Clin Exp Immunol 1997; **108**:366–374

Somatic hypermutation of immunoglobulin genes in human neonates

J. RIDINGS*‡, I. C. NICHOLSON*, W. GOLDSWORTHY†, R. HASLAM†, D. M. ROBERTON‡ & H. ZOLA*‡
Child Health Research Institute, †Women's and Children's Hospital and ‡Department of Paediatrics, University of Adelaide, Adelaide, Australia

(Accepted for publication 16 January 1997)

SUMMARY

The antibody response in the young infant is limited in several ways; in particular, responses generally are of low affinity and restricted to IgM. This raises the question whether the affinity maturation process, consisting of somatic mutation of immunoglobulin genes coupled with selection of high-affinity variants, is operative in the neonate. Re-arranged V_H6 genes were amplified by polymerase chain reaction (PCR) from cord blood and from peripheral blood of infants. Heteroduplex analysis detected mutation in only 2/18 cord blood samples, while mutations were seen from about 10 days of age onwards. Cloning and sequencing of mutated neonatal V_H6 genes showed that mutated sequences contained relatively few mutations (one to three mutations per sequence) compared with published values of about 10 in adult IgM sequences. Selection was not evident in the majority of neonatal samples. Thus mutation can occur in the human neonate, but is minimal and generally not accompanied by selection. The age at which affinity maturation develops effectively is yet to be defined.

Keywords immunoglobulin somatic mutation neonatal antibody response affinity maturation immunoglobulin genes

INTRODUCTION

Human neonates have a limited ability to generate effective antibody responses. They make largely IgM antibodies, respond poorly to certain types of antigen, and do not develop an effective memory response when exposed to antigen [1]. This immunological immaturity reflects in part a lack of immune stimulation *in utero*, but also is, in part, developmental. Most neonatal B cells belong to the $CD5^+$ subset, which is associated, in the adult, with low-affinity, cross-reactive and often autoreactive antibodies, usually restricted to the IgM class [2]. The observed immunological immaturity implies a deficit in the chain of events that leads to the generation of memory, of high-affinity antibody and of class-switched antibody. This chain of events includes cognate interaction between T and B cells and the germinal centre reaction.

The process of cognate interaction depends on adequate expression and function of a number of ligand-pairs on the interacting cells [3]. Among the known properties of neonatal cells that may contribute to unproductive cognate interaction are the relative lack of CD45RO T cells [4,5] and the inability of neonatal T cells to express CD40 ligand, under certain activation conditions [6,7]. However, numerous other phenotypic differences between neonatal and adult cells may also be involved, including a relative deficit, in the neonate, of cytokine receptor expression [8] and an activated B cell phenotype [9].

Correspondence: Professor Heddy Zola, Child Health Research Institute, Women's and Children's Hospital, North Adelaide 5006, Australia.

Affinity maturation is the process by which the initial, low-affinity antibody response changes to a predominantly high-affinity response. The increase in affinity is achieved by mutation of antibody variable region genes and subsequent selection of those cells which make high-affinity antibody. Mutation and selection take place principally in germinal centres, and depend on cognate interaction [10]. Although generally associated with $CD5^-$ B cells and downstream immunoglobulin isotypes, mutations are found in $CD5^+$ B cells [11–14], and in IgM [15–17]. Within the germinal centre, the onset of somatic mutation precedes isotype switching [18].

We [19] and others [14,15] have shown that B cells in cord blood from healthy full-term human infants lack mutations in the immunoglobulin genes. Somatic mutations of immunoglobulin genes have been demonstrated at 4 years of age [20], but knowledge of mutations at time points between birth and 4 years is lacking. The aim of this study was to determine to what extent human neonates could mutate and select immunoglobulin genes.

MATERIALS AND METHODS

Samples

Cord blood (up to 10 ml) was collected into heparin. Peripheral blood samples (120–600 μl) were obtained from infants aged from 1 day to 2 months, who either were hospitalized with acute infection or were free from apparent infection and were being

Table 1. Polymerase chain reaction (PCR) amplification of V_H6 sequences derived from peripheral blood lymphocytes from infants. Detection of mutated V_H6 sequences using heteroduplex analysis

Sample code	Age (days)	Gestation† (weeks)	Transfused‡	Diagnosis§	V_H6 rearrangements detected¶	V_H6 mutations detected**
NI-13*	1	36	−	Congenital CMV	+	−
NI-8 (C10)*	1	34	−	Healthy	+	−
NI-19	2	31	−	Mother, Hep C Ab^+	+	−
NI-3*	2	33	−	GBS septicaemia and meningitis	+	−
NI-14*	3	31	−	Healthy	+	−
NI-7*	3	24	+	Group F streptococcal septicaemia	+	−
NI-11	4	33	−	Mother, Hep B Ag^+, neonate immunized at 48 h	+	−
NI-1	4	32	+	GBS sepsis	+	−
NI-16	5	33	−	GBS sepsis	+	−
NI-18 (C15)*	5	40	−	Mother, Hep B Ag^+, neonate immunized at 5 h	+	−
NI-26*	6	27	+	Presumed sepsis, cultures negative	+	−
NI-9	6	34	−	*Escherichia coli* septicaemia	+	−
NI-3a*	7	33	+	GBS sepsis	−	
NI-4	7	34	−	Mother, Hep B Ag^+, neonate immunized at 5 h	+	−
NI-5	7	34	−	Mother, Hep B Ag^+, neonate immunized at 5 h	+	−
NI-27*	7	25	+	GBS septicaemia and meningitis	+	−
NI-21	10	30	+	Healthy	+	−
NI-10	10	26	+	*Staphylococcus epidermidis* septicaemia	+	−
NI-23	10	38	−	Healthy	+	−
NI-22	10	34	+	*Herpes simplex* type 2	+	+
NI-17	10	30	+	Thrombophlebitis (i.v. site)	+	+
NI-12	10	35	−	*E. coli* urinary tract infection	+	+
NI-20*	14	31	+	Necrotising enterocolitis, peritonitis	+	+
NI-13a*	15	36	+	Congenital CMV	+	+
NI-24	16	33	−	Thrombophlebitis (i.v. site)	−	
NI-20a*	16	31	+	Necrotising enterocolitis, peritonitis	+	+
NI-25	17	38	+	Healthy	+	+
NI-26a*	18	27	+	Presumed sepsis, negative cultures	+	+
NI-27a*	18	25	+	GBS septicaemia and meningitis	+	+
NI-6	20	38	−	*S. aureus*, skin abscess	+	+
NI-20b*	22	31	+	Necrotising enterocolitis, peritonitis	+	+
NI-14a*	40	31	+	Respiratory syncytical viraemia	+	−
NI-2	45	28	+	*S. epidermidis* septicaemia	+	+
NI-7a*	50	24	+	Group F streptococcal septicaemia	+	+
P-16	60	26	−	Healthy	+	−
P-17	60	26	−	Healthy	+	+
P-18	60	38	−	Healthy	+	+
P-19	60	40	−	Healthy	+	+

