000 dilution. One of the CHB sera, which contained reactivity to  $\alpha$ -enolase at 1:100, had not been reactive with p200.



**Figure 1.** Anti- $\alpha$ -enolase reactivity in mothers of children with CHB (RRNL), patients with SLE, and healthy controls (HC). Y axis shows optical densities (OD) of sera at 1:100 and 1:1000 dilutions. All the RRNL sera tested were known to contain anti-Ro52 reactivity and were tested in duplicate for anti- $\alpha$ -enolase and anti-Ro52 reactivity in the same plate. A. Results for each group with their respective medians using sera diluted 1:100. No significant differences were found between CHB mothers and patients with SLE, or between CHB mothers and controls ( $p = 0.07$ ;  $p = 0.26$ , respectively) or between patients with SLE and controls ( $p = 0.43$ ) at this dilution. B. Results for each group for samples diluted 1:1000. None of the comparisons between groups were significant (CHB vs SLE,  $p = 0.46$ ; CHB vs controls,  $p = 0.72$ ; and SLE vs controls,  $p = 0.64$ ). RRNL: Research Registry for Neonatal Lupus.

The specificity of the anti- $\alpha$ -enolase response in the CHB sera was confirmed by blocking with  $\alpha$ -enolase (Figure 2A). Preincubation with  $\alpha$ -enolase inhibited binding of the positive control anti- $\alpha$ -enolase antibody to its cognate antigen by 82.2%, which corresponded to a decrease in the mean  $\alpha$  SD OD from  $0.99 \pm 0.01$  to  $0.17 \pm 0.04$  ( $p = 0.002$ , paired Student's *t* test). Similarly, preincubation of the CHB serum containing anti- $\alpha$ -enolase reactivity with  $\alpha$ -enolase inhibited the respective binding to  $\alpha$ -enolase by 71.1% ( $p = 0.025$ ). In



**Figure 2.** Inhibition ELISA showed no significant cross-reactivity between anti- $\alpha$ -enolase and anti-Ro52 in an anti- $\alpha$ -enolase-positive CHB serum. A. Samples were preincubated with either recombinant human  $\alpha$ -enolase or Ro52 or absent recombinant antigen prior to addition to the  $\alpha$ -enolase ELISA plate. Preincubation with  $\alpha$ -enolase inhibited binding of the  $\alpha$ -enolase antibody or the CHB serum containing anti- $\alpha$ -enolase to its cognate antigen. Preincubation with recombinant Ro52 had no effect on anti- $\alpha$ -enolase reactivity. B. Format similar to A, using a plate coated with Ro52. There was no binding by anti- $\alpha$ -enolase to Ro52 in any of the 3 conditions tested. Preincubation with Ro52 decreased binding to the cognate antigen by 70% (mean  $\pm$  SD of the CHB serum for Ro52 were  $0.96 \pm 0.16$  and  $0.29 \pm 0.05$  for the uninhibited and inhibited conditions, respectively;  $p = 0.034$ ). Incubation of the serum with  $\alpha$ -enolase had no effect on the Ro52 reactivity.

contrast, preincubation of these CHB sera with recombinant Ro52 had no effect on anti- $\alpha$ -enolase reactivity with  $\alpha$ -enolase ( $p = 0.19$ ). The anti- $\alpha$ -enolase polyclonal antibody did not bind Ro52. Moreover, preincubation of a serum containing anti-Ro52 antibodies with  $\alpha$ -enolase had no effect on binding to Ro52 ( $p = 0.192$ ; Figure 2B).

## DISCUSSION

The strong association of anti-Ro52 antibodies with CHB and the potential cross-reactivity of this response with  $\alpha$ -enolase<sup>7</sup> prompted the search for this novel response in a large cohort of mothers with affected infants. Although all of these mothers had detectable antibodies to Ro52, reactivity to  $\alpha$ -enolase

was infrequent. Cross-blocking experiments did not support a shared epitope between these 2 antigens, thus suggesting separate antibody specificities in those select patients with anti- $\alpha$ -enolase antibodies.

Whether anti-Ro52 antibodies contribute to the initiation of cardiac injury by binding to surface  $\alpha$ -enolase remains to be evaluated in a cell-based system. Given the intracellular location of the Ro/SSA complex, cross-reactivity between anti-Ro antibodies and a surface molecule would account for a mechanism by which anti-Ro antibodies might be pathogenic. Precedent for this consideration is the observation that cardiomyocytes from pups with conducting abnormalities born to Ro52-immunized rabbits have a lower L-type Ca channel density than controls and protein levels are downregulated<sup>17</sup>. In addition, it has been reported that anti-Ro52 antibodies recognize the serotonergic-5-HT<sub>4</sub> receptor and induce heart block antagonizing serotonin-induced L-type Ca channel activation on the human heart<sup>18</sup>. Moreover, pups from mice immunized with peptides from this receptor showed bradycardia and first- and second-degree heart block<sup>19</sup>. However, as in the case of anti- $\alpha$ -enolase antibodies, anti-5-HT<sub>4</sub> antibodies are also uncommon in the sera of mothers whose children have CHB<sup>20</sup>.

Given the reported increase of apoptosis in the hearts of CHB fetuses<sup>21</sup> and substantial experimental evidence suggesting that many autoantigens are susceptible to modifications during cell death<sup>22</sup>, an alternative explanation for the recognition of Ro52 by circulating maternal autoantibodies may be the exposure of this intracellular antigen on the surface of fetal cardiocytes during apoptosis<sup>23,24</sup>. Once opsonized by maternal anti-Ro/SSA antibodies, apoptotic cells might be phagocytosed by macrophages and stimulate tumor necrosis factor- $\alpha$  and transforming growth factor- $\beta$  production, leading to inflammation and fibrosis in the fetal heart<sup>25,26</sup>. The hypothesis that autoantigens contained in apoptotic blebs become immunogenic has been evaluated before. Available data demonstrate that both necrotic and apoptotic cells release material that induces  $\alpha$ -interferon production in plasmacytoid dendritic cells (pDC). In both cases RNA was necessary for this response<sup>27</sup>. hYRNAs associated with Ro when mixed with lipofection are capable of triggering  $\alpha$ -interferon production from monocytes<sup>28</sup>. Interestingly, there is evidence supporting the role of uridine-rich sequences from hYRNA in activating Toll-like receptor 7 and 8 pathways and promoting  $\alpha$ -interferon release from pDC<sup>29</sup>. Using transfection as the delivery mechanism, it has been shown that small single-stranded RNA molecules generate TNF- $\alpha$  induction in human macrophages and human monocytic cell lines<sup>30</sup>.

CHB is a disease with a high mortality and morbidity. While it is a valuable initiative to seek novel markers and more specific antibodies in mothers at risk of having children with this disease, anti- $\alpha$ -enolase antibodies are likely to be contributory only in a rare subset of mothers.

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