The diversity of rearranged immunoglobulin heavy chain variable region genes in peripheral blood B cells of preterm infants is restricted by short third complementarity-determining regions but not by limited gene segment usage

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The immunoglobulin diversity is restricted in fetal liver B cells. This study examined whether peripheral blood B cells of extremely preterm infants show similar restrictions (overrepresentation of some gene segments, short third complementarity-determining regions [CDR3]). DNA of rearranged immunoglobulin heavy chain genes was amplified by polymerase chain reaction, cloned, and sequenced. A total of 417 sequences were analyzed from 6 preterm infants (25-28 weeks of gestation), 6 term infants, and 6 adults. Gene segments from the entire VH and DH gene locus were rearranged in preterm infants, even though the DH7-27 segment was overrepresented (17% of rearrangements) compared to term infants (7%) and adults (2%). CDR3 was shorter in preterm infants (40 ± 10 nucleotides) than in term infants (44 ± 12) and adults (48 ± 14) (P < .001) due to shorter N regions. Somatic mutations were exclusively found in term neonates and adults (mutational frequency 0.8% and 1.8%). We conclude that preterm infants have no limitations in gene segment usage, whereas the diversity of CDR3 is restricted throughout gestation.

Amplification and sequencing of VDJ rearrangements
DNA (0.5-1.0 μg) extracted from heparinized blood samples was used for the polymerase chain reaction (PCR) amplification of the rearranged IgH chain variable region with a nested primer PCR previously established by our group. For the first amplification a mixture of family-specific primers for framework region 1 was used in conjunction with a consensus JH primer; for the reamplification framework region 2 family-specific primers with another consensus JH primer. Positive (B-cell line Raji) and negative (H2O) controls were carried out with each PCR.

The amplificates (length 230-280 nucleotides) were isolated and cloned (TOPO TA cloning kit, Invitrogen, Leek, The Netherlands). Twenty-five to 35 randomly selected clones of each subject were sequenced (ABI 377A, Applied Biosystems, Weiterstadt).

Sequence analysis
The germline VH, JH, and DH segments were identified using GenBank (release 98) and VBASE directory. Only functional rearrangements were further analyzed. For DH identification we used the criteria of Shiokawa, but accepted no DIR segments, inverted D segments, or D-D recombinations. CDR3 was defined according to Kabat (amino acids 93-102).

Study design

Patients
We collected cord blood of 6 preterm infants (25-28 weeks of gestation, birth weight 470-1120 g), 6 term infants (39-42 weeks), and peripheral blood of 6 adults, aged 26 to 43 years. Infections were ruled out in all individuals (normal clinical examination, blood count, and C-reactive protein). The study protocol had been approved by the institutional review board and written consent was obtained.

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Database accession nos. AF 235505-235642; from 6 term infants, 142 sequences, AF 235643-235784; and from 6 adults, 137 sequences, AF 235785-235921. Strengths of our study are that we always studied groups of individuals and that we investigated the same cell population, mature B cells, in each group.

The combinatorial diversity was similar in the 3 groups. All individuals, even each of the most immature preterm infants, used V_H, D_H, and J_H gene segments from the entire gene locus. V_H family usage roughly corresponded to V_H family size. Only the V_H4 family was used significantly more often in preterm infants (P < 0.01) (Figure 1). The number of different V_H gene segments used was not smaller in preterm infants (31 V_H gene segments), than in term infants (28) or adults (30) and the V_H6-1 gene segment (V_H6 family) was only slightly overrepresented in preterm infants compared to adults (5.5% versus 2.0% of sequences). Each group used 18 of the 27 D_H gene segments, even though the D_H7-27 segment (D_H7 family) was overrepresented in preterm infants (17% of rearrangements) compared to term infants (7%), and adults (2%) (Figure 1).

