Somatic Mutation in the Neonatal Mouse

Carol A. Giorgetti and Joan L. Press

*J Immunol* 1998; 161:6093-6104; 
http://www.jimmunol.org/content/161/11/6093
Somatic Mutation in the Neonatal Mouse

Carol A. Giorgetti and Joan L. Press

Several mechanisms that diversify the adult immune repertoire, such as terminal deoxynucleotidyl transferase-dependent N region addition, are not available to the neonatal mouse. One important process that contributes to protective immunity in the adult is somatic mutation, which plays a major role in the generation of high affinity memory B cells. It is not clear whether B cells in the neonatal mouse can activate the somatic mutation machinery. To investigate this, we immunized neonates with poly(L-Tyr, L-Glu)-poly-(L-Ala-poly-L-Lys complexed with methylated BSA, or (4-hydroxy-3-nitrophenyl)acetetyl coupled to chicken gamma-globulin. Eight to fourteen days after priming, V(D)J rearrangements of known V_H genes (V_H SM7 family) were screened for mutations using a temperature-melt hybridization assay and oligonucleotide probes specific for complementarity-determining regions I and II; possible mutations were confirmed by sequence analysis. More mutations per sequence were found in heavy chains from neonates immunized with (4-hydroxy-3-nitrophenyl)acetetyl coupled to chicken gamma-globulin than in those from neonates immunized with poly(L-Tyr, L-Glu)-poly-(L-Ala-poly-L-Lys complexed with methylated BSA. Mutations were found in heavy chains lacking N regions, suggesting that B cells of the putative fetal lineage can somatically mutate and diversify an initially limited repertoire. Since neonates immunized as early as 1 or 2 days after birth had mutations, the somatic mutation machinery can be activated soon after birth, suggesting that early vaccination should result in affinity maturation and protective immunity in the neonate.

Received for publication May 8, 1998. Accepted for publication July 24, 1998.

E

arlier studies established that B cells in the neonatal mouse are competent to respond to T cell-dependent (TD)1 antigenic stimulation (1–5). More recent studies have shown that in vivo neonatal immunization can generate B cell memory, defined by the production of IgG Abs to secondary challenge weeks after priming (6, 7), and that neonatal T cells are competent to provide helper as well as cytotoxic T cell activity if appropriately stimulated (7–9). These studies indicate that neonatal and adult primary B cells are functionally comparable by parameters such as Ab secretion and isotype switching. However, adult and neonatal B cell populations differ in other respects. One important difference is the change in expression of the enzyme terminal deoxynucleotidyl transferase (TdT) during ontogeny, which has a significant impact on Ig repertoire expression and diversification. During V(D)J recombination, TdT contributes to the N-region diversity in CDR III found in adult B cell heavy chain gene rearrangements (10–12). The V(D)J joins of fetal and early neonatal B cells generally lack N regions, probably as a result of little or no TdT expression during fetal and early neonatal ontogeny (13–16). In the absence of N regions, rearrangement of V_H, D, and J_H genes with homology matches at coding ends is favored (11, 12, 17, 18), contributing to the preferential usage or increased frequency of certain V_H genes and junctional sequences among fetal and early neonatal B cells (17, 19–21). Thus, the absence of TdT limits repertoire diversity in the early neonatal primary B cell pool. Moreover, the delayed ontogenetic expression of TdT illustrates that a temporal difference can exist in molecular mechanisms available to B cells in the adult vs fetal or early neonatal mouse.

In adult mice the molecular mechanism giving rise to somatic mutation is an important component of B cell memory generation. Although the process by which somatic mutation occurs is unknown, it results in the introduction of nucleotide changes in the variable regions of Ig genes, generating mutated products from which are selected high affinity Abs characteristic of the secondary response (22–26). Since somatic mutation plays an integral role in affinity maturation in adults (27–30), if activation of the somatic mutation machinery were to occur only late in ontogeny (as does TdT expression), there would be an impact both on repertoire diversification in the neonate and on the neonate’s ability to generate protective immunity. For example, Ab concentration as well as avidity is important for antiviral protection (31). Higher affinity Abs can bind antigenic epitopes even when Ab concentrations are low. This may be of consequence for neonatal mice, who have fewer B cells and produce less Ab per clone than adults (1, 2) and whose Ig repertoire is restricted (14, 15, 17, 20). We therefore asked whether neonatal B cells can activate the machinery required for somatic mutation.