* Multiple blood samples were taken from these infants over the duration of the infection. Letters a and b immediately following sample code denote second and third samples, respectively. NI-8 and NI-18 samples were obtained from the same individual as cord blood samples C-10 and C-15, respectively.

† Gestation was determined from the mother's last menstrual period. Term neonates have a gestation of 37–40 weeks, and premature neonates are less than 37 weeks.

‡ Those infants who received blood transfusions (+) were administered reconstituted packed cells at least once, and often multiple platelet transfusions. All blood products were gamma-irradiated before use.

§ All infants showed evidence of clinical infection, unless specified as healthy, or were born to mothers who were positive for hepatitis B surface antigen or hepatitis C antibody. Confirmation of infection was based on clinical symptoms, a blood film appearance consistent with sepsis, and a positive microbiological report (in most cases). Thrombophlebitis is a presumed localized *S. epidermidis* infection. GBS, Group B streptococcus.

¶ Two-round PCR protocol followed by agarose gel electrophoresis as described in Materials and Methods.

** Heteroduplex analysis of V_H6 PCR products as described in Materials and Methods.

bled for reasons unrelated to this project. Informed consent was obtained and samples were taken by heel/finger prick or by arterial catheter where possible. All blood samples were anti-coagulated with lithium heparin (500 U for cord blood and 15 U for peripheral blood samples). Infants were assessed clinically and serologically to determine their infection status (see footnotes to Tables 1 and 2). The studies were approved by the Ethics Committee of the Queen Victoria Hospital (Women's and Children's Hospital), working under the principles of the Helsinki declaration.

Preparation of cells

Mononuclear cell fractions were isolated by density centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway). Small blood samples (< 600 μl) were centrifuged at 350 ***g*** for 4 min in a

Table 2. Frequency of mutated V_H6 genes from cloned V_H6 rearrangements

Sample code	Age (days)	Diagnosis	Number of mutated clones*	%
C-13	Cord blood	Mother, Hep C Ab^+	6 of 27	(22)
NI-13	1	Congenital CMV	7 of 70	(10)
NI-12	10	*Escherichia coli* urinary tract infection	9 of 60	(15)
NI-17	10	Thrombophlebitis (i.v. site)	5 of 69	(7)
NI-13a	15	Congenital CMV	27 of 80	(34)
NI-6	20	*Staphylococcus aureus*, skin abscess	5 of 30	(17)
NI-2	45	*S. epidermidis*, septicaemia	4 of 21	(19)
NI-7a	50	Group F streptococcal septicaemia	0 of 35	(0)
Control VH6 -A†	—	—	3 of 52	(6)
Control VH6 -B†	—	—	3 of 32	(9)

* Detection of mutated V_H6 sequences from cloned rearrangements using heteroduplex analysis (see Fig. 3).

† Known germ-line V_H6 rearrangements (control V_H6 -A and -B) underwent the same amplification protocol, cloning steps as the samples (Fig. 1) and V_H6 clones were analysed by the heteroduplex procedure (Fig. 3). The frequency of these heteroduplex-positive clones gives an indication of mutations potentially introduced by the amplification system.

microfuge. Mononuclear cell layers were collected and washed once with PBS (3800 ***g*** for 2 min), erythrocytes were lysed with 1 ml of hypotonic ammonium chloride [21] and mononuclear cell fractions were washed again in PBS (3800 ***g*** for 2 min).

Genomic DNA extraction

Genomic DNA was prepared from the mononuclear cell fractions using the method described previously [22]. Cell pellets were resuspended in 400 μl of lysis buffer (50 mM KCl, 10 mM Tris–HCl pH 8·3, 2·5 mM $MgCl_2$, 0·1 mg/ml gelatin, 0·45% Nonidet P-40, 0·45% Tween 20) and were incubated with 80 μg Proteinase K (Boehringer, Mannheim, Germany) for 5 h or overnight at 55°C with mixing. The DNA was ethanol-precipitated in the presence of 3·5 mM $MgCl_2$, 20 mM sodium acetate and 20 μg glycogen and pelleted by centrifugation at 15 000 ***g*** for 20 min at 4°C, washed in 70% ethanol and centrifuged at 15 000 ***g*** for 10 min. The DNA pellet was dried (Speedyvac centrifugal drier; Savant Instruments, Farmingdale, NY) and resuspended overnight at 4°C in 12 μl of sterile water.

Polymerase chain reaction amplification of rearranged V_H6-D-J_H genes

A two-round polymerase chain reaction (PCR) protocol was used to amplify genomic V_H6-D-J_H rearrangements. PCR reactions were performed in 25-μl volumes that contained 50 ng of each primer, 50 mM KCl, 10 mM Tris–HCl pH 9·0, 2 mM $MgCl_2$, 0·2 mM of each dNTP, 0·5 U Taq DNA polymerase (Promega, Madison, WI) and 5·0 μl of DNA template. All reactions were performed using a Perkin Elmer/Cetus Model 480 Thermal Cycler. The amplification protocol for each round comprised 35 cycles of 1 min at 94°C (denaturation), 1 min at 60°C (annealing), and 2 min at 72°C (extension). The first cycle was preceded by an extra 5 min at 94°C, and the final extension step was continued for 10 min.

