T-Cell Receptor β-Chain Gene Rearrangement and Expression During Human Thymic Ontogenesis

By Antonio Bonati, Paola Zanelli, Sergio Ferrari, Anna Plebani, Bruno Starcich, Mario Savi, and Tauro M. Neri

T-cell receptor (TCR) β-chain proteins appear early (~15th week of gestation) during human thymic ontogenesis. These β-chain proteins, which appear before terminal deoxynucleotidyl transferase (TdT), could be an expression of a fully rearranged (V-D-J), incompletely rearranged (D-J), or germ-line TCR β-chain gene. The aims of this study, performed from the 15th week onward, were the following: (1) to investigate whether or not TCR β gene rearranges at an early stage during human thymic ontogenesis; (2) to investigate whether complete presumptive functional (1.3 kb) TCR β gene transcript is present at these early stages of development, or if incomplete (1 kb) or germ-line (1.1 kb) transcripts are expressed; (3) to examine the phenotype of TCR β-chain cells with two-color fluorescence using monoclonal antibody (MoAb) βF, and MoAbs that recognize CD1, CD2, CD3, CD4, CD8, CD5, and CD7 antigens (rabbit anti-calf TdT antiserum was used to detect TdT); and (4) to demonstrate whether or not β gene N-diversity regions are detectable as early as the 15th week and whether or not N-nucleotide insertions correlate to TdT expression. Fifteen- to 22-week fetal thymuses and pediatric thymuses were investigated. We demonstrated that TCR β-chain gene rearranged as early as the 15th week in human thymus and that a complete functional TCR β gene transcript was expressed at these early stages of human development. No other analyses to date have investigated TCR β gene expression in human thymus using molecular biology techniques. No significant differences were detectable between phenotypic analysis of fetal and pediatric samples, except for TdT expression, which appeared after the 20th week. Essentially all mCD3⁺ (OKT3⁺) cells were β-chain⁺ at the different weeks investigated. A significant percentage of CD4⁺ cells were β-chain⁺, and the percentage increased along with the age of development. After the 20th week, we identified three main populations: TdT⁺, mCD3⁺, βF⁺ (early thymic precursors); TdT⁺, CD1⁺, βF⁺ (intermediate maturity cortical thymocytes); and TdT⁺, mCD3⁺, βF⁺ (mature medullary thymocytes). Given these values, we may consider β-chain expression an ordered process. β gene N-nucleotide insertions were correlated to TdT expression, since N-regions increased considerably after the 20th week. A further increase of N-nucleotide insertions was detected from the 22nd week to the 32nd week.

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not TCR β gene rearranges at an early stage during human thymic ontogenesis; (2) to investigate whether complete presumptive functional (1.3 kb) TCR β gene transcript (which is the expression of a complete productive V-D-J β gene rearrangement\(^{20,21}\)) is present at these early stages of development, or if incomplete (1 kb) or germ-line (1.1 kb) transcripts are expressed; (3) to examine the phenotype of TCR β chain* cells with two-color fluorescence by using monoclonal antibody (MoAb) BF\(_1\) (which recognizes a constant region epitope localized at the inner [cytoplasmic] face of the cell surface membrane\(^{23}\)) and MoAbs that recognize CD1, CD2, CD3, CD4, CD8, CD5, and CD7 antigens (rabbit anti-calf TdT antisera was used to detect TdT); and (4) to demonstrate whether or not β gene N-diversity regions are detectable as early as the 15th week and whether or not N-nucleotide insertions correlate to TdT expression.

**MATERIALS AND METHODS**

We included in this study 12 fetal thymuses of a gestational age between 15 and 22 weeks, one case at the 32nd week, and pediatric thymuses and T-ALL samples as a control. Fetal tissues were obtained from abortions performed by dilatation and evacuation on women from 18 to 33 years old at the Milan and Parma University Gynaecological Clinics (one sample was obtained from the Reggio Emilia Hospital Gynaecological Division); the fertilization age was calculated by the ultrasound method.\(^{24}\) The case at the 32nd week was provided by Parma University Gynaecological Clinics. This fetus had died a few hours after a premature birth.

The terminations were performed according to Italian law. Permission from the Ethics Committee of the University of Parma was obtained for the fetal tissue experiments. Fetuses were not affected by congenital diseases and none of the mothers was involved in pharmacological trials. Pediatric thymuses were obtained from children 2 to 4 years old undergoing cardiac surgery at the Institute of Heart Surgery of Parma University.

T-cell ALL was represented by four cases classified on the basis of the expression of thymic antigens\(^{25}\) provided by the Hematological Unit of Modena University. In particular, we examined three cases of common T-ALL, TdT*, CD7*, CD5*, CD1*, CD3* and one case of mature T-ALL, TdT*, CD7*, CD5*, CD3*, CD1*. Clinical and immunological diagnoses had been performed before the samples were analyzed by us. Leukemic samples were investigated to obtain easily recognizable RNA transcript bands as a control, not to add novel information or to make a comparison between leukemic and normal ontogeny.

Six fetal thymic samples (two 15-week cases, two 17-week cases, one 19-week case, and one 22-week case), two pediatric thymocytes (4 years old), and four T-ALL were analyzed first. Fetal and pediatric samples were analyzed to detect DNA rearrangement. RNA transcript, cytoplasmic β-chain protein, and nuclear TdT expression. T-ALL samples were analyzed to detect RNA transcript as a control.

