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Primary Structure of the Human VkII Regions Elicited by *Haemophilus influenzae* Type b Polysaccharide Vaccines; The J Gene Usage Is Restricted in Child Antibodies Using the A2 Gene

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The *Haemophilus influenzae* type b (Hib) has been a major cause of bacterial meningitis in children who are less than two years old. The variable (V) region repertoire of adult Caucasian antibodies (Abs) to Hib polysaccharide (PS) has been characterized well. The majority of adult antibodies against Hib uses VL that is derived from the Vk gene A2 and have arginine at the N region. In order to explore the possibility those antibody responses to Hib-PS is variable in various age groups, we examined the VL regions of the antibodies to Hib-PS in Korean adults and children. We immunized Korean adults (n = 8) and children (n = 39) with Hib tetanus conjugated vaccines, isolated RNAs from the peripheral lymphocytes, and amplified the A2-derived VL regions by RT-PCR. The PCR products were subcloned and sequenced. Forty-seven out of 54 independent clones from children used the Jκ2, or Jκ3 gene in preference. The adults, however, used all of the Jκ genes evenly. With respect to the amino acid sequences of variable regions, adult A2-Jκ recombinants have a germline sequence. But, the 76th codon (AGC) of child A2-Jκ2 recombinants was substituted with CGC (arginine) in most cases (88%) and 77 percent of child clones using the A2-Jκ3 genes have isoleucine-109 at the junction of Jκ-Cκ instead of threonine that is found in a germline sequence. These results suggest that the mechanism of antibody production in young children is different from that of adults.

Keywords: CDR-3, *Haemophilus influenzae* type b, J gene.

Introduction

The *Haemophilus influenzae* type b (Hib) has been a leading cause of bacterial meningitis in young children (Murphy *et al.*, 1992; Trollfors *et al.*, 1992). Antibodies to the Hib polysaccharide (polyribosyl-ribitol phosphate; PRP) induced a protective immunity in older children, but it was not immunogenic in the younger population that is most susceptible to Hib disease (Trollfors *et al.*, 1992). Several vaccines, which Hib polysaccharide conjugated to protein carrier molecules, were developed and these induced anamnestic antibody responses in infants as young as 2 months (Granoff *et al.*, 1993). Concurrent with the development of the Hib vaccine, much has been learned about the human antibody response to Hib polysaccharide. For instance, although the antibody response to Hib polysaccharide (PS) of an individual is oligoclonal, there is a considerable V region heterogeneity (Scott *et al.*, 1991). Molecular studies of anti-Hib polysaccharide V regions have shown that at least two VHIII genes are expressed with considerable D and N region heterogeneity and that a minimum of eight different VL can be expressed in this antibody response (Scott *et al.*, 1991). The repertoire can be divided into two types based on the VL expression. One type of antibody repertoire contains antibodies that use the product of a single VκII gene, A2, which is dominantly expressed among anti-Hib-PS antibodies. The A2-VκII in anti-Hib polysaccharide antibodies appears to be germline in its sequence and has arginine at a complementarity determining region-3 (CDR-3) as an N region amino acid (Scott *et al.*, 1989). This amino acid is necessary for the strong binding of the antigen that has a negative charge under physiological conditions. In contrast, the other antibody repertoire is comprised of a multiple VL, which includes products of at least 2 VκI genes, another VκII gene identified in one subject, a VκIII gene, a VκIV gene, and at least one Vl gene. All of these contain somatic mutations.

The immune response is variable with different age groups. For instance, the anti-Hib antibody response in young children is weaker than that in adults and the avidity of child anti-Hib antibodies is far below that of adults (Schlesinger and Granoff,