Previous studies described an overrepresentation of the V_H6-1 gene segment (25%) and the D_H7-27 gene segment (50%) in early fetal liver B cells (8-10 weeks of gestation).3,4 These gene segments are located at the 3' end of their respective gene locus, which makes them more readily available for recombination. This bias moderately decreased in later stages of gestation (15-19 weeks of gestation).3,4,13 Our study demonstrates that the overrepresentation was dramatically reduced in preterm infants (V_H6-1: 5.5%; D_H7-27: 17%) and did not affect the overall combinatorial diversity any more.

The preterm infants we studied preferentially expressed J_H3 and J_H4 (Figure 1). This is in agreement with developmental trends observed previously. With increasing age J_H usage shifts from the D_H proximal J_H1/J_H2 gene segments in the fetus to J_H4/J_H6 in adults.4,14 The mean length of V_H rearrangements considerably increased by 4 nucleotides from preterm infants to term infants and by 8 nucleotides from preterm infants to adults (Figure 2, P < .001, ANOVA). The paramount cause of this increase was addition of N-nucleotides. This suggests that the activity of the terminal deoxynucleotidyl transferase (TdT), that introduces N-nucleotides, is regulated in close correlation to gestational age. D_H length and the contribution of J_H to CDR3 were not reduced in the infants, indicating that overrepresentation of D_H7-27, the shortest D_H germline segment, and use of shorter J_H gene segments,4 to which short CDR3 had been attributed in the fetus, did not affect CDR3 length in infants (Figure 2).

Our finding that the CDR3 of preterm infants was 6 N-nucleotides shorter than in adults means a 400-fold reduction in potential CDR3 diversity because the nontemplated N-nucleotides are randomly inserted and therefore each codon added increases the potential diversity of the repertoire 20-fold.13 The short CDR3 has been implicated with the polyreactive low affinity binding of antigen observed in fetus and neonate.3 The short CDR3 in preterm infants might therefore contribute to their increased susceptibility to infection. Yet, the significance of CDR3 length for immunologic competence is not completely understood. Mice with a null mutation in the TdT gene and no N-nucleotides had surprisingly normal immune responses,15 whereas exceptionally long CDR3

Figure 1. Rearranged gene families. Frequency of rearranged V_H gene families, D_H gene families, and J_H gene segments in preterm infants (filled bars), term infants (hatched bars), and adults (open bars). The V_H6 family contains the single gene segment V_H6-1, and the D_H7 family the single gene segment D_H7-27. The line indicates the frequency of usage expected from the germline composition. Values are mean ± SD; ** P < .01; * P < .05 (ANOVA).

Figure 2. Mean length (nucleotides) of the different components of CDR3 in preterm infants, term infants, and adults. Data are mean ± SD; ** P < .001 versus term infants and versus adults; * P < .05 versus adults (ANOVA).
regions were found in X-linked agammaglobulinemia and nonfunctional IgH chain rearrangements. We analyzed all rearrangements for somatic mutations. The nucleotide exchange rate was very low in preterm infants (0.3%) and 75% of the rearrangements were completely identical to the germline. The modifications in preterm infants most likely represent Taq polymerase errors (0.076% in our study) or interindividual polymorphisms or both. Although the overall nucleotide exchange rate was also low in term infants (0.8%), the presence of 15 rearrangements each with more than 3 nucleotide exchanges (range, 4-12 exchanges) clearly indicates their introduction by a somatic mutation mechanism. Moreover, in term infants only 49% of the rearrangements were completely identical to the germline. In blood samples from adults, which were investigated for control and comparison, 38 rearrangements had more than 3 nucleotide exchanges (range, 4-16 exchanges) and thus displayed clear indication for somatic mutations with an overall nucleotide exchange rate of 1.8%. This latter result agrees with previous investigations of blood samples from human adults.

In conclusion, we demonstrate that there is an unrestricted gene segment usage in preterm as well as in term infants. However CDR3 is significantly shorter in preterm than in term infants or adults. Mutations are absent in preterm infants but are already present at a low frequency in term infants even without prenatal or perinatal infection. Our findings suggest that developmentally regulated limitations in rearranged IgH chain variable regions of neonatal peripheral blood B cells affect junctional diversity and mutational frequency longer than combinatorial diversity.

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References
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