Six additional fetal thymic samples (two 15-week cases, two 17-week cases, two 22-week cases) and two pediatric samples (2 and 4 years old) were then examined to analyze the phenotype of TCR β-chain* cells and to detect β gene N-nucleotide insertions. These samples were also analyzed for DNA rearrangement, RNA transcript, TdT, and β-chain protein expression. One case at the 32nd week was analyzed to detect β gene N-nucleotide insertions.

Fetal thymus and pediatric cell suspensions for DNA analysis were prepared by gently teasing the tissues on a stainless steel mesh. Mononuclear cell pellets were used for DNA extraction after washing and centrifugation. The thymic tissues used for isolating RNA were minced on ice and homogenized with a solution containing guanidinium thiocyanate and 2-mercaptoethanol\(^{26}\) by a manual glass homogenizer.

High-molecular weight DNA was extracted as described by Ferrari et al.\(^ {27}\) Genomic DNA was digested by restriction endonucleases HindIII and EcoRI, electrophoresed on 0.8% agarose gels, and transferred to Gene Screen membrane (NEW Research Products, Boston, MA). The amount of DNA loaded varied depending on the different amount of material available and ranged from 20 µg (granulocytes) to 7 µg to 5 µg (fetal and pediatric samples). After prehybridization, the filters were hybridized at 65°C with a TCR β gene probe. After washing in 0.2 x SSC (1 x SSC = 0.15 mol/L NaCl + 0.015 mol/L sodium citrate) and 0.5% sodium dodecyl sulfate (SDS) at 65°C for several hours, the filters were exposed at -80°C with intensifying screens.

Total RNA of the cases examined first was extracted by a single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction using the technique of Chomczynsky and Sacchi.\(^ {28}\) Ten micrograms of RNA for each lane was loaded onto a 1% agarose/formaldehyde gel in 1 x Mops buffer [Mops: 3-(N-morfolino)propanesulfonic acid; Mops buffer: 0.1 mol/L Mops (pH 7), 40 mmol/L sodium acetate, 5 mmol/L EDTA (pH 8)]. The RNA was transferred to Hybond (Amersham, Buckinghamshire, UK) membrane using the electrol blotting procedure.\(^ {29}\) The membrane was prehybridized, hybridized with "P"-labeled probes as described by Ferrari et al., and washed with 0.1 x SSC and 0.5% SDS at 65°C for several hours. The autoradiography was performed using an intensifying screen at -80°C.

The TCR β gene probe was a purified Ig/ECBV fragment from the TCR β gene cDNA\(^ {30}\) and was obtained from Dr T.W. Mak (Ontario Cancer Research Institute, Toronto, Canada).

All the thymic samples examined (except for one case at the 32nd week) were also analyzed for TdT and TCR β chain expression by double indirect immunofluorescence assay as described.\(^ {18,31}\) Thymic mononuclear cell suspensions were pelleted by a cytocentrifuge (Shandon Cytospin, Cheshire, UK) on glass slides, fixed in methanol (4°C, 30 minutes), and stained. The MoAb βF\(_{1}\), kindly provided by Dr B. Brenner (Dana-Farber Cancer Institute, Boston, MA), was used to detect the β-chain of the TCR heterodimer. Rabbit anti-TdT antiserum was used to identify nuclear TdT (Supertech, Bethesda, MD). The binding of the antibodies was visualized with a goat anti-mouse F(ab')\(_2\), immunglobulin tetramethyl rhodamine isothiocyanate (TRITC; Technogenetics, Torino, Italy) and goat anti-rabbit fluorescein isothiocyanate (FITC; Supertech).

Six additional fetal thymuses (15 to 22 weeks) and two pediatric thymuses (2 to 4 years old) were investigated to detect the phenotype of TCR β-chain protein* cells by double indirect immunofluorescence assay as described by Bonati.\(^ {32}\)

The following reagents were used: CD1a clone Na1/34, isotype IgG\(_2a\) (Serootech, Oxford, England); CD2, clone MB21, isotype IgG\(_2a\) (Seraotec); CD4, clone B519, isotype IgG\(_2a\) (Seralab, Crawley Down, Sussex, England); CD8, clone T8 11, isotype IgG\(_2a\) (Seralab); CD5, clone F936 D9, isotype IgG\(_1\) (Seralab); CD7, clone 3A1E-12H7, isotype IgG\(_2a\) (Seralab); CD3, clone OKT3, isotype IgG\(_2a\) (Ortho Diagnostics, Raritan, NJ). (OKT3 is an anti-CD3e MoAb capable of staining approximately 50% of thymocytes at membrane level, primarily medullary cells.\(^ {33}\) OKT3 recognizes an epitope present in the perinuclear and Golgi area of thymocytes, identifiable by cytoplasmic staining, after fixation.\(^ {34,35}\))

As second-step reagents, we used goat anti-mouse IgG\(_2a\) TRITC (Seralab) and goat anti-mouse IgG\(_2a\) TRITC (Seralab).

Briefly, 10\(^ 6\) thymic mononuclear cells in 50 µL of phosphate-buffered saline containing 0.2% bovine serum albumin-[BSA] and...
0.2% sodium azide (PBSA) were incubated at 4°C for 30 minutes with the MoAbs described above at saturating concentration. Irrelevant MoAbs of identical isotype were analyzed as a control in parallel samples. After being washed twice in PBSA, the cells were stained with a second-step reagent represented by a goat anti-mouse immunoglobulin conjugated to TRITC fluorochrome and specifically reacting against the isotype of the MoAb previously used. After a 30-minute incubation at 4°C, the cells were washed twice and pelleted onto glass slides in a cytocentrifuge. The cytosin preparations were fixed in acetone (20°C, 15 minutes), then washed in PBS and stained with βF, MoAb. To stain βF, we used as a second-step reagent goat anti-mouse IgG, FITC (Serlabah).