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1992; Lucas *et al.*, 1993; Chung *et al.*, 1995). There are differences in the degree of the affinity maturation of Igs between adults and children. The process of affinity maturation of antibodies includes isotype switching, junctional flexibility at the CDR-3, somatic mutations and the addition of N nucleotides. The usage of variable (V), diversity (D) or joining (J) genes is restricted in the antibody production of fetal and neonatal mice (Feeney, 1992a; Victor *et al.*, 1994), or piglets (Sun *et al.*, 1998). The restriction of J gene usage in the early ontogeny of human beings was also reported (van Es *et al.*, 1993). Child and adult Ab repertoires differ strikingly at the molecular level in N region diversification and Ig CDR-3 size distribution, as well as in the immunoglobulin (Ig) gene utilization. Immunoglobulins from neonatal mice have germline in their V sequences and they have no N region amino acids (Feeney, 1990, 1992b). Most of the larval immunoglobulin samples in *Xenopus laevis* contained a shorter CDR-3 than that for adults (Desravines and Hsu, 1994) and this difference in Ig CDR-3 size distribution was also observed in human beings (Gokmen *et al.*, 1998). The somatic hypermutation of Ig genes, and selection of cells expressing mutations with improved affinity for antigens, are the molecular and cellular processes underlying the maturation of antibody affinity. The mutation frequency increased with age (Riding *et al.*, 1997, 1998). This shows the sequence, or length variability at CDR-3 regions during ontogeny. The variability in the CDR-3 of anti-Hib antibodies seems to be taken into account for the low affinity of the child anti-Hib Abs.

Antibodies using the VκII-A2 gene segment predominate the human antibody repertoire to Hib-PS (Scott *et al.*, 1989, Han *et al.*, 1998, Lucas *et al.*, 1998). We therefore, hypothesized that there is a restriction of the J gene usage for the formation of kappa light chains of anti-Hib antibodies in children. We also expected that child kappa chains do not have arginine as an N region amino acid at the CDR-3 region of child anti-Hib light chains. This accounts for the low affinity of child antibodies. The restriction of J gene usage, and the lack of N region amino acids in child anti-Hib Abs, was tested in this paper. However, since it is very difficult to get enough sera for the purification of clonal antibodies from children less than two years of age, we cloned and sequenced cDNAs obtained from individuals immunized with Hib antigens.

Materials and Methods

Immunization and Cell Preparation After obtaining an informed consent, 39 children and 8 adults were immunized with the Hib-Tetanus toxoid (Hib-TT) vaccine (Trial HIT 34494; Pasteur Merieux) according to the manufacturer's protocol. Children were boosted two times with the same vaccine at a month's interval. Immune sera were obtained 4 weeks after the final immunization. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Histopaque 1077 (Sigma) purification.

Reverse Transcription-Polymerae Chain Reaction (RT-PCR)

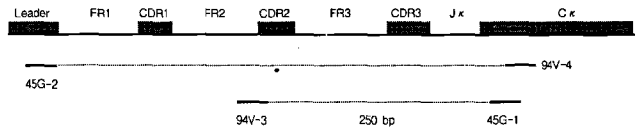

Fig. 1. Primer binding sites for the amplification of A2-Jκ-Cκ recombinants. 45G-2 and 94V-4 are the primers for the first PCR. 94V-3 and 45G-1 are the primers for the second nested PCR.

RNAs were isolated from 10^6 PBMCs by the modified method of Chomczynski (Chomczynski and Sacchi, 1987). Briefly, 1 ml of Trizol (Gibco BRL) was added to each cell pellet. After incubation at room temperature for 5 min, 0.2 ml of chloroform was added and vortexed. The samples were centrifuged at 10,000 × g for 15 min. The aqueous phase containing the RNA was transferred to a clean tube, mixed with 1 ml of isopropyl alcohol and placed in ice for 10 min. Precipitated RNA was pelleted by centrifugation, washed once with 75% ethyl alcohol, dried and resuspended in 20 μl of diethylpyrocarbonate-treated water. A cDNA synthesis was performed in 40 μl of reverse transcriptase buffer containing RNase inhibitor (Sigma), 4 μg of oligo$(dT)_{15}$, 1 mM deoxynucleotide triphosphate (Boehringer Mannheim) and 200 U of MMLV reverse transcriptase (Gibco BRL). After incubation at 42°C for 60 min, the samples were briefly boiled in order to stop the cDNA synthesis and then stored at -20°C. For the amplification of the A2-derived kappa light chain variable region genes, nested PCR was applied. The 5' sense and 3' antisense primers for the first PCR were GGGAATTCATG GACATG (AG)(AG)(AGT)(CT)CC(ACT)(ACG)G(CT)(GT)CA (CG)CTT (45G-2) and CAGATTTCAACTGCTCAT (94V-4), respectively. The primers for the second nested PCR were CTGATCTATAAGTTTCCAAC (94V-3) and CAACGGATCCTC TGATGGCGGGAAGAT (45G-1), respectively. Figure 1 represents the binding sites of the PCR primers to the κ region of the human immunoglobulin gene. For PCR, 2.5 ml of the cDNA were amplified in 0.5 ml GeneAmp (Perkin Elmer) tubes containing PCR buffer and the appropriate sense and antisense primers (0.2 mM each) in a 50 ml final volume. PCR tubes were overlaid with mineral oil and amplified for 40 cycles using a thermal reactor (Hybaid). Each sample was denatured at 94°C for 1.25 min, annealed at 55°C for 1.5 min, and extended at 72°C for 2 min. PCR products and molecular weight markers were separated in 2% NuSieve GTG agarose (FMC) using a Tris-borate buffer.

