

# Transforming Growth Factor $\beta$ 1 in the Pathogenesis of Autoimmune Congenital Complete Heart Block

## Lesson From Twins and Triplets Discordant for the Disease

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**Objective.** Clinical evidence and experimental evidence suggest that anti-Ro/La autoantibodies are necessary but not sufficient for the development of congenital complete heart block (CCHB). Maternal, fetal, and environmental factors may also contribute to heart damage in CCHB. The aim of our study was to investigate polymorphisms of transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) genes in twins and triplets discordant for CCHB whose mothers are anti-Ro/La positive.

**Methods.** We studied 2 families in which 1 of the mothers gave birth to triplets and the other gave birth to twins. Only 1 child in each family was affected by CCHB, although 1 of the triplets had incomplete heart block. We analyzed TNF $\alpha$  and TGF $\beta$ 1 polymorphisms in the 2 babies with CCHB and their siblings. TNF $\alpha$  polymorphisms at the promoter region and TGF $\beta$ 1 polymorphisms at codons 10 and 25 were determined using polymerase chain reaction–restriction fragment length polymorphism analysis. In addition, the production of TGF $\beta$ 1 and TNF $\alpha$  by resting or mitogen-stimulated peripheral blood mononuclear cells in cell

culture supernatants was evaluated by enzyme-linked immunosorbent assay.

**Results.** The profibrotic TGF $\beta$ 1 genotype was detected in the twin with CCHB but not in the healthy twin, while all of the triplets displayed the same TGF $\beta$ 1 genotype at codon 10. Peripheral blood mononuclear cells from the children with CCHB displayed higher spontaneous and mitogen-stimulated TGF $\beta$ 1 secretion than was observed in their siblings. No differences regarding TNF $\alpha$  polymorphisms and secretion of TNF $\alpha$  were observed.

**Conclusion.** The results of this study suggest that, besides anti-Ro/La autoantibodies, a fetal profibrotic response might contribute to the development of CCHB, but additional pathogenic mechanism(s) are also likely to play a role.

Neonatal lupus is a model of passively acquired autoimmune disease; its major clinical manifestation is congenital complete heart block (CCHB) (1,2). In almost all cases, CCHB in the absence of congenital cardiac malformations is linked to the presence of autoantibodies against the nuclear antigens SSA/Ro and SSB/La. Results of numerous experimental studies support the evidence that the clinical manifestations of neonatal lupus not only are associated with but also are causally related to the presence of these autoantibodies. However, it is not known why only a minority of mothers with anti-Ro and anti-La antibodies give birth to an affected child (3) nor why heart block almost always affects only the fetus and not the mother. Moreover, the disease discordance in twins, both monozygotic and dizygotic, is intriguing (4). It has therefore been suggested that the presence of anti-Ro/La antibodies represents a condition that is necessary but not sufficient for

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disease development. It has been hypothesized that maternal, fetal, and environmental factors are also contributory (5).

Cytokines that lead from inflammation to fibrosis, such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and transforming growth factor  $\beta$  (TGF $\beta$ ), have been suggested to be involved in the pathogenesis of CCHB. Polymorphisms of these cytokines were evaluated in a recent large study (6), and a significantly increased frequency of the -308A (high producer) allele of TNF $\alpha$  was observed in all of the patients with neonatal lupus, independently of the clinical manifestations. In contrast, the frequency of the TGF $\beta$  polymorphism Leu-10 (which is associated with increased fibrosis) was significantly higher in children with CCHB than in unaffected offspring and controls. This finding, together with the demonstration that macrophages secrete high levels of TGF $\beta$  when cocultured with apoptotic human fetal cardiocytes sensitized by anti-Ro antibodies, suggests that both proinflammatory and profibrotic cytokine environments in the fetus might be factors favoring the occurrence of CCHB (7). In the current study, we tested this hypothesis in twins and triplets discordant for CCHB.

## PATIENTS AND METHODS

**Patients.** We recently encountered 2 families in which 1 mother gave birth to triplets, and the other mother gave birth to twins. Of the triplets, 1 had CCHB, the second had minor-degree (i.e., incomplete) heart block, and the third child was healthy. In the other family, 1 of the twins had CCHB, and the other twin was healthy (8,9). Informed consent was obtained from the mothers, and the protocol was approved by the ethics committee of the treating center (Clinica Pediatrica, Milan, Italy).