Other experiments were performed with mononuclear cells directly cytocentrifuged, fixed with acetone (20°C, 15 minutes), and stained with OKT3 and βF. We found indirect immunofluorescence assay in order to detect cytoplasmic (c) CD3 and TCR β-chain in the same cell. As second-step reagent, we used goat anti-mouse IgG, TRITC to stain OKT3 and goat anti-mouse IgG, FITC to stain βF. Suspensions of CD1+ and membrane (m) CD3+ (OKT3) thymocytes were cytocentrifuged, fixed in methanol-acetone at 4°C for 15 minutes, and stained with rabbit anti-TdT antiserum as described above.

All immunological samples were examined using a Zeiss D-7082 microscope equipped with epifluorescence condenser III RS, selective filters for FITC and TRITC excitation, and a 40× phase objective. Twenty high-power fields for each smear, corresponding to approximately 3,000 cells, were examined. We considered positive only the cells with a clear patch formation (when membrane staining of cells that had been stained before fixation was examined), with a perfect ring-like pattern (when βF, cytoplasmic antigen that had been stained after fixation was examined), with nuclear staining (when TdT was examined), and with bright fluorescence intensity distinctly greater than the negative control.

Six fetal thymuses (two 15-week cases, two 17-week cases, two 22-week cases), one case at the 22nd week, and two pediatric samples (2 and 4 years old) were used for cloning and sequencing experiments. We amplified TCR V regions using degenerate primers.

The synthetic oligonucleotides were synthesized on a 38-1A DNA synthesizer (Applied Biosystem, Foster City, CA) and purified on a C-18 Sep-Pack cartridge (Waters Associates, Milford, MA) according to the manufacturer's instructions.

Total RNA was prepared by the guanidium isothiocyanate method according to Chirgwin et al. Six micrograms of total RNA was used to analyze RNA transcript of the samples as described above.

Three to five micrograms of total RNA and 50 ng of a specific C β primer (5’T GTTGCAGGGAAGCCTGTG 3’) were heated in a 20-μL volume at 55°C for 10 minutes and allowed to cool at room temperature. The RNA mixture was retrotranscribed in a final volume of 50 μL in 50 mmol/L Tris-HCl, pH 8.3, at 37°C, 50 mmol/L KCl, 6 mmol/L MgCl2, 10 mmol/L DTT, 1 mg/mL BSA, 0.2 mmol/L of dATP, dCTP, dGTP, and dTTP each, and 0.5 U of Moloney murine leukemia virus reverse transcriptase (Bohringer, Mannheim, Germany) at 37°C for 1 hour. At the end of the incubation, the mixture was heated at 68°C for 5 minutes to inactivate the enzyme and diluted to 250 μL with double distilled water.

Fifteen microliters of the first strand solution was made up to a final volume of 100 μL in 0.2 mmol/L of dATP, dCTP, dGTP, dTTP each, 10 mmol/L Tris-HCl, pH 8.3, at 25°C, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.01% gelatin, 200 ng of a second specific C β primer (5’T C C T T T T G G G G T T G G G A G A T C 3’), 200 ng of a V β degenerate primer (5’T CTGGAGTCRCCAGAVNCC 3’, where R = A or T; V = A or C; N = A, C, G or T) complementary to conserved sequences at the 5’ end of the TCR V gene and 2.5 U of native Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). The mixture, overlaid with two drops of sterile mineral oil, was subjected to two cycles of 1 minute at 94°C, 2 minutes at 50°C, and 2 minutes at 72°C, followed by 40 cycles of 1 minute at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C, with a final extension of 15 minutes at 72°C. In all experiments, negative controls (without template) were included.

The polymerase chain reaction (PCR) products were ethanol-precipitated, resuspended in water, and fractionated on a 3% agarose gel (Nu-Sieve 3:1, FMC, Rockland, ME). The bands from 300 to 500 bp were isolated, purified from agarose with Qiagen resin (Qiagen, Studio City, CA), and introduced into Escherichia coli TG1 cells made competent with calcium chloride. Bacterial colonies were transferred to nylon filters (Gene Screen, Dupont, Boston, MA), floated 3 minutes on 10% SDS; 5 minutes on 0.5 mol/L NaOH, 1.5 mol/L NaCl; 5 minutes on 0.5 mol/L Tris-HCl, pH 7.4, 1.5 mol/L NaCl; and 5 minutes on 2× SSPE. The DNA was fixed by baking the filters for 2 hours in an oven at 80°C. Recombinant colonies were screened with an oligonucleotide probe complementary to the 5’ portion of the C β region (5’T GTGTTGGGAGATCTCTGTTCGTG 3’) in 3 mol/L tetramethylammonium chloride (TMAC), 50 mmol/L Tris-HCl, pH 8, 2 mol/L EDTA, pH 8, 5× Denhart solution, and 100 μg/mL sonicated salmon sperm DNA at 50°C for 2 hours. The filters were washed twice for 10 minutes with 2× SSPE, 0.1% SDS; once for 10 minutes in 3 mol/L TMAC, 50 mmol/L Tris, pH 8, 2 mol/L EDTA, pH 8 (TMAC solution) at room temperature; and, finally, twice for 10 minutes in TMAC solution at 60°C. They were then air-dried and exposed at ~−80°C to an autoradiography film (X-Omat AR, Kodak, Rochester, NY) with an intensifying screen.