Subcloning of PCR Products The PCR products were cloned using a TA cloning kit (Invitrogen). Briefly, 1 μl of PCR products were inserted into the lacZ alpha region of a pCR 2.1 vector using 4 units of T4 DNA ligase at 14°C for 12 hours. Competent cells, INValphaF, were pretreated with 2 μl of 0.5 M 2-mercaptoethanol and mixed with 2 μl of ligates. The mixtures were incubated on ice for 30 minutes and subjected to heat shock at 42°C for 30 sec. The transformed cells were incubated in 450 μl of a SOC medium by rotating at 37°C for an hour and then selected on a LB plate that contained Ampicilin and x-gal. The bacteria of white colonies were cultured in a liquid LB-Ampicillin media and then harvested. Plasmids were prepared using a Wizard-miniprep kit

(Promega) and the insert size was checked by *EcoRI* digestion.

DNA Sequencing and Analysis DNA sequencing was performed using a Sequenase version 2.0 (U.S. Biochemicals) kit following the manufacturer's protocols. Briefly, 3 μg of plasmid DNA was denatured in a NaOH solution and precipitated in ethanol. Dried DNA was then dissolved in 6 μl of sterile H_2O. Dissolved DNA was mixed with one picomole of primer (94V-3) and 2 μl of sequenase buffer at 65°C for 2 min. It was then annealed slowly to below 35°C for 30 min. The labeling reaction was performed by mixing 10 μl of the annealed DNA mixture with a labeling mixture that contained 1 μl of DTT, 0.5 μl of [^{35}S]dATP and T7 DNA polymerase. Mixtures were incubated at room temperature for 5 min and 3.5 μl aliquots of labeled DNA were transferred to each termination tube (G,A,T,C). They were then incubated at 37°C for 5 min and the reaction stopped by adding 4 μl of a stop solution to each reaction tube. Samples were heated to 75°C for 2 min and loaded onto a 6% polyacrylamide gel containing 8 M urea that was warmed to 50°C. After the electrophoresis, gels were dried in a vacuum heat gel dryer and exposed to X-ray film at -70°C. Part of the samples was sequenced with an automatic sequencer ABI 377 (Perkin Elmer) after the labeling of samples with a dye sequencing kit (Boehringer Mannheim). These sequences were then compared to those reported previously (Cox *et al.*, 1994; Johnson *et al.*, 1995; Atkinson *et al.*, 1996).

Results

Usage of the J gene In the murine system, usage of the V, D, or J genes is restricted during the Ab formation in fetal and neonatal mice. Though the immune response against Hib in young children differs from that in adults (Schlesinger and Granoff. 1992; Chung, *et al.*, 1995), both use the A2 gene for the synthesis of Vκ. We, therefore, expected a different usage pattern of the Jκ genes for the production of anti-Hib kappa chains between children and adults. We examined the usage of the Jκ genes for the synthesis of anti-Hib kappa light chains. In order to test the usage of the Jκ genes, we isolated 26 cDNA clones from 4 adults and 54 clones from 12 children. The partial V sequences of these clones were almost (95-100%) homologous to the A2 germline sequence. The usage of five Jκ genes in adult clones was distributed evenly, but child clones used the Jκ2 and Jκ3 genes predominantly (87%) among the five Jκ genes (Table 1). Among clones we tested, one of the child A2-Jκ2 recombinants and two of the adult A2-Jκ2 recombinants have termination codons within their sequences.