**Polymorphisms and in vitro production of cytokines by peripheral blood mononuclear cells (PBMCs).** TNF $\alpha$  polymorphisms at the promoter region and TGF $\beta$ 1 polymorphisms at codons 10 and 25 were determined using polymerase chain reaction–restriction fragment length polymorphism analysis, as previously described (6). The DNA amplification primers were as follows: for TNF $\alpha$ , 5'-AGGCAATAGGTTTGAGGGT-CAT-3' (forward) and 5'-ACACTCCCCATCCTCCCTG-CTC-3' (reverse); for TGF $\beta$ , 5'-TTCCCTCGAGGCCCTCCTA-3' (forward) and 5'-GCCGCAGCTTGGACAGGATC-3' (reverse). Amplified DNA (4  $\mu$ l) was digested in a 10- $\mu$ l reaction mixture using buffer and temperatures recommended by the manufacturer. The enzymes used were as follows: for TNF $\alpha$ , *Nco* I; for codon 10 of TGF $\beta$ , *Msp* A1I; and for codon 25 of TGF $\beta$ , *Fse* I (all from New England Biolabs, Ipswich, MA). The analysis was performed using DNA from the triplets, the twins, and a healthy control baby.

Isolated PBMCs were cultured for 48 hours in a humidified atmosphere with 5% CO<sub>2</sub>, in medium alone or in the presence of phorbol myristate acetate (5 ng/ml) plus ionomycin (500 ng/ml) (Sigma-Aldrich, St. Louis, MO). TNF $\alpha$

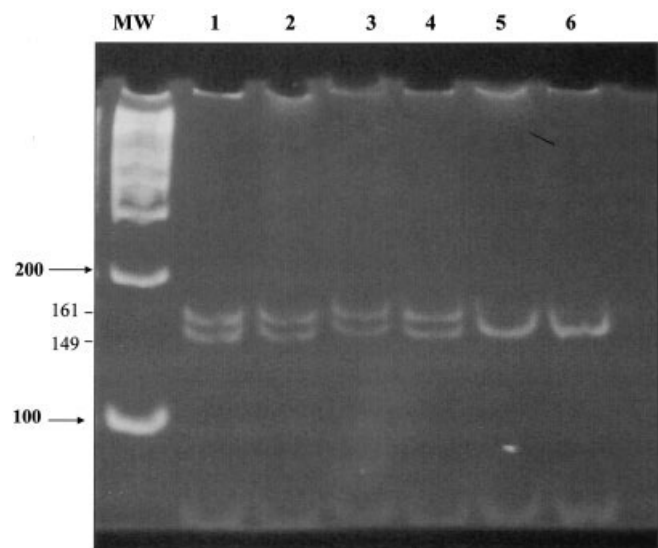
and TGF $\beta$ 1 levels in cell culture supernatants were determined by enzyme-linked immunosorbent assay, according to the manufacturers' instructions (BioSource, Nivelles, Belgium, and R&D Systems, Minneapolis, MN, respectively).

## RESULTS

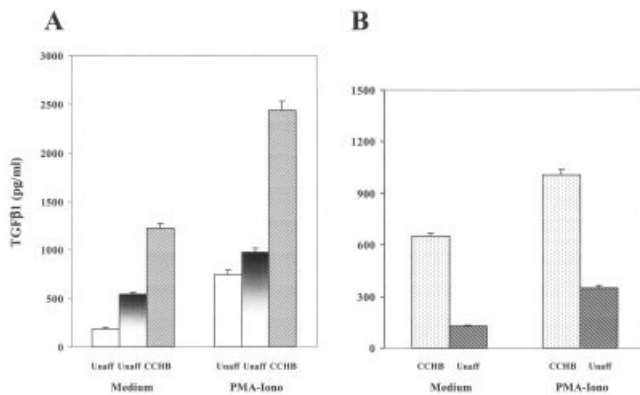
Both mothers were positive for anti-52-kd Ro, anti-60-kd Ro, and anti-La. The mother of the triplets was asymptomatic, and the mother of the twins had subacute cutaneous lupus erythematosus. Despite the presence of these pathogenic autoantibodies, only 1 of the triplets and 1 of the twins had CCHB.

In the family with twins, the TGF $\beta$ 1 profibrotic genotype was identified in the child with CCHB but not in the healthy sibling (Figure 1). In the family with triplets, all 3 siblings showed the same TGF $\beta$ 1 genotype at codon 10. No differences in the TGF $\beta$ 1 polymorphism at codon 25 were observed between patients with CCHB and their siblings (data not shown).