Bacterial colonies with the correct insert were used for sequence analysis by dideoxy chain termination method. Double-strand DNA sequence reactions were performed on plasmids from minipreps with the Taq track sequencing kit from Promega, Madison, WI. The primer used was the same C β primer of the PCR amplification. We sequenced four clones derived from pediatric samples, four clones from thymocytes at the 32nd week, eight clones at the 22nd week, eight clones at the 17th week, and nine clones at the 15th week. Sequences of D and J regions were compared and aligned with the germ-line sequences already published. N-regions were identified according to Kimura et al. and Concannon et al. For each thymic cDNA sample, sequences were obtained from clones obtained with separate PCR amplifications and ligations.

RESULTS

Before investigating TCR RNA transcript of our samples, we analyzed whether or not an early rearrangement of TCR β gene occurred. Figure 1 reports our results: in the fetal thymuses examined, an early rearrangement of β gene was detectable, since we found the loss of germ-line configuration at the 15th week. New rearranged bands were not seen, as we examined normal polyclonal tissues. No differences were evident between the fetal and pediatric samples examined. The configuration of the bands indicated that the major part of both fetal and pediatric thymocytes were rearranged. EcoRI digestion showed in thymic samples the complete disappearance of 11-kb bands, so that unequivocal rearrangement of C-β region was detected. Both 11-kb and 4-kb bands were preserved in granulocytes with the same intensity. The different intensity of the 4-kb band in the different samples examined was due to a different amount of DNA loaded. The 8-kb band defined an EcoRI

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Fig 1. Analysis of DNA from thymuses at different ages of development by the probe Cβ, after digestion with EcoRI (A) and HindIII (B). Lane 1 represents the germ-line configuration of TCR β gene detected in granulocytes. Lane 2 shows that an early rearrangement of TCR β gene occurred at the 15th week of gestation, since we observed the disappearance of germ-line configuration. New rearranged bands were not seen, as we examined normal polyclonal tissues. Lane 3 shows a pediatric sample that expressed a DNA pattern like that identified at the 15th week. In particular, EcoRI digestion (A) shows a complete disappearance of the 11-kb band. Both the 11-kb and 4-kb bands were preserved in granulocytes with the same intensity. The different intensity of the 4-kb band in the different samples examined was due to a different amount of DNA loaded. The 8-kb band was partly digested. HindIII digestion (B) in thymic samples showed an evident decrease of intensity of the 8-kb band with respect to the 6.5-kb and 3.5-kb bands. The 8-kb, 6.5-kb, and 3.5-kb bands had the same intensity in germ-line granulocytes. Granul, granulocytes; th, thymus.
site partly resistant to normal digestion as described previously.\textsuperscript{33} HindIII digestion in thymic samples showed an evident decrease of intensity of the 8-kb band with respect to the 6.5-kb and 3.5-kb bands. This means that rearrangement of the C-\(\beta\)\textsubscript{2} region was present.\textsuperscript{41} The three bands (8 kb, 6.5 kb, 3.5 kb) had the same intensity in germ-line granulocytes.

The presence of an early rearrangement of \(\beta\)-chain gene in the major part of thymic cells induced us to investigate if these rearrangements could produce a complete presumptive functional 1.3-kb or an incomplete 1-kb \(\beta\) gene transcript.

Our results showed that a predominant complete 1.3-kb RNA transcript was expressed in thymic cells as early as the 15th week. This was also seen in the fetuses examined at a later period during ontogenesis, as well as in the pediatric thymocytes we examined. Figure 2 shows a fetal thymic sample at the 15th week of gestation and a 4-year-old pediatric sample, which expressed a primarily complete 1.3-kb transcript and a less significant 1-kb incomplete band. Both bands are compared with an isolated 1.3-kb band expressed by a T-ALL. This is a case of common T-ALL; the other three cases of T-ALL analyzed presented both 1.3-kb and 1-kb transcripts (data not shown).

Table 1 summarizes our results with regard to TCR \(\beta\)-chain expression; a correlation with TdT\textsuperscript* cells is reported. The data show that the major portion of thymocytes (~60%) expressed \(\beta\) chain as early as the 15th week, when only occasional TdT\textsuperscript* cells could be seen.

Figure 3 represents the different expression of TdT and TCR \(\beta\)-chain during human ontogenesis. Occasional TdT\textsuperscript* cells were detectable around the 15th week of gestation when \(\beta\)F\textsubscript{1} was expressed in 50% to 60% of thymocytes. A coordinated expression of TdT and \(\beta\)F\textsubscript{1} appeared only around the 20th week.

Figure 4 shows that few TdT\textsuperscript* blast thymocytes (mainly large cells) did not express TCR \(\beta\)-chain. The other cells represented in pediatric thymus were small TdT\textsuperscript* (cortical) thymocytes, which were \(\beta\) chain\textsuperscript*, and TdT\textsuperscript* (medullary) thymocytes, which were strongly \(\beta\) chain\textsuperscript*. \(\beta\)-chain protein expression increased along with the loss of TdT, so that an orderly expression of TdT and TCR \(\beta\)-chain was present.

Phenotypic analysis of TCR \(\beta\)-chain protein\textsuperscript* cells at different ages of development is reported in Table 2. A significant percentage of thymic cells expressed membrane T-cell-associated antigens as early as the 15th week.

At the 15th week, approximately 60% of thymic cells were CD1\textsuperscript{+}, 35% mCD3\textsuperscript+ (OKT3), 80% CD4\textsuperscript+, 75% CD8\textsuperscript+, and 90% CD2\textsuperscript+ CD5\textsuperscript+, and CD7\textsuperscript+. Essentially all thymic cells were CD3\textsuperscript+.