Somatic Mutations of the A2 gene The anti-Hib antibodies with the A2 gene product have a germline sequence generally. However, many somatic mutations were observed in the framework regions, as well as in CDRs of anti-Hib Abs. It was particularly interesting that the rate of somatic mutations in Korean adults was 4.3 mutations out of 100 bases. This is almost twice the mutation rate of the child Abs (Table 2). The

Table 1. Jκ usage of A2-Jκ recombinants in Korean antibodies elicited by a Hib vaccine.

Jκ usage	Number of Clones (%)	
	Adults	Children
Jκ1	3 (12)	2 (4)
Jκ2	4 (15)	25[a] (46)
Jκ3	8 (31)	22 (41)
Jκ4	4 (15)	2 (4)
Jκ5	7[b] (27)	3 (5)

[a]One of these clones has a termination codon in A2 region.
[b]Two of these clones have termination codons in their V regions.

Table 2. Somatic mutations of A2-Jκ recombinants in Korean antibodies elicited by a Hib vaccine.

Jκ USAGE	Mutations per base(10^{-2}) (Mutations/clones)	
	Children	Adults
Jκ1	3.3 (6/2)	4.1 (11/3)
Jκ2	2.5 (57/25)	3.6 (13/4)
Jκ3	2.2 (43/22)	3.1 (22/8)
Jκ4	1.1 (2/2)	3.3 (12/4)
Jκ5	1.5 (4/3)	6.8 (43/7)
Average	2.3 (112/54)	4.3 (101/26)

Jκ-dependent somatic mutation also could be seen (shown in Figure 2.) The A2-Jκ2 and A2-Jκ3 recombinants have a bias toward a specific pattern of somatic mutations, whereas somatic mutations were dispersed in A2-Jκ5 recombinants. The somatic mutation was the highest in the adult antibodies with Jκ5, whereas such Jκ dependency of somatic mutation was not observed in child antibodies (Figure 2, Table 2). As expected, the somatic mutation was the highest at the CDR-3 region. The rate of somatic mutations in an adults CDR-3 region was higher than a child's in this region (Table 3). Replacement over the Silent mutation (R/S) ratio was also higher in adults than in children. Interestingly, the pattern of somatic mutations found in children who have Jκ2 or Jκ3 was almost identical in all of the clones. Somatic mutations in them were found in the framework-3 region and the constant region, as well as in the CDR-3 region. Especially, serine-76 and serine-91 were substituted with arginine and glycine, respectively, in children who have Jκ2. Adult clones, however, were not mutated at these sites (Figure 3).

Variability of CDR-3 region The recombination of V and J genes, somatic mutation of V regions and/or the addition of N region amino acids form antibody diversity, especially the diversity of light chains. Here, we focused on the A2 gene that is a dominant gene for the anti-Hib Ab response. Jκ gene usage was restricted in child antibodies as described in a