The 2 children with CCHB displayed both spontaneous and mitogen-stimulated secretion of TGF $\beta$ 1, the level of which was higher than that observed in their siblings. Interestingly, the level of TGF $\beta$ 1 secretion was lowest in the child without the profibrotic TGF $\beta$ 1 polymorphism (Figure 2). All of the children displayed the presence of the -308A TNF $\alpha$  allele, and no differences



**Figure 1.** Transforming growth factor  $\beta$ 1 Leu-10 polymorphisms in triplets (lanes 1, 2, and 3), twins (lanes 4 and 5 [lane 4 represents the twin with congenital complete heart block]), and a healthy baby (lane 6). The profibrotic genotype is associated with a double DNA line.



**Figure 2.** Transforming growth factor  $\beta 1$  (TGF $\beta 1$ ) secretion by resting or activated peripheral blood mononuclear cells from patients with congenital complete heart block (CCHB) and their siblings. **A**, Cytokine levels in culture supernatants from triplets. The middle column represents the triplet with incomplete heart block. **B**, Cytokine levels in culture supernatants from twins. Values are the mean + 3 SD of triplicate determinations. Unaff = unaffected; PMA-Iono = phorbol myristate acetate–ionomycin.

in TNF $\alpha$  secretion by PBMCs between CCHB patients and their siblings were observed (data not shown).

The age of the children at the moment of blood drawing was 30 months for the triplets and 20 months for the twins. We chose not to perform the cytokine production experiments during the first year of life, because these babies had been exposed in utero to maternal dexamethasone treatment, which was given to the mothers because of the presence of CCHB and could have temporarily influenced the immune systems of the newborns.

## DISCUSSION

Besides anti-Ro/La autoantibodies, additional maternal, fetal, and environmental factors have all been implicated as contributing to the development of CCHB (5). For example, a diverse production of cytokines has been suggested to play a possible role in explaining the differences in disease expression. In this regard, histologic evaluation of hearts affected by CCHB raised the possibility of a pathogenic role of TNF $\alpha$ , as suggested by the increased presence of this cytokine in tissue lesions (6). It has been hypothesized that a higher prevalence of TNF $\alpha$  polymorphisms associated with increased cytokine production could have a role. However, Clancy et al (6) did observe a higher frequency of the –308A (high producer) allele of TNF $\alpha$  in 88 children and 74 mothers from the Research Registry for Neonatal Lupus, but without any association with CCHB. Our data for both

triplets and twins did confirm the lack of a correlation between TNF $\alpha$  polymorphisms and CCHB; moreover, levels of this cytokine in cell supernatants were not significantly different between patients with CCHB and their siblings.

The contribution of TGF $\beta$  to CCHB has also been recently reviewed (10). TGF $\beta$  is a multifunctional cytokine that modulates the proliferation and differentiation of many cell types, and it is considered to play a critical role in fibrotic conditions (11). The human gene encoding TGF $\beta$  is located on chromosome 19q13 and is highly polymorphic. Two of its 5 known polymorphisms, i.e., those at positions 869 and 915, which change codon 10 (T or C, Leu $\rightarrow$ Pro) and codon 25 (G or C, Arg $\rightarrow$ Pro), are associated with interindividual variation in TGF $\beta$  production. For both polymorphisms, the alleles encoding proline are generally associated with lower TGF $\beta$  synthesis in vitro and in vivo. The genetic and histologic study by Clancy et al convincingly linked TGF $\beta$  to the pathogenesis of CCHB (6). In fact, the contribution of TGF $\beta$  polymorphism Leu-10 was significantly higher in children with CCHB than in unaffected offspring and controls, while there were no significant differences between controls and other groups of patients with neonatal lupus (i.e., those without CCHB). For TGF $\beta$  polymorphism Arg-25, there were no significant differences between neonatal lupus groups and controls.

In the present study, we corroborated these findings in the family with twins but not in the family with triplets. Interestingly, PBMCs from the 2 patients with CCHB displaying the profibrotic genotype secreted levels of TGF $\beta 1$  in vitro that were higher than those of their siblings, in either resting or mitogen-stimulated conditions. Although as a whole these findings suggest that TGF $\beta 1$  secretion might play a role in the pathogenesis of CCHB, this is not sufficient to explain the differences in disease expression.