In the first round, the V_H6 family specific VH6/Pst primer (5′-CAGGTACAGCTGCAGCAGTCAGG-3′ [23]) and the consensus Jcon/Bam primer (5′-TGACCAGGATCCCTTGGCCC-CAG-3′ [22]) were used to amplify V_H6 rearrangements. DNA samples were titrated in the first round to determine the dilution which maximized the product in the second round. The second round primers (Fig. 1) amplified the V_H6 region using an internal V_H6-specific primer (VH6/ND; 5′-CCTGTGCCATCTCCGGG-GACAGTG-3′; [24]) and a consensus primer complementary to the final 20 bases of the germ-line V_H6 sequence (FWR3-anti; 5′-ACAGTAATACACAGCCGTGT-3′; [25]). First round DNA products were diluted 1 : 10 in sterile water before addition to the second round reaction. The primer locations are shown in Fig. 1.

Heteroduplex analysis of amplified V_H6 DNA

Second round PCR product (15 μl) was mixed with 5 μl of loading dye (40% sucrose, 0·25% bromophenol blue, 0·01% xylene cyanol). The mixture was denatured at 95°C for 10 min and then re-annealed on ice for 40 min. A 15-cm gel consisting of 10% acrylamide (37·5 : 1 acrylamide : bisacrylamide; BioRad, Hercules, CA) in 1·5 × Tris-borate-EDTA buffer was pre-electrophoresed in 0·5 × Tris-borate-EDTA buffer at 270 V for 1 h or until the buffer temperature reached 26–29°C, a range which gave best separation of heteroduplex bands. The entire mixture (20 μl) was loaded on the gel and electrophoresed at 270 V for 2 h at room temperature. Gels were stained with ethidium bromide and the DNA was visualized using ultraviolet transillumination.

Cloning of V_H6 rearrangements

V_H6-D-J rearrangements (first round PCR products) were amplified with VH6/ND and Jcon/Bam primers (Fig. 1). The PCR products were purified using Wizard PCR Preps (Promega) and ligated into the pGem-T vector (Promega). The ligation was used to transform competent *Escherichia coli* TG1 cells and recombinant colonies were selected by blue/white screening. Colonies were screened for the presence of an insert containing the V_H6 gene by PCR amplification using the internal V_H6-specific primers (VH6-ND and FWR3-anti). The product gave a band at 241 bp and was analysed for mutations using the heteroduplex method.

Heteroduplex analysis of cloned V_H6 [25]

Cloned V_H6 amplified product (5 μl) was mixed with unmutated V_H6 DNA (5 μl) which had been amplified with identical primers (VH6/ND and FWR3-anti). Loading buffer (5 μl) was added to the mixture and the conditions thereafter were as described above.

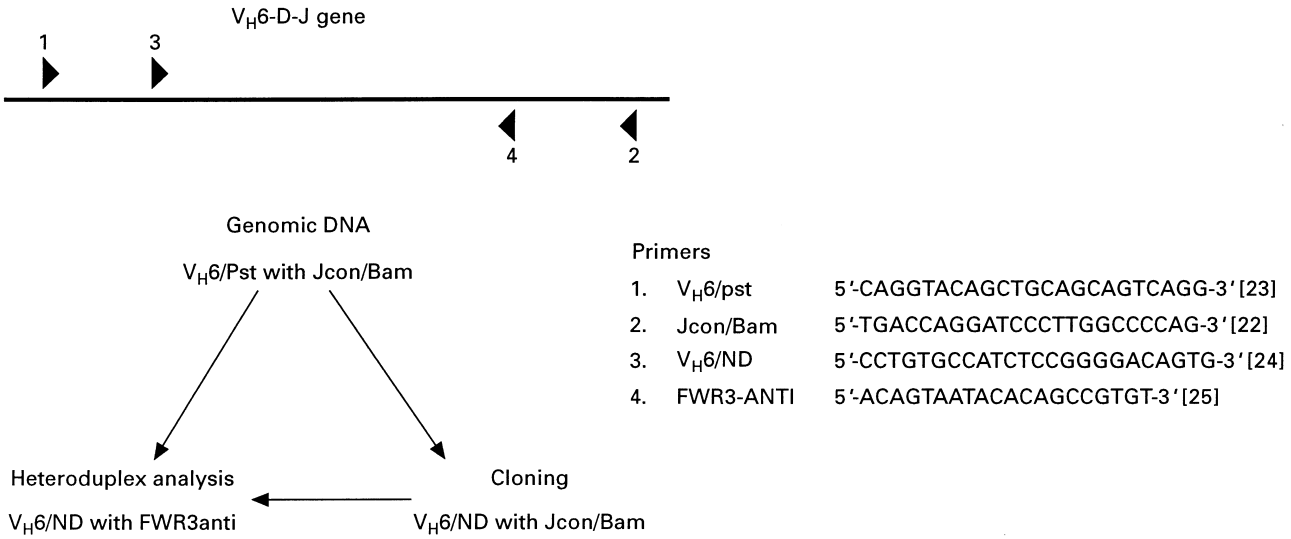

Fig. 1. Location of primers and amplification strategy used for isolation of V_H6-D-J rearrangements from genomic DNA, and for subsequent heteroduplex analysis of mutations and cloning for DNA sequence analysis.

Analysis of V_H6-D-J_H sequences

Clones which appeared mutated by heteroduplex analysis were selected for sequencing; for each donor one clone which did not give heteroduplexes was also sequenced. Sequencing templates were prepared from cloned V_H6-D-J_H inserts by PCR amplification using the M13-USP (5′-GTAAAACGACGGCCAGT-3′) and M13-RSP (5′-CACACAGGAAACAGCTATGACCATG-3′) primers. Each insert was sequenced in both directions using M13-USP and M13-RSP primers and the Taq DyeDeoxy Terminator Cycle Sequencing Kit on the Applied Biosystems 373 A Automated Sequencer (Perkin Elmer, Foster City, CA) operated by the Flinders University of South Australia (Adelaide).