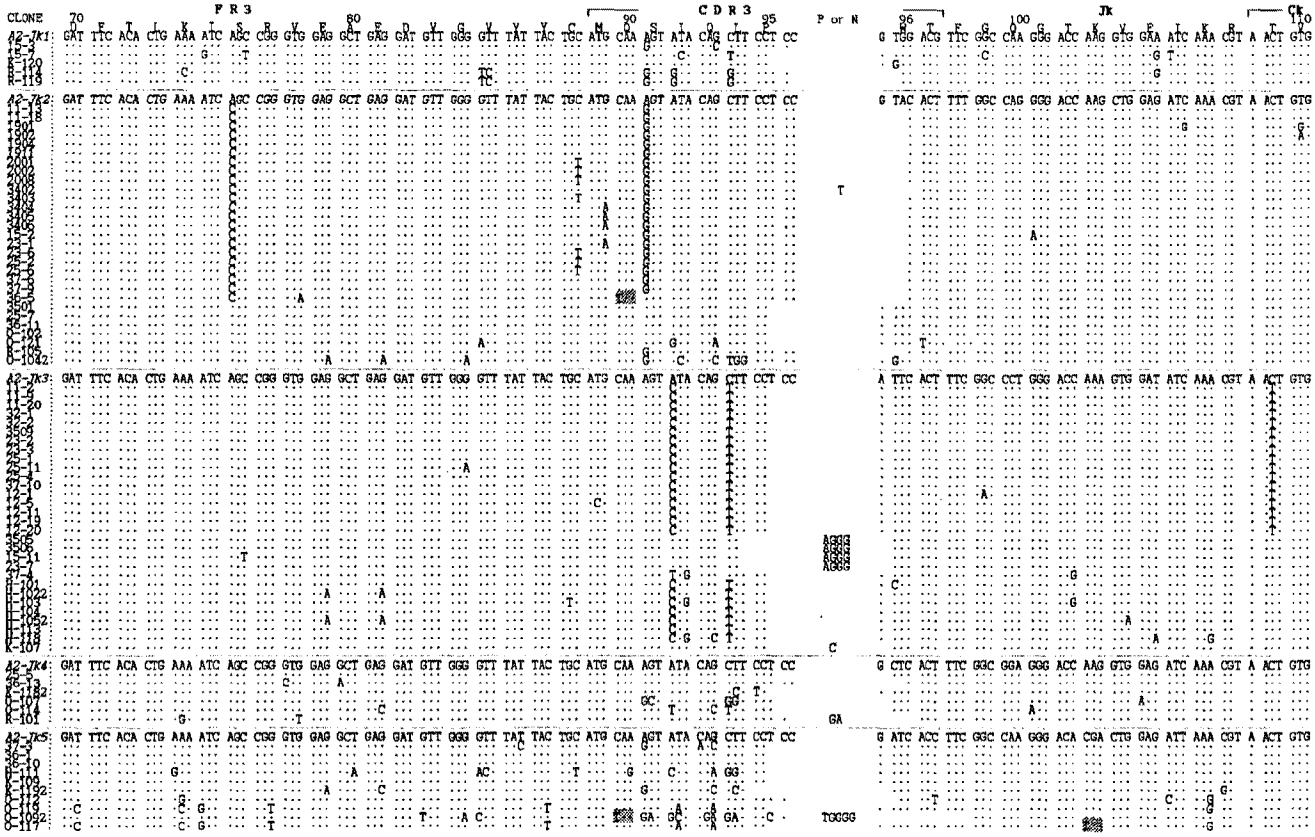

Fig. 2. Partial nucleotide sequences of cloned A2-Jκ-Cκ recombinants. Clones with initial numbers are clones from child samples. Those clones with initial alphabets are from adult samples. The center space is P or N region in which nucleotides belong to neither the A2 (left side) nor Jκ (right side) genes. Numbering of amino acids is according to Johnson *et al.* (1995) and stop codons are shaded.

Table 3. Site-dependent somatic mutations of A2-Jκ recombinants in Korean antibodies elicited by a Hib vaccine.

Group	Mutations per base(10^{-2}) (Replacement/silent mutation)				
	FR3	CDR3	Jκ	Cκ	Mean
Children	1.0(2.1)	6.5(17.5)	3(2.5)	1.5(9.0)	1.7(6.0)
Adults	2.2(1.0)	11.4(9.3)	7(0.9)	1.1(7.0)	3.1(2.7)

previous section. The analysis of clonal antibodies from adult Caucasians showed that they have invariant arginine at the joint of A2, as well as J gene products as an N region amino acid (Scott *et al.*, 1989). This basic amino acid helps with the binding of negatively charged antigens to antibodies. We expected that there would be no arginine at the joint between A2 and Jκ gene products of child Abs since the child anti-Hib abs showed a low affinity to the antigen (Schlesinger and Granoff, 1992). We found arginine in the CDR-3 regions only in A2-Jκ1, A2-Jκ4 and A2-Jκ5 recombinants, however, these were not formed by the base addition but by base substitutions. An A2-Jκ5 clone, named O-109, has a codon for arginine in the CDR-3 region, but the sequence also has a stop codon in the upstream of the CDR-3 coding region (Figure 3). Therefore, only A2-Jκ1 and A2-Jκ4 recombined clones have arginine in their CDR-3 regions. Arginine was found in one clone of child antibodies (out of four), but half of the adult antibodies (3/6 of A2-Jκ1, A2-Jκ4 clones) had arginine in their CDR-3 regions (shown in Figure 3.) These arginines are not formed by the N region addition of nucleotides, but by somatic mutations of A2 or J genes.