Approximately 50% of CD1\textsuperscript+ cells, 90% of mCD3\textsuperscript+ cells, 70% of CD4\textsuperscript+ cells, 70% of CD8\textsuperscript+ cells, and 60% of CD2\textsuperscript+, CD5\textsuperscript+, CD7\textsuperscript+ expressed cytoplasmic \(\beta\)-chain. Sixty percent of CD3\textsuperscript+ cells were \(\beta\)-chain\textsuperscript*. Approximately 50% of \(\beta\)-chain\textsuperscript* thymocytes were CD1\textsuperscript+, 50% mCD3\textsuperscript+, 70% CD4\textsuperscript+, 70% CD8\textsuperscript+, 90% CD2\textsuperscript+, CD5\textsuperscript+, and CD7\textsuperscript+. Essentially all \(\beta\)-chain\textsuperscript* cells were CD3\textsuperscript+.

No substantial differences were detectable between the samples examined at the 15th week, fetuses taken at a later gestational age, and pediatric thymuses, except for some progressive increase in the percentage of \(\beta\)-chain\textsuperscript* cells, particularly at the level of the CD1\textsuperscript* subset.

The percentages clearly demonstrated that \(\beta\)-chain\textsuperscript* cells were detectable essentially in all the cells of the mCD3\textsuperscript* subset and in a significant percentage of the cells of the CD1\textsuperscript* subset. Seventy-nine percent to 92% (range of the percentages observed in the different cases examined after the 20th week) of the mCD3\textsuperscript* cells were TdT\textsuperscript* (primarily mature medullary subset), and 83% to 97% of CD1\textsuperscript* cells were TdT\textsuperscript* (primarily intermediate maturity cortical subset). The majority of \(\beta\)-chain\textsuperscript* cells expressed CD4, CD8, CD2, CD5, and CD7, as these antigens were widely represented in thymic cells as early as the 15th week.

Figure 5 shows three main populations that we distinguished in human thymus after 20 weeks of gestation with regard to the phenotype of \(\beta\)-chain\textsuperscript* cells.

After having demonstrated that a complete functional TCR \(\beta\) gene transcript was expressed at the 15th week, we completed our investigation in order to know whether or not \(\beta\) gene N-diversity region expression was correlated to...
Table 1. TCR β Gene Rearrangement, Transcript, and Protein Expression in Human Thymus at Different Ages of Development: Correlation With TdT

<table>
<thead>
<tr>
<th>Cells</th>
<th>β Gene</th>
<th>β Transcript</th>
<th>β-Chain*</th>
<th>TdT*</th>
<th>TdT*/β-Chain*</th>
<th>β-Chain*/TdT*†</th>
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<tr>
<td>Thymus (15 wk, n = 4)</td>
<td>R</td>
<td>1.3 kb</td>
<td>60.5</td>
<td>0.3</td>
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<td>‡</td>
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<td></td>
<td></td>
<td>(predominant)</td>
<td>(54-68)</td>
<td>(0.1-0.6)</td>
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<tr>
<td>Thymus (17 wk, n = 4)</td>
<td>R</td>
<td>1.3 kb</td>
<td>65.75</td>
<td>0.55</td>
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<td></td>
<td></td>
<td>(predominant)</td>
<td>(58-73)</td>
<td>(0.2-0.8)</td>
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</tr>
<tr>
<td>Thymus (19 wk, n = 1)</td>
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<td>1.3 kb</td>
<td>62</td>
<td>1</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(predominant)</td>
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<tr>
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<td>87.66</td>
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<tr>
<td></td>
<td></td>
<td>(predominant)</td>
<td>(78-87)</td>
<td>(76-82)</td>
<td>(84-91)</td>
<td>(70-80)</td>
</tr>
<tr>
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<td>79</td>
<td>87.5</td>
<td>74</td>
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<td></td>
<td>(predominant)</td>
<td>(77-94)</td>
<td>(72-85)</td>
<td>(85-93)</td>
<td>(68-84)</td>
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</table>

The results are expressed as mean values. Numbers in parentheses express the range. TCR β gene rearrangement, functional transcript (1.3 kb), and protein were expressed at an early stage in human thymus, when only occasional TdT+ cells were detectable. A coordinated expression of TdT and β-chain appeared after the 20th week.

Abbreviation: R, rearranged.

*TdT+ cells that expressed β-chain.
†β-Chain+ cells that expressed TdT.
‡No value is given due to the low percentage of TdT+ cells.

To our knowledge, no other analysis to date has investigated TCR β gene expression by molecular biology technique in early human tissues. TCR β-chain proteins identified by us and others at an early stage during ontogenesis could be expression of an incomplete nonfunctional TCR β gene transcript. Incomplete nonfunctional D-J rearrangements might produce incomplete aberrant proteins, that could have been recognized by an MoAb such as βF1, active against an epitope codified by TCR β gene constant region; moreover, βF1 recognizes cytoplasmic β-chains still unlinked to the α-chains, to produce the 90-Kd α/β functional heterodimer on the surface membrane. WT31, analyzed by others in thymic ontogenesis investigations, was recently demonstrated to be directed to the CD3 subunit, but not to the α/β heterodimer as previously suggested. Therefore, our findings of the early appearance of a primarily complete functional 1.3-kb TCR β transcript in human thymus are a significant novelty, and contribute to highlight the processes of intrathymic maturation.

A study performed in thymic subpopulations both in fetal and pediatric samples with the separation of T3-, CD1- and T3+, CD1+ fractions demonstrated by densitometric scanning that only approximately 25% of the cells in T3-, CD1+ fractions were β gene unrearranged, but the type of rearrangement and the β gene transcripts were not investigated.