The consensus sequence was determined for each clone from the complementary M13-USP and M13-RSP sequences using the SeqEd software (Applied Biosystems). This sequence was compared with the germ-line V_H6 sequence 6-1G1 [26] using the IBI MacVector software (Kodak, Rochester, NY), and any point mutations were identified.

RESULTS

Heteroduplex analysis of V_H6 DNA

V_H6 gene rearrangements could be detected in 18 of 20 cord blood specimens studied. Of these, only two samples showed mutations by heteroduplex analysis of V_H6 DNA amplified from polyclonal cell populations. Both of these samples were from neonates born to mothers who had tested positive for hepatitis C antibody; one of the

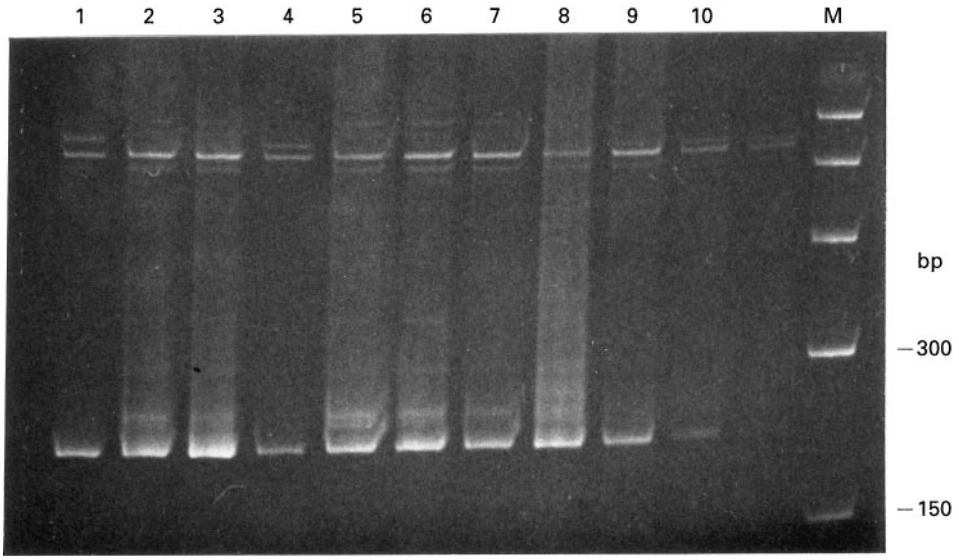

Fig. 2. Heteroduplex formation in uncloned DNA from neonatal and cord blood samples. The primers used to amplify V_H6 sequences without the CDR3 region, so that sequence variations can be attributed to mutation, are shown in Fig. 1. Lane 1, a cloned control (no heteroduplex); lane 2, a 4-month-old (heteroduplex-positive); lanes 3–8, infant samples; lanes 9, 10, cord blood samples. Lanes 2, 3, 5, 6, 7 and 8 show heteroduplex formation.

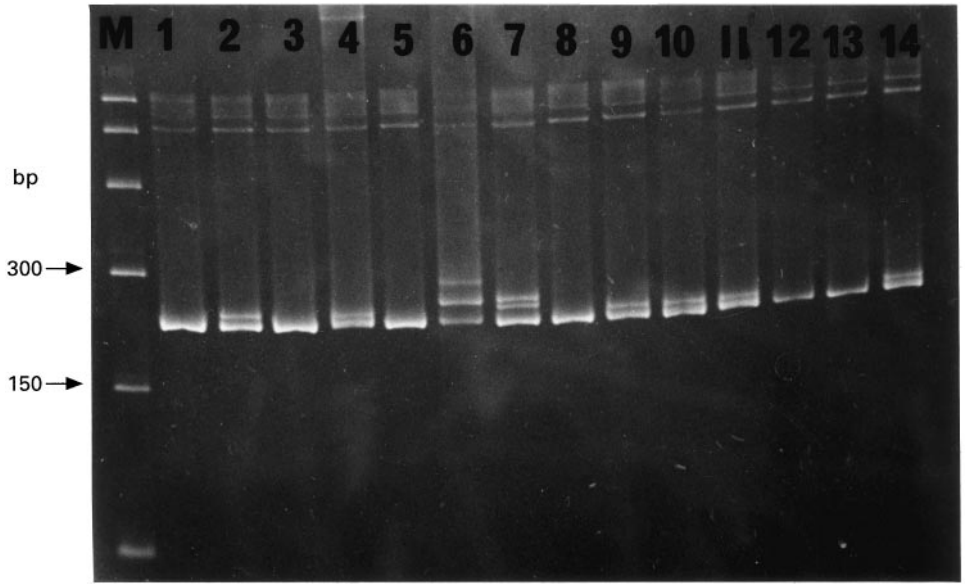

Fig. 3. Heteroduplex formation between unsequenced V_H6 clones and a known germ-line V_H6 sequence (cord 03) (lanes 2, 4, 6, 7, 9, 10 and 11). The sample in lane 6 was of higher molecular weight than expected for the amplified region of V_H6. Lane 13, cord 03 only; lane M, polymerase chain reaction (PCR) markers (Promega); lane 14, positive control: V_H6 clone (PBL14) known to contain five point mutations mixed with cord 03; lane 12, PBL14 only. Clones corresponding to lanes 2–11 were subsequently sequenced and heteroduplex-positive clones were confirmed as mutated, whilst heteroduplex-negative clones (lanes 1, 3, 5 and 8) were confirmed unmutated.

mothers also was positive for hepatitis B surface antigen. There was no evidence of infection in the infants.

Table 1 shows that V_H6 gene rearrangements could be detected in 36 of 38 samples from infants ranging in age from 1 to 60 days. Figure 2 shows a heteroduplex analysis gel. Mutations were not detected by heteroduplex analysis in neonates less than 10 days old. In infants 10 days or older, mutations were detected in 16 of 21 samples. Of four healthy infants tested at 2 months of age, three showed mutations by heteroduplex analysis.