Discussion

Children are the ones who are the most susceptible to Hib diseases. However, the studies on the human antibody response to Hib polysaccharide have been done mainly in adults. The anti-Hib Ab repertoire is divided into two groups based on VL expression (Scott *et al.*, 1989). One type of antibody repertoire contains antibodies that use the product of a VκII gene, A2. The A2-VκII in anti-Hib-PS Ab appears to be germline in its sequence. In contrast, the other antibody repertoire is comprised of multiple, less frequently used VL. This includes products of the 2 VκI genes, another VκII gene, a VκIII gene, a VκIV gene, and a Vl gene, all of which contain somatic mutations. Among Vκ genes, the A2 gene is

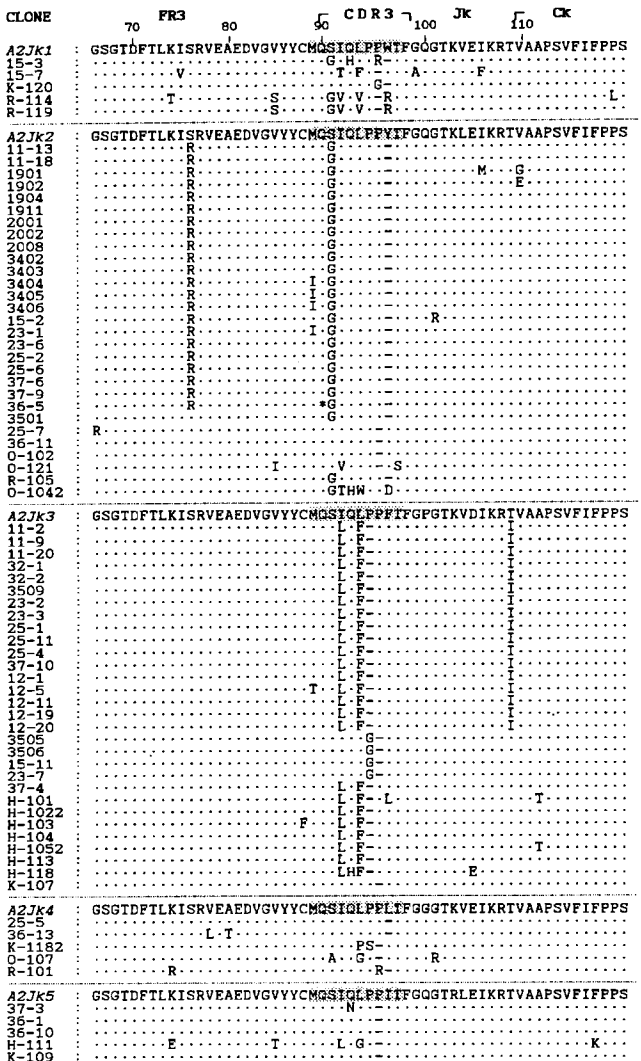

Fig. 3. Translated amino acid sequences of A2-Jκ-Cκ recombinants from Korean children and adults. Germline sequences were given on the top of recombinant groups. Asterics denote stop codons. Dashed lines means a lack of corresponding amino acid at that site.

expressed predominantly in Caucasian anti-Hib Abs. The A2 gene product not only has a germline sequence, but it also has an arginine at the CDR-3 region as an N region amino acid. This amino acid seems to be necessary for the strong binding of Hib antigen that has negative charges under physiological conditions. The A2 gene expression is also predominant in Korean anti-Hib Abs, even though it is different from the Caucasians genetically. The differences of haplotypes result in the different expression of the A2 gene (Chung *et al.*, 1993).