Other important reports were produced concerning TCR β-chain gene ontogenesis in mouse thymic cells. In the mouse, significant levels of the 1.3-kb β gene transcript were seen at day 17, and not in correlation to the presence of TdT (a unique DNA polymerase that acts as a somatic mutagen, by adding extra random nucleotides at the D-J joining region during the process of rearrangement, contributing to the generation of antibody and TCR protein diversity [N-diversity]), as TdT levels in fetal thymocytes were found to be greatly lower than in adult thymocytes. An analysis performed at the single-cell level using in situ...
hybridization technique confirmed these early genetic events in T-cell development of the mouse. 

By investigating the expression of TdT and TCR β-chain protein in fetal and pediatric thymocytes, we found that a coordinated expression of TdT and TCR β-chain occurred in humans starting around the 20th week of gestation (Fig 3) (a similar sequence of coordinated events with respect to TdT and cytoplasmic μ-chain was observed in postnatal regenerating human bone marrow, in fetal liver and bone marrow, and in fetal lymphonodes). Furthermore, we showed that the β-chain proteins identified around the 15th week before TdT appearance were an expression of complete functional transcript. It has been stated well both in mice and in humans that the functional 1.3-kb transcript was produced by a complete V-D-J gene rearrangement, whereas the 1-kb incomplete product was produced by an incomplete D-J rearrangement. Given these values, the β-chain proteins identified by us were an expression of complete 1.3-kb transcript and of complete V-D-J rearrangement so that a functional activity was suggested.

Our results are not in contrast with those of Greenberg and Kersey, who showed that TdT expression can precede TCR β-chain rearrangement in T-ALL, but might indirectly confirm it. TdT T-leukemic cells were probably the pathological counterpart not of TdT fetal thymic cells, but of late (after the 20th week) fetal or adult thymic cells.

Fig 4. Pediatric mononuclear cells double-stained by TdT and MoAb βF1. The fields stained by TdT (a) and βF1 (b), and the phase-contrast field (c) are presented. Large arrows indicate four TdT' blasts (see a). βF1' (see b). Smaller arrows indicate two small TdT' (cortical thymocytes) (see a). βF1' (see b). Arrowheads indicate two TdT' (medullary) thymocytes (see a), strongly βF1' (see b).
which commonly express TdT. Among these cells, the small subset (5% to 10%) of TdT+ large thymic blasts that are β-chain− (see Fig 4), and presumably β gene rearrangement− is the putative counterpart of the T-leukemic cell that was described did not have β gene rearrangement.

The appearance of terminal transferase in the thymus around the 20th week during human ontogenesis was confirmed by other investigators using both biochemical and immunological assays.53 TdT− cells are not detectable before the 15th week, as was demonstrated by an analysis performed as early as 10.5 weeks.

The phenotype of TCR β-chain+ cells was studied to understand at which level of the intrathymic maturation process these cells were recognizable. In addition to the analysis of TdT and βF1, we considered that a reliable method to investigate this was the analysis of cytoplasmic β-chains in cytocentrifuged thymocytes previously stained with membrane markers of T-cell differentiation, cCD3 and βF1, expression in the same cell was also investigated. We did not examine membrane α/β heterodimer, since the MoAb available (WT31) was recently demonstrated to stain non-α/β heterodimer, but the invariant CD3, subunit,23 as reported above.

It is of interest that essentially all mCD3+ (OKT3+) cells were β-chain+ at the different weeks investigated. A significant percentage of CD1+ cells were β-chain−, and the percentage increased along with the age of development. mCD3+ (OKT3) thymocytes are primarily mature medullary thymocytes,31,32 and CD1+ thymocytes are primarily cortical thymocytes.33 We think we stained only a low percentage of the TdT+, CD1+, mCD3− cells of intermediate maturity between cortical and medullary thymocytes that have been described by others,49 because mCD3+ (OKT3) cells were primarily TdT−.

No significant differences were detectable between phenotypic analysis of fetal and pediatric samples except from TdT expression. This is in agreement with the fact that as early as the 10th week a clear distinction between cortical and medullary thymic area was demonstrated.

The data taken from the analysis of TdT, cCD3, and membrane T-cell–associated antigens double-stained with βF1 prompted us to identify three main populations after the 20th week, as reported in Fig 5. These populations corresponded to the three main populations identified by Reinherz et al52 in human thymus. The first population was represented by TdT+ large thymic blasts that were β-chain− (see Fig 4). These cells were cCD3+, since essentially all thymic cells expressed this antigen into the cytoplasm. The second population is TdT+, CD1+ βF1− (intermediate maturity cortical thymocytes). The third population is TdT+, mCD3+, βF1++ (mature medullary thymocytes). We may consider β-chain expression an ordered process, since both the percentage of positive cells (see Table 2) and the level of expression (see Fig 4) were progressively increasing along intrathymic T-cell maturative lineage.