Cloning followed by sequencing

Samples from infants with heteroduplex-positive mutations were studied further by cloning the amplified V_H6 rearrangements. In order to focus sequencing on mutated rearrangements, clones were screened for the formation of heteroduplexes with cloned V_H6 DNA known to be unmutated (Fig. 3). Clones showing heteroduplexes, together with a selection of heteroduplex-negative clones (at least one per donor), were sequenced. Table 2 provides a summary of the frequency of clones showing mutations by heteroduplex analysis. Table 3 summarizes the results of sequencing studies, and Fig. 4 shows the mutations. A small number of clones gave equivocal results and are omitted from Table 3 and Fig. 4. Of 52 clones which were classified as mutated by heteroduplex analysis, 48 were confirmed to be mutated by sequence analysis. Of 13 clones which did not show heteroduplex patterns but were sequenced for confirmation, one showed a single mutation. In two experiments, cloned germ-line V_H6 DNA was subjected to PCR amplification in order to detect mutations introduced by the procedure. The amplified DNA was cloned and analysed by heteroduplex analysis. These samples (Table 2) gave heteroduplex-positive clone frequencies of 6% and 9%.

Table 3. Summary of sequence analysis of mutated V_H6 rearrangements isolated from cord blood and infant peripheral blood lymphocytes

Sample code	Age (days)	Number of unique sequences	Range of mutations*	Mutation frequency†
C-13	Cord blood	4	1–3	1/121
NI-13	1	5	1–2	1/201
NI-12	10	6	1–3	1/121
NI-17	10	3	1–2	1/181
NI-13a	15	13	1–5	1/98
NI-6	20	1	1	1/241
NI-2	45	3	2	1/121
A	Adult	7	1–8	1/58

* Range of single point mutations identified in the V_H6 sequences for each of the samples cloned.
† Frequency of point mutations observed per base, calculated using mutated V_H6 sequences only.

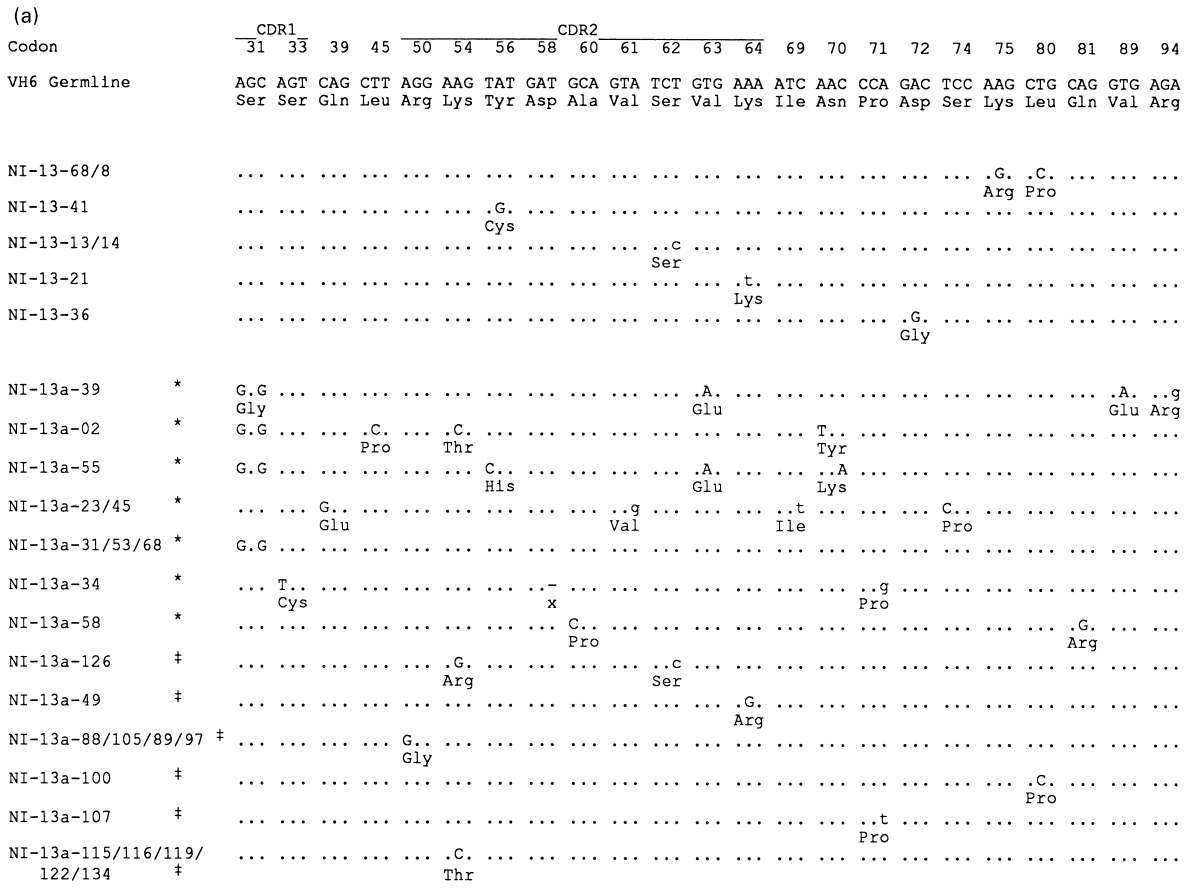

Fig. 4. (Continued on next page.) Nucleotide sequences of cloned V_H6 genes (see Tables 2 and 3 for donor details). (a) NI-13 and NI-13a are sequential samples taken at day 1 and day 15, respectively. (b) Samples NI-12, NI-17, NI-2 and C13. For all samples, clones are compared with the germ-line V_H6 sequence 6-1G1 [26]. Unmutated codons are not shown. Dots indicate nucleotide identity, upper case letters represent replacement mutations, lower case letters represent silent mutations, × indicates a single base deletion. Corresponding amino acids are shown below the mutated codon. CDR regions and codons are numbered according to Kabat *et al.* [37]. These sequence data are available from EMBL/GenBank/DDBJ under accession number U59327–U59374. *,‡ Clonally related sequences (i.e. sequences with an identical CDR3).