Additional fetal factors have been reported. For example, the persistent presence of maternal cells in the fetal heart (microchimerism) has been suggested to be involved in the pathogenesis of CCHB by eliciting a graft-versus-host reaction or an allogenic response that may induce local inflammatory damage of the atrioventricular cardiac conduction tissue (9,12). Polymorphisms of the human Fc $\gamma$  receptor (Fc $\gamma$ R) have also been proposed as representing an additional fetal factor that is potentially responsible for the development of CCHB (13). Actually, Fc $\gamma$ R polymorphisms are known to be associated with different affinity for the Fc domain of IgG, affecting in vivo immune complex clearance and

modulating antibody ligation to Fc $\gamma$  receptors and their subsequent downstream effects on the cells (14). We previously analyzed Fc $\gamma$ RIIA and Fc $\gamma$ RIIIB genotyping in these triplets (15), and the baby with CCHB showed a His/His-131 genotype for Fc $\gamma$ RIIA, while the other 2 had a heterozygous Arg/His-131 genotype. Also, the Fc $\gamma$ RIIIB NA1/NA2 genotype was observed in the affected child but not in the siblings (NA2/NA2 genotype). The genotypes in the affected baby, in particular His/His-131, are known to display higher affinity for IgG and could have contributed to a stronger activation of phagocytes favoring the local inflammatory process in the atrioventricular conduction tissue.

In conclusion, our findings in a spontaneous *in vivo* model confirm that a profibrotic response linked to increased TGF $\beta$ 1 secretion might be involved in the pathogenesis of CCHB. However, this does not seem to be the unique fetal factor, and additional mechanisms are apparently contributory. It is worth noting that we previously demonstrated a correlation between the presence of microchimerism and CCHB only in the triplet family (9), while in the present study we observed a correlation with TGF $\beta$ 1 polymorphisms in the twin family. Together with our data on Fc $\gamma$ R genotyping, these findings suggest not only that a mosaic of maternal, fetal, and possibly environmental factors might be involved in inducing CCHB, but also that the combination of such factors might be variable among different patients.

## REFERENCES

- Buyon JP, Clancy RM. Neonatal lupus syndromes. *Curr Opin Rheumatol* 2003;15:535–41.
- Buyon JP, Rupel A, Clancy RM. Neonatal lupus syndromes. *Lupus* 2004;13:705–12.
- 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.
- Solomon DG, Rupel A, Buyon JP. Birth order, gender and recurrence rate in autoantibody-associated congenital heart block: implications for pathogenesis and family counseling. *Lupus* 2003;12:646–7.
- Buyon JP, Kim MY, Copel JA, Friedman DM. Anti-Ro/SSA antibodies and congenital heart block: necessary but not sufficient [editorial]. *Arthritis Rheum* 2001;44:1723–7.
- Clancy RM, Backer CB, Yin X, Kapur RP, Molad Y, Buyon JP. Cytokine polymorphisms and histologic expression in autopsy studies: contribution of TNF- $\alpha$  and TGF- $\beta$ 1 to the pathogenesis of autoimmune-associated congenital heart block. *J Immunol* 2003;171:3253–61.
- Clancy RM, Buyon JP. Clearance of apoptotic cells: TGF- $\beta$  in the balance between inflammation and fibrosis. *J Leukoc Biol* 2003;74:959–60.
- Fesslova V, Mannarino S, Salice P, Boschetto C, Trespidi L, Acaia B, et al. Neonatal lupus: fetal myocarditis progressing to atrioventricular block in triplets. *Lupus* 2003;12:775–8.
- Stevens AM, Hermes HM, Lambert NC, Nelson JL, Meroni PL, Cimaz R. Maternal and sibling microchimerism in twins and triplets discordant for neonatal lupus syndrome congenital heart block. *Rheumatology (Oxford)* 2005;44:187–91.
- Buyon JP, Clancy RM. Autoantibody-associated congenital heart block: TGF- $\beta$  and the road to scar [review]. *Autoimmun Rev* 2005;4:1–7.
- Awad MR, El-Gamel A, Hasleton P, Turner DM, Sinnott PJ, Hutchinson IV. Genotypic variation in the transforming growth factor  $\beta$ 1 gene: association with transforming growth factor TGF- $\beta$ 1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. *Transplantation* 1998;66:1014–20.
- Stevens AM, Hermes HM, Rutledge JC, Buyon JP, Nelson JL. Myocardial-tissue specific phenotype of maternal microchimerism in neonatal lupus congenital heart block. *Lancet* 2003;362:1617–23.
- Miranda-Carus ME, Kimberly RP, Edberg JC, Buyon JP. Fc $\gamma$ R allelic polymorphisms: a candidate fetal factor in the development of congenital heart block (CHB) [abstract]. *Arthritis Rheum* 2001;44 Suppl 9:S340.
- Karassa FB, Trikalinos T, Ioannidis JP. The role of Fc $\gamma$ RIIA and IIIA polymorphisms in autoimmune diseases. *Biomed Pharmacother* 2004;58:286–91.
- Cimaz R, Fesslova V, Trespidi L, van Royen A, Kuis W, Meroni P. Neonatal lupus in triplets: new insights in the pathogenesis of complete congenital heart block [abstract]. *Arthritis Rheum* 2002;46 Suppl 9:S319.