The percentage of T-cell–associated antigens observed by us is similar to that detected by others both in fetal and in pediatric samples.50,51,52,55

New technical improvements using degenerate primers provided the possibility of constructing pan-Vβ primers in order to amplify TCR V regions.53 This is an important step for amplifying genes like TCR β, which have greater combinatorial diversity than TCR γ and δ genes.54 Using this strategy, we answered the question whether β gene N-diversity is correlated to the expression of TdT during human thymic ontogenesis. Our results showed that this correlation is present, since N-regions increased significantly in the samples examined after the 20th week. The occasional isolated random nucleotides detected at an early stage might be either an expression of low levels of TdT activity produced by the occasional TdT+ cells identifiable at the 15th week or a residual of the heptamer regions. Our results suggest that the period between 20 to 30 weeks is of critical importance for the addition of β gene N-regions and the acquisition of N-diversity. No differences were detectable between 30 weeks and pediatric period. The observation of two negative clones at the 22nd week might depend

### Table 2. Phenotype of βF1− Cells in Human Thymocytes

<table>
<thead>
<tr>
<th>Cells</th>
<th>CD1+</th>
<th>CD2</th>
<th>mCD3+</th>
<th>cCD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD5</th>
<th>CD7</th>
<th>pβF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>15 wk, n = 2</td>
<td>61.5 55.0 49.0</td>
<td>92 62.0 93.5</td>
<td>34.5 92 51.5</td>
<td>99.0 61.5 99.0</td>
<td>80.5 89.5 71.0</td>
<td>74.0 72.0 73.0</td>
<td>92.0 57.5 95.0</td>
<td>94 61.0 93.5</td>
</tr>
<tr>
<td>Thymus</td>
<td>17 wk, n = 2</td>
<td>63.0 59.0 53.5</td>
<td>94 65.0 94.5</td>
<td>38.0 95 49.0</td>
<td>100.0 65.0 100.0</td>
<td>82.0 88.0 74.0</td>
<td>70.0 74.5 69.0</td>
<td>95.0 61.0 93</td>
<td>90 58.5 95.0</td>
</tr>
<tr>
<td>Thymus</td>
<td>22 wk, n = 2</td>
<td>64.5 82.5 72.0</td>
<td>93 84.5 96.0</td>
<td>32.5 98 34.0</td>
<td>98.0 83.5 100.0</td>
<td>85.0 88.0 75.0</td>
<td>73.0 89.0 72.5</td>
<td>98.5 83.0 90</td>
<td>98 80.5 92.0</td>
</tr>
<tr>
<td>Thymus (pediatric)</td>
<td>n = 2</td>
<td>72.5 86.5 76.5</td>
<td>98 82.0 95.0</td>
<td>33.5 99 36.0</td>
<td>99.5 84.0 100.0</td>
<td>79.5 85.0 77.0</td>
<td>77.0 85.5 70.0</td>
<td>98.0 85.0 92</td>
<td>96 82.0 94.0</td>
</tr>
</tbody>
</table>

The results are expressed as mean values. The tests were performed by double immunofluorescent assay combining MoAbs of different specificities.

<table>
<thead>
<tr>
<th>TF</th>
<th>30 weeks</th>
<th>22 wk. n</th>
<th>17 wk. n</th>
<th>15 wk. n</th>
</tr>
</thead>
<tbody>
<tr>
<td>TdT+</td>
<td>79%</td>
<td>63.0</td>
<td>76.5</td>
<td>93</td>
</tr>
<tr>
<td>CD1+</td>
<td>82.5</td>
<td>72.0</td>
<td>77.0</td>
<td>96</td>
</tr>
<tr>
<td>mCD3+</td>
<td>85.5</td>
<td>70.0</td>
<td>74.0</td>
<td>90</td>
</tr>
<tr>
<td>cCD3</td>
<td>95.0</td>
<td>61.0</td>
<td>65.0</td>
<td>53.5</td>
</tr>
<tr>
<td>CD4</td>
<td>64.5</td>
<td>59.0</td>
<td>53.5</td>
<td>51.5</td>
</tr>
<tr>
<td>CD8</td>
<td>77.0</td>
<td>74.5</td>
<td>70.0</td>
<td>67.5</td>
</tr>
<tr>
<td>CD5</td>
<td>98.0</td>
<td>95.0</td>
<td>93.5</td>
<td>90</td>
</tr>
<tr>
<td>CD7</td>
<td>93.5</td>
<td>92.0</td>
<td>91.0</td>
<td>87.0</td>
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<tr>
<td>pβF1</td>
<td>82.5</td>
<td>80.5</td>
<td>77.0</td>
<td>74.0</td>
</tr>
</tbody>
</table>

![Fig 5. The three main populations we identified in human thymus after the 20th week of gestation with regard to TdT, CD1, CD3, and βF1 expression. These populations corresponded to the three main populations described in human thymus by Reinherz et al. The expression of TdT, CD antigens, and βF1 was ordered, since βF1 increased along with the loss of TdT, which disappeared at the level of the mCD3 subset.](image-url)
<table>
<thead>
<tr>
<th>V</th>
<th>N1</th>
<th>D</th>
<th>N2</th>
<th>J</th>
<th>D9</th>
<th>J9</th>
<th>C9</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 wk</td>
<td>CCAGCACTTA</td>
<td>GGCCACG</td>
<td>GGAC</td>
<td>ACGGTAAGGG</td>
<td>2.1</td>
<td>2.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CCACT</td>
<td>GACAGG</td>
<td></td>
<td>TAGCAATGCGCTTCTT</td>
<td>1.1</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>32 wk</td>
<td>CCAGCGCG</td>
<td>CCGCAT</td>
<td>GACTA</td>
<td>CAGATAGCGAGTATTT</td>
<td>2.1</td>
<td>2.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CCAGCA</td>
<td>GGAA</td>
<td>GACTAGGG</td>
<td>TGGAAGGG</td>
<td>2.1</td>
<td>2.3</td>
<td>2</td>
</tr>
<tr>
<td>22 wk</td>
<td>CCAGCA</td>
<td>GGCA</td>
<td>CCCTTA</td>
<td>TAGCAATCAGCGCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.1</td>
<td>1.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>17 wk</td>
<td>CCAGCAGTTT</td>
<td>C</td>
<td>GGGGG</td>
<td>TAGCAGATACGCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.1</td>
<td>2.4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>15 wk</td>
<td>CCAGCAGCTT</td>
<td>C</td>
<td>GC</td>
<td>TACAGTACGTTT</td>
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<td></td>
<td></td>
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<tr>
<td></td>
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<td>2.1</td>
<td>2</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TAGCAATGCGCTT</td>
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<td></td>
<td></td>
<td></td>
<td>2.1</td>
<td>2.7</td>
<td>2</td>
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</tr>
</tbody>
</table>