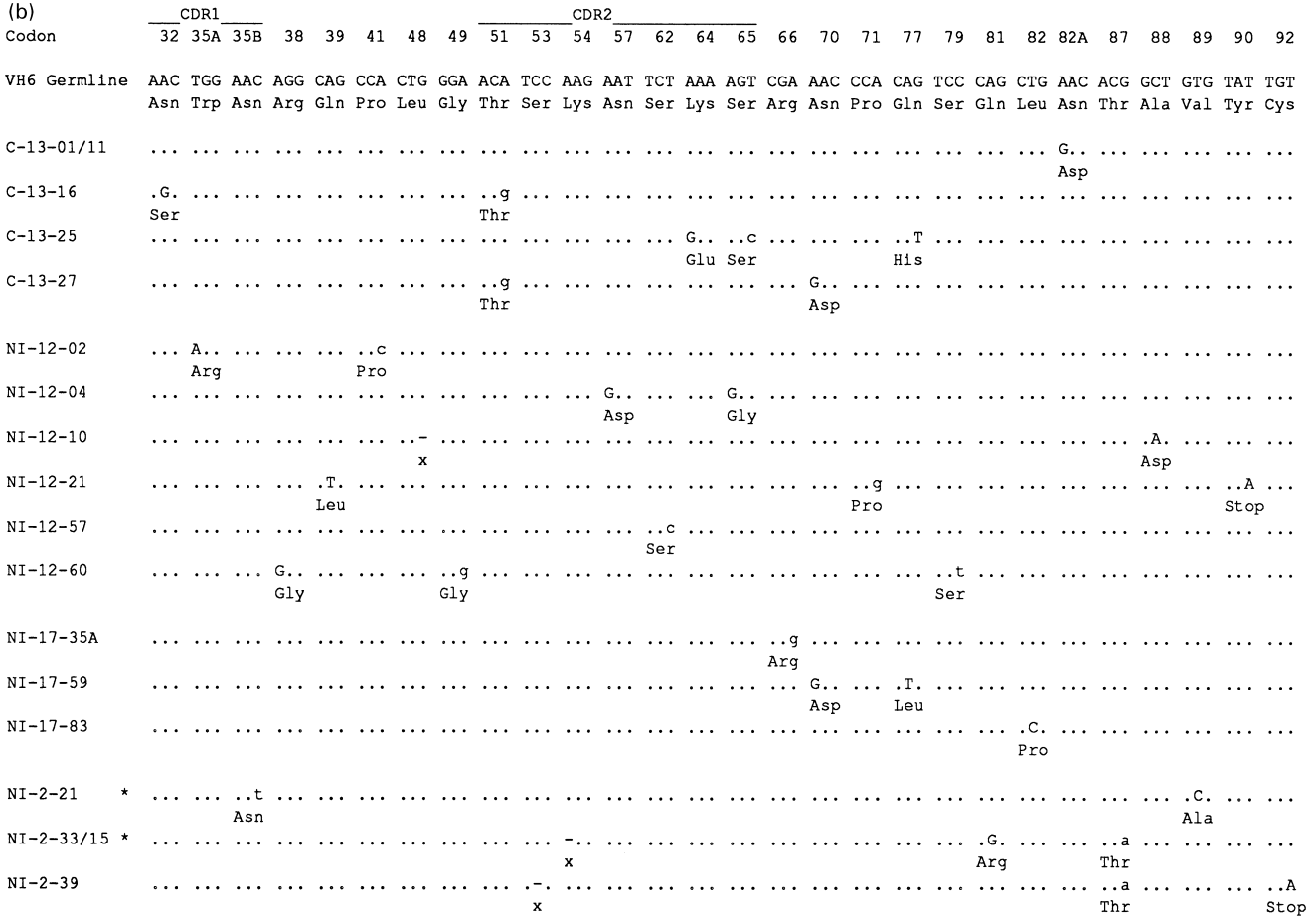

Fig. 4. (Continued).

DISCUSSION

The purpose of this study was to examine the development of the capacity for mutation and selection of immunoglobulin genes, an essential process in the formation of high-affinity antibodies. The neonatal period, when the infant is exposed to many infectious agents for the first time, is of particular clinical relevance. A number of published studies have shown that cord blood B cells have very few if any mutations in the immunoglobulin genes, whilst adult cells have many [14,15,19]. Mortari [27] found that mutations were rare in cord blood IgM sequences, but significantly higher in IgA and IgG sequences. However, IgG and IgA recombinants were rare, comprising 6·7% of the total immunoglobulin recombinants (the remainder being IgM). It would be difficult to rule out some contribution from maternal blood; maternal cells have been found at a frequency of $10^{-4}-10^{-5}$ in 10/10 cord blood samples tested [28]. One study has demonstrated the occurrence of mutations in tonsil B cells from a 4-year-old child [20]. This is to be expected, since by that age children are capable of making high-affinity antibody responses. The study reported here shows that mutations are detectable in many infants from the second week of life, but mutations are infrequent and show minimal evidence of selection.

Heteroduplex analysis of V_H6 sequences amplified from genomic DNA was used to identify the time periods which would merit more detailed analysis. Only two of 18 cord samples with re-arranged V_H6 showed mutation by heteroduplex analysis. In the infant samples, mutations were not detected before the age of 10 days. In infants 10 days or older, mutations were found in most samples.

V_H6 rearrangements from eight samples were cloned for sequencing. Clones which showed the presence of mutations by heteroduplex analysis were sequenced and examined for mutation, evidence of clonal relationship and selection. The frequency of heteroduplex-positive clones was 6–9% in control experiments designed to evaluate mutation introduced by the amplification procedures. Two of the eight samples analysed showed frequencies of heteroduplex-positive clones of 9% or less (Table 2).