Earlier studies from our laboratory showed that primary B cells from 1- to 2-week-old BALB/c neonatal mice (Igh^a-allotype) can respond to the branched polypeptide Ag poly(L-Tyr, L-Glu)-poly-(L-Ala-poly-L-Lys (C106), AF059261 (8.1.c), AF059702–AF059709 (8.3.a, N1.1.a, N1.2.a, N1.2b, N1.1.b, N2.1.a, N2.2.a, N2.3.a), AF061559–AF061561 (9.3.a, 9.3.c, 9.4.a), AF061915–AF061917 (6.1.a, 9.4.b, 6.1.b), AF062382 (A2.a), and AF062756–AF062760 (A2.a, A3.a, A3.c, A1.a, A2.b, A3.b).

1 Address correspondence and reprint requests to Dr. Joan L. Press, Brandeis University, Rosenstiel Research Center, Mailstop 029, Waltham, MA 02454-9110. E-mail address: press@hydra.rose.brandeis.edu

2 Abbreviations used in this paper: TD, thymus-dependent; TdT, terminal deoxynucleotidyl transferase; CDR, complementarity-determining residue; (T,G)-A–L, poly(L-Tyr, L-Glu)-poly-(L-Ala-poly-L-Lys, mBSA, methylated BSA, Nip, 5-iodo-(4-hydroxy-3-nitrophenyl)acetetyl; CGG, chicken gamma-globulin; Nip, 5-iodo-(4-hydroxy-3-nitrophenyl)acetetyl; GTG, chicken gamma-globulin.
mice with (T,G)-A–L conjugated to methylated BSA (mBSA) were analyzed by dieoxynucleotide sequencing of RNA. Of the nine V(D)J sequences reported in that study, two differed from the germline H10 sequence, by 1 and 2 bp, respectively. We resequenced those two hybridomas by RT-PCR and found that they were unmutated (J. Press, unpublished observations). Since others have shown that mutations begin to accumulate after the first week of priming (29, 39, 40), the absence of mutations in this small sample of neonatal primary day 7 Abs was not unexpected.

Our earlier studies of the neonatal response to Ag were not designed to ask whether neonatal B cells can somatically mutate and how early in ontogeny this can occur. To determine these, we have examined heavy chain gene rearrangements of the H10 Vh gene from mice immunized at varying ages after birth with (T,G)-A–L-mBSA. We also analyzed heavy chain gene rearrangements in neonates immunized with the hapten (4-hydroxy-3-nitrophenyl) acetyl conjugated to chicken γ-globulin (Np-CGG), which in adult Ig h mice can elicit Abs using the H10 Vh4 gene (41–43). Somatic mutations were found in B cells from neonates immunized within 1 or 2 days after birth. Thus, it seems likely that in response to Ag or vaccination, the neonate’s initially limited primary B cell repertoire can further diversify by somatic mutation, with concomitant production of and selection for higher affinity Abs and memory B cells.

Materials and Methods

Ags, mice, and immunizations

BALB.B and BALB.K mice (Igh a allotype) were bred by brother-sister matings. The synthetic branched polypeptide pol-(t-Tyr-glutamyl-poly-ol-Ala-poly-l-Lys) (T,G)-A–L (lot MC11) was purchased from Miles-Yeda (Rehovot, Israel). The mBSA and CGG were obtained from Sigma (St. Louis, MO). The NP-CGG was the gift from Dr. Thereza Imanishi-Kari, Tel Aviv University (Rehovot, Israel). The mBSA and CGG were obtained from Sigma (St. Louis, MO). The mBSA and CGG were obtained from Sigma (St. Louis, MO). The NP-CGG was the gift from Dr. Thereza Imanishi-Kari, Tel Aviv University (Rehovot, Israel). The mBSA and CGG were obtained from Sigma (St. Louis, MO). The NP-CGG was the gift from Dr. Thereza Imanishi-Kari, Tel Aviv University (Rehovot, Israel). The mBSA and CGG were obtained from Sigma (St. Louis, MO).

Preparation of DNA and RNA

Lymphocytes were prepared from mice sacrificed 8–14 days after primary immunization. Usually, an individual’s spleen and draining lymph nodes were pooled for analysis. One-third to one-half of the cells collected were used to prepare genomic DNA; the rest were frozen as a pellet for RNA preparation. DNA was prepared as previously described (32, 34). The number of cells used to prepare DNA ranged from 20–200 × 10^6, and 200–10^6 cells yielded 1 μg of genomic DNA. Total RNA was prepared by the method of Chomczynski-Sacchi as previously described (33, 34), giving 50–100 μg of total RNA from 50–100 × 10^6 cells. For hybridomas, polynucleic mRNA was prepared as previously described (32, 34).