In animal models, there are restrictions of the V(D)J gene usage in infants (Feeney 1992; Victor *et al.*, 1994). Young animals have few somatic mutations in their V region. They also have no N region amino acids in the CDR-3 region (Feeney, 1990, 1992a). It has not been reported whether or not this phenomenon also takes place in human systems. The anti-Hib response in young children is weak and they are divided into two groups in terms of antibody affinity. One group has a relatively strong affinity and the other has a very weak affinity to the antigen (Schlesinger and Granoff, 1992). We assumed, therefore, that the anti-Hib response in children might be different than that of adults as they do not have arginine in their CDR-3 region. This can be verified by the amino acid sequencing of cloned antibodies. However, since it is very difficult to get enough sera for the purification of antibodies from children under two years old, we amplified and cloned cDNAs obtained from children immunized with Hib antigens and sequenced them. We immunized 8 adults and 39 children with a Hib-TT vaccine and isolated PBMC from the blood taken from these individuals. Although we tried to amplify the V region by RT-PCR, we identified no bands on the gel. We, therefore. tried to amplify the internal sequence again by a nested PCR. Using this method, we obtained 250 bp of fragments. We then inserted the fragments into cloning vectors and cloned PCR fragments and sequenced them. In this study, we were unable to verify whether or not the A2 sequences are from the B cells that produce the antibodies that are specific to Hib polysaccharide. There are more than 70 V genes on chromosome 2 on which the genes of the immunoglobulin kappa chain locate. The A2 gene is used predominantly for the formation of anti-Hib Ab (Scott *et al.*, 1989) and the lack of the A2 gene is correlated with the high incidence of Hib-related diseases (Feeney *et al.*, 1996). Moreover, Abs using the A2 gene segment predominates the human antibody repertoire to the Hib polysaccharides (Lucas *et al.*, 1998). This implies that the A2 gene expression is predominant in individuals exposed to the Hib antigens, but this does not mean that all A2 genes amplified are from the B cells that produce the antibodies specific to Hib antigens. Few of the child clones used Jκ1, Jκ4 or Jκ5, but most of them used Jκ2 and Jκ3. This is the first case that shows a restriction in J gene usage in young children. Child clones with Jκ2 or Jk3 showed unique characteristics. Their somatic mutation was uniform, whereas, the somatic mutations of clones with Jκ1, Jκ4 or Jκ5 were dispersed. We found termination codons in parts of A2-Jκ2 recombinants. Several types of hypermutation were reported in the kappa light chain V region and a stop codon was identified in the open reading frame of the sequence (Kosmas *et al.*, 1996). Nevertheless, they identified no Jk dependent somatic mutations.

One goal of this study was to test whether or not there is an arginine as an N region amino acid in the child anti-Hib Abs. The lack of N region amino acid can be deduced by the low affinity of child anti-Hib antibodies and the limited N region diversity of neonatal mice (Carlsson and Holmberg, 1990; Feeney, 1990). In all of the child clones we tested, we found no additional arginine that is positioned between the V and J sequences, but one clone had arginine in the CDR-3 region.

This amino acid was not an N region amino acid and it was formed by somatic mutation of a preexisting gene segment. We also analyzed adult clones and many of them had arginine in their CDR-3 regions, but these were also formed by somatic mutation of preexisting sequences. An interesting observation is that the pattern of somatic mutation was different between groups using different Jκs. The Jκ-dependent somatic mutation has not yet been reported. We also analyzed the A2/A18 genomic sequences in order to compare somatic mutations between genomic DNAs and processed RNAs. All of the genomic sequences obtained were compared with the germline sequences described previously (Atkinson *et al.*, 1996). The fragments we obtained, however, had recombination signal sequences. This means that the genomic DNAs we obtained are germline DNAs. We, therefore, could not determine if the base substitutions found in the A2 gene is formed during the transcriptional stage. In this study, several clones can arise from a single individual clone, but this possibility was eliminated by the comparison of sequences between clones. We analyzed sequences from Korean individuals in this study. As mentioned, the expression of the A2 gene can be affected by the haplotype. Therefore, such restriction in Jκ gene usage and somatic mutation may have been caused by the differences between the races. This possibility should be studied further.

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