The cord blood sample sequenced came from a child born to a mother who was hepatitis C-positive. Hepatitis C is transmitted vertically at a low and variable rate, approximating 10%, usually during delivery but possibly *in utero* [29]. There was no evidence of infection in this particular infant. The number of mutations ranged from zero to three per clone, with an overall mutation frequency, in the mutated clones, of one mutation per 121 bases of V_H6 sequence. The overall frequency of mutation was higher than found previously for healthy cord (1/250 in our previous study [19]; 1/300 published by van Es *et al.* [15]). The frequency of mutations per clone is low compared with memory B cells from adults, which contain an average of 10 mutations per clone [15,17], although our approach examined a segment of 241 bp, whilst the other studies examined the entire V_H6 sequence of 303 bp.

In infant samples, mutations were seen as early as day 10. Mutations are seen in adult mice from 6 days after immunization, increasing during the following week [30]. This finding is consistent with the possibility that the mutations detected in some of these infants resulted from infection during birth (see Table 1). However, there is not necessarily any connection between the mutations detected and the infections diagnosed in any of the patients; we selected patients with infection in order to maximize the opportunity for immune stimulation. Overall mutation frequencies in this group of patients ranged from 1/98 to 1/241, whilst mutations per clone ranged up to five. All of the sequences with four or more mutations per clone came from the day 15 sample (NI-13a) of a patient with confirmed *in utero* cytomegalovirus (CMV) infection. Thus the neonatal samples show that mutation is possible at this age, but, with the possible exception of NI-13a, the response is rudimentary. There was little evidence of clones receiving sequential mutations, with most of the clones having unique CDR3 regions, which shows that they originated from different parent clones. Mixing of cells from various lymphoid tissues in the blood would make the detection of clonally related cells improbable, except in a situation where the immune response was dominated by a particular stimulus. Sample NI-13a did show a mixture of shared and unique mutations in clones with an identical CDR3 sequence, suggesting intraclonal diversification (Fig. 4a). This sample gave a replacement mutations/silent mutations (R/S) ratio of 9 for CDR regions, higher than the expected value in the absence of selection (2·9 [31]), and 2·3 for the framework regions, indicating selection. The sum of all the other samples gave R/S ratios less than 2·9 for both framework and CDR regions. Thus, with the exception of one sample, there is no evidence for selection.

The sequential samples NI-13 and NI-13a merit closer examination. This infant was born at 36 weeks gestation and was ill at birth, with marked liver dysfunction. CMV antigen was detected in the urine on the day of birth. The first sample, taken 12 h after birth, showed a low frequency of mutated clones, a low frequency of mutations in mutated clones, and all mutated clones showing different CDR3 sequences. The mutation frequency is marginally higher than Taq polymerase error; there is no evidence for selection or for cells re-entering the mutation reaction. The second sample was obtained 15 days after birth, and shows extensive mutation, evidence of selection, and intraclonal diversification. The infant had received a packed cell transfusion on the day of birth (after the blood sample was taken) and several platelet transfusions thereafter. The last transfusion, of irradiated platelets, was given 6 days before collection of the second blood sample. Whilst it is highly unlikely that mutated clones detected in the blood derived from the transfused cells, it is difficult to rule this out absolutely. Although transfused cells are cleared very rapidly from the circulation [32,33], this is not always the case in neonates [34].

It would be expected that virtually all of the antibody produced in these young infants is IgM. van Es *et al.*, Insel *et al.* and Pascual *et al.* [15–17] have demonstrated unequivocally that μ-chain-associated V_H6 can be mutated. van Es *et al.* found mutation rates in adult IgM-associated V_H6 averaging 10 mutations per clone [15], while Pascual *et al.* [17] found on average 5·7 mutations per clone in IgM-associated V_H6.

The finding of low levels of mutation, with little evidence of selection, may be consistent with an extrafollicular site of mutation. Whilst the germinal centre is clearly the major site for mutation and selection, evidence for extrafollicular mutation has been provided, most clearly in patients with X-linked hyper-IgM syndrome, who lack germinal centres as a consequence of a disabling mutation in CD40 ligand [35]. Alternatively, the mutations may derive from germinal centres which are able to support mutation but not selection. Ziegner *et al.* [36] have shown, by analysis of individual germinal centres, that some centres show expanded populations of cells lacking mutation, some show high-level mutations with evidence of selection, while an intermediate

group show only low-level mutation and no evidence of selection. This latter group may predominate in the neonate.

ACKNOWLEDGMENTS

These studies were supported by grants from the Queen Victoria Hospital Research Trust and the National Health and Medical Research Council, Australia. We are grateful to the clinical staff of the Women's and Children's Hospital, Women's and Babies' Division for help with cord and perinatal blood samples, to Ms Leonie Dinan for help with blood samples from infants enrolled in a vaccination trial, and to Kelly Lenton and Debbie Little for technical assistance.