Fig 6. Nucleotide sequences of V-D-J junctions from pediatric and 32-, 22-, 17-, and 15-week human fetal thymuses. Sequence alignments are made as described in the text. At top center, the germ-line sequences of Dp 1.1 and Dp 2.1 germ-line regions are shown. Below are aligned the 5' end of the J regions. Beside each sequence, the DJ-C combinations used are shown. In-frame joins are labeled with +, out-of-frame joins with -. The J and 5' end of the J regions, Beside each sequence, the D-J-C combinations used are shown. In-frame joins are labeled with +, out-of-frame joins with -. The J and 5' end of the J regions. Beside each sequence, the D-J-C combinations used are shown. In-frame joins are labeled with +, out-of-frame joins with -. The J and 5' end of the J regions.

on the fact that a minor percentage of thymocytes has still to begin the process of N-nucleotide incorporation at this week.

An analysis of TCR γ/δ transcripts in human thymic ontogenesis similarly demonstrated very low levels of N-nucleotide insertions around the 15th week, which dramatically increased in pediatric samples in both sequences of δ and γ transcripts. A study of δ gene in mice showed that N-region addition correlated well with the presence of TdT, as the presence of N-regions in adult sequences, but not in fetal, was parallel to the low TdT activity found in fetal thymocytes compared with adult cells. Others suggested that the absence of N-regions in newborn mice with immunoglobulin V-D-J functional sequences was due to the fact that TdT does not reach maximum levels until some time after birth in mouse B cells, similar to the slow increase in thymocytes.

Our results in humans were in agreement with those shown in mice, since in both species a correlation between TdT and N-nucleotide insertions was seen. The observations that N-nucleotides were increased after the 20th week in humans differs from the observations in mice, as in this species N-regions are detectable only in adults. This interesting discrepancy could be present, since, in contrast to human ontogeny, T-cell immunocompetence in rodents is established later during development.

In conclusion, immunological and molecular results of our experiments showed that TCR β-chain gene, as early as at the 15th week, had a behavior similar to the pediatric thymus, except for the presence of N-diversity regions that were increasing along with TdT expression after the 20th week of gestation.

The results of our study could lead not only to the improvement of theoretical knowledge of thymic ontogenesis, but also to practical approaches, eg, to investigate if the β gene is normally expressed during ontogenesis in the cases of different immunodeficiency diseases or in diseases with an immunological pathogenesis. In athymic mice, greatly reduced levels of β but not γ transcript were observed. Recently, an approach like this was carried out in different immunological diseases and interesting prospects have been opened.

We were not able to analyze fetal tissues before the 15th week of gestation, and in particular to detect the size of TCR β gene transcript expressed by the subset (30% of the cells) of CD7+, CD2+, cCD3+, TdT+, thymocytes which first showed the appearance of β-chain protein in the cytoplasm at 9.5 weeks. Similarly, we did not investigate the few
CD7+, cCD3+, TdT− cells in fetal liver at 10.5 weeks, which have been described to present β-chain. We may postulate that in the earliest stages of T-cell development, a prevalent precursor may present p-chain. We may postulate that in the earliest stages of T-cell development, a prevalent precursor may present p-chain. We may postulate that in the earliest stages of T-cell development, a prevalent precursor may present p-chain. We may postulate that in the earliest stages of T-cell development, a prevalent precursor may present p-chain. We may postulate that in the earliest stages of T-cell development, a prevalent precursor may present p-chain. We may postulate that in the earliest stages of T-cell development, a prevalent precursor may present p-chain. We may postulate that in the earliest stages of T-cell development, a prevalent precursor may present p-chain. We may postulate that in the earliest stages of T-cell development, a prevalent precursor may present p-chain.

We focused our work on β-chain gene analysis, since the behavior of this gene is parallel to that of α-chain gene, in order to produce a functional α/β heterodimer in approximately 90% of peripheral blood T lymphocytes. Whether γ/δ chain proteins, which are represented only in rare thymic cells through the whole span of human thymic development, might have the ontogenic seed in other tissue(s) is still unknown.

Notwithstanding the high frequency of TCR δ/γ rearrangements shown in B-cell precursor ALL, an analysis of CD10/surface μ− and CD10/surface μ+ cells in fetal bone marrow did not demonstrate TCR γ or δ rearrangements in these early B-cell subsets. This suggests a potential difference in patterns of gene rearrangement that distinguish normal and leukemic precursors. Therefore, to analyze the human hematopoietic ontogenesis, it may be more useful to study directly normal precursors, rather than to extrapolate data taken from the study of leukemic precursors.

ACKNOWLEDGMENT

We are very grateful to Professor Guido Lucarelli (Division of Hematology and Bone Marrow Transplantation Unit, Pesaro, Italy), who kindly helped and advised us; to Dr Mauro Buscaglia (Gynecological Clinics, University of Milano), Dr Roberto Tredade (Gynecological Clinics, University of Parma), and Dr Alessandro Ventura (Gynecological Division of Reggio Emilia Hospital) for providing fetal thymuses; and to Dr Dante Medici (Heart Surgery Institute, University of Parma) for providing pediatric thymocytes.

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T-cell receptor beta-chain gene rearrangement and expression during human thymic ontogenesis

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