REFERENCES

1 Gathings WE, Kubagawa H, Cooper MD. A distinctive pattern of B cell immaturity in perinatal humans. Immunol Rev 1981; **57**:107–26.
2 Kipps TJ. The CD5 B cell. Adv Immunol 1989; **47**:117–85.
3 Janeway CA Jr, Bottomly K. Signals and signs for lymphocyte responses. Cell 1994; **76**:275–85.
4 Sanders ME, Makgoba MW, Sharrow SO *et al.* Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-*c* production. J Immunol 1988; **140**:140–17.
5 Zola H, Flego L, Macardle PJ, Donohoe PJ, Ranford J, Roberton D. The CD45RO (p180, UCHL1) marker: complexity of expression in peripheral blood. Cell Immunol 1992; **145**:175–86.
6 Brugnoni D, Airo P, Graf D *et al.* Ineffective expression of CD40 ligand on cord blood T cells may contribute to poor immunoglobulin production in the newborn. Eur J Immunol 1994; **24**:1919–24.
7 Fuleihan R, Ahern D, Geha RS. Decreased expression of the ligand for CD40 in newborn lymphocytes. Eur J Immunol 1994; **24**:1925–8.
8 Zola H, Fusco M, Macardle PJ, Flego L, Roberton D. Expression of cytokine receptors by human cord blood lymphocytescomparison with adult lymphocytes. Pediatric Res 1995; **38**:397–403.
9 Small TN, Keever C, Collins N, Dupont B, O'Reilly RJ, Flomenberg N. Characterization of B cells in severe combined immunodeficiency disease. Hum Immunol 1989; **25**:181–93.
10 MacLennan ICM. Germinal centres. Ann Rev Immunol 1994; **12**: 117–39.
11 Ebeling SB, Schutte ME, Logtenberg T. The majority of human tonsillar $CD5^+$ B cells express somatically mutated V kappa 4 genes. Eur J Immunol 1993; **23**:1405–8.
12 Ebeling SB, Schutte MEM, Logtenberg T. Peripheral human $CD5^+$ and CD5 B cells may express somatically mutated VH5- and VH6-encoded IgM receptors. J Immunol 1993; **131**:6891–9.
13 Hashimoto S, Dono M, Wakai M *et al.* Somatic diversification and selection of immunoglobulin heavy and light chain variable region genes in IgG^+ $CD5^+$ chronic lymphocytic leukemia B cells. J Exp Med 1995; **181**:1507–17.
14 Cai J, Humphries C, Richardson A, Tucker PW. Extensive and selective mutation of a rearranged VH5 gene in human B cell chronic lymphocytic leukemia. J Exp Med 1992; **176**:1073–81.
15 Van Es JH, Gmelig Meyling FHJ, Logtenberg T. High frequency of somatically mutated IgM molecules in the human adult B cell repertoire. Eur J Immunol 1992; **22**:2761–4.
16 Insel RA, Varade WS, Marin E. Human splenic IgM immunoglobulin transcripts are mutated at high frequency. Mol Immunol 1994; **31**: 383–92.
17 Pascual V, Liu YJ, Magalski A, de Bouteiller O, Banchereau J, Capra JD. Analysis of somatic mutation in five B cell subsets of human tonsil. J Exp Med 1994; **180**:329–39.
18 Liu YJ, Malisan F, de Bouteiller O *et al.* Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation. Immunity 1996; **4**:241–50.
19 Nicholson IC, Brisco MJ, Zola H. Memory B lymphocytes in human tonsil do not express surface IgD. J Immunol 1995; **154**:1105–13.
20 Klein U, Kuppers R, Rajewsky K. Variable region gene analysis of B cell subsets derived from a 4-year-old child: somatically mutated memory B cells accumulate in the peripheral blood already at young age. J Exp Med 1994; **180**:1383–93.
21 Kanof ME, Smith PD, Zola H. Preparation of human mononuclear cell populations and subpopulations. In: Unit 7.1. Coico R, Coligan JE, Margulies DH, Shevach EM, Strober W, Kruisbeek AM, eds. Current protocols in immunology. New York: John Wiley, 1996.
22 Brisco MJ, Condon J, Hughes E *et al.* Prognostic significance of detection of monoclonality in remission marrow in acute lymphoblastic leukemia in childhood. Australian and New Zealand Children's Cancer Study Group. Leukemia 1993; **7**:1514–20.
23 Marks JD, Tristem M, Karpas A, Winter G. Oligonucleotide primers for polymerase chain reaction amplification of human immunoglobulin variable genes and design of family specific oligonucleotide probes. Eur J Immunol 1991; **21**:985–91.
24 Deane M, Pappas H, Norton JD. Immunoglobulin heavy chain gene fingerprinting reveals widespread oligoclonality in B-lineage acute lymphoblastic leukaemia. Leukemia 1991; **5**:832–8.
25 Nicholson IC, Zola H. Rapid detection of immunoglobulin gene somatic hypermutation using heteroduplex formation. Mol Immunol 1994; **31**:1213–8.
26 Berman JE, Mellis SJ, Pollock R *et al.* Content and organization of the human Ig VH locus: definition of three new VH families and linkage to the Ig CH locus. EMBO J 1988; **7**:727–38.
27 Mortari F, Wang J-Y, Schroeder HW. Human cord blood antibody repertoire. J Immunol 1993; **150**:1348–57.
28 Petit T, Gluckman E, Carosella E, Brossard Y, Brison O, Socie G. A highly sensitive polymerase chain reaction method reveals the ubiquitous presence of maternal cells in human umbilical cord blood. Exp Hematol 1995; **23**:1601–5.
29 Matsubara T, Sumazaki R, Takita H. Mother-to-infant transmission of hepatitis C virus: a prospective study. Eur J Pediatr 1995; **154**:973–8.
30 Berek C. Somatic mutation and memory. Curr Opin Immunol 1993; **5**:218–22.
31 Jukes TH, King JL. Evolutionary nucleotide replacements of DNA. Nature 1979; **281**:605–6.
32 Adams PT, Davenport RD, Reardon DA, Roth MS. Detection of circulating donor white blood cells in patients receiving multiple transfusions. Blood 1992; **80**:551–5.
33 Lee TH, Donegan E, Slichter S, Busch MP. Transient increase in circulating donor leukocytes after allogeneic transfusions in immuno-competent recipients compatible with donor cell proliferation. Blood 1995; **85**:1207–14.
34 Hutchinson DL, Turner JH, Schlesinger ER. Persistence of donor cells in neonates after fetal and exchange transfusion. Am J Obstet Gynec 1971; **109**:281–4.
35 Chu YW, Marin E, Fuleihan R *et al.* Somatic mutation of human immunoglobulin V genes in the X-linked hyperIgM syndrome. J Clin Invest 1995; **95**:1389–93.
36 Ziegner M, Steinhauser G, Berek C. Development of antibody diversity in single germinal centers: selective expansion of high-affinity variants. Eur J Immunol 1994; **24**:2393–400.
37 Kabat EA, Wu TT, Perry HM, Gottesman KC, Foeller C. Sequences of proteins of immunological interest. Bethesda: US Department of Health and Human Services, 1991.