Primers and PCR conditions

RT-PCR was conducted on total RNA or hybridoma polynucleic mRNA as previously described (6, 35), using the Invitrogen kit (San Diego, CA). For RT of the heavy chain, the 5′ primer (5′-d(CCGAATTCGCAATCTA AAGGTT)-3′) was used with a Csa I constant region primer (5′-d(CCGAATTCGCTTCTCGGAGGACAC)-3′) or a Cy3 constant region primer (5′-d(AGGAAGCAAGAGGATGAC)-3′). The 5′ primer spans the leader –3 to +2 codon of the H10 germline gene in the Vh-SM7 family (36); it amplifies heavy chain rearrangements of H10 and two other Vh SM7 germline genes: H4a, which is identical with H10 in the –3 to +2 region, and H2b, which differs by one nucleotide, in the –3 codon (36). For PCR with genomic DNA from kidney or spleen and lymph nodes, most reactions used EcoRI-cut DNA. Template amounts ranged from 250 ng to 8 μg; most experiments used 500 ng to 2 μg of template. The 5′ primer was the same as that used for RT-PCR. The 3′ primer (5′-d(CCGAATTCGCAATCTA AAGGTT)-3′) used for PCR with lymphocyte DNA contained 15 nucleotides of the Jh region, which are the same for the 3′ exon. The 3′ primer used for kidney PCR (5′-d(CCGAATTCGCTTCTCGGAGGACAC)-3′) corresponded to the Vh sequence of H10, codons 93–98. All primers contain an EcoRI site. Primer oligonucleotides were purchased from DNA International (Lake Oswego, OR).

PCR was conducted in 100 μl with final concentrations of 0.125 mM dNTPs, 2.5 μM of each primer, and 1/200 enzyme buffer in an Ericon (San Diego, CA) thermal cycler. Magnesium concentrations and enzymes used varied. Taq polymerase (Promega, Madison, WI) was used with 0.5–2.0 mM Mg2+. The Expand/Long system, a mixture of Taq and Pwo polymers (Boehringer Mannheim, Indianapolis, IN) was used with 1.75 mM Mg2+. Pwo polymerase (Boehringer Mannheim) by itself and Pfu polymerase (Stratagene, La Jolla, CA) were used with 1.5–2 mM Mg2+, respectively. The Pwo and Pfu polymerases possess a 3′ to 5′ proofreading exonuclease activity that is supposed to improve the fidelity of cDNA during amplification (44). For PCR with Taq, Pwo, or Pfu, the cycling conditions were one cycle of 94°C (6 min), 50°C (3 min), and 72°C (2 min); 15 cycles of 94°C (2 min), 55°C (2 min), and 72°C (2 min); and one cycle of 72°C (7 min). For the Expand/Long PCR system, the cycling conditions were one cycle of 94°C (2.5 min); 10 cycles of 94°C (30 s), 55°C (30 s), and 72°C (2 min); 15 cycles of 94°C (1 min), 55°C (2 min and 5 s), and 72°C (2 min); and one cycle of 68°C (7 min).

PCR contamination control measures

All reagent stocks were prepared and aliquoted under sterile conditions; aliquots were used once and discarded. Pipetmen dedicated to PCR reagents (no nucleic acid) were used to set up master mixes for the reactions; other pipettes, for use only with genomic DNA (no plasmid DNA), were used for the preparation of DNA and its addition to reactions. Pipet microtips containing filter plugs (ART tips, Molecular Bio-Products San Diego, CA) were used on all Pipetmen. Preparation and aliquoting of PCR reagents were conducted at a work station physically separated from the area for the preparation of template DNA, nucleic acid manipulations, and pipetting, and work stations were UV irradiated for 20 min before use. Negative controls (reagents and primers only, no template DNA), run in all but one of the PCR reactions, did not give PCR products detectable on ethidium bromide-stained gels.

Cloning and hybridization of PCR products

PCR cDNA was EcoRI cut, purified on a 1.5% low melt agarose gel, and cloned into the EcoRI site of pUC 18 as previously described (6, 35). After transformation of JM83, colonies were picked, and DNA minipreps were prepared, EcoRI cut, run on 1.5% agarose gels, and blotted onto nitrocelulose or Nytran (Schleicher & Schuell, Keene, NH) for Southern blot analysis as previously described (6, 35). Blots were screened for PCR products containing H10 mutations by a temperature-melt hybridization assay using 32P-labeled oligonucleotides for hybridization. Oligonucleotide (DNA International) with sequence complementarity for regions (CDRI or CDRII) in the H10 or H4a and H2b germline genes in the Vh-SM7 family (see Fig. 1) were end labeled using T4 polymerase kinase (New England Biolabs, Beverly, MA) and [32P]ATP (New England Nuclear, Boston, MA) and centrifuged through BioSpin 6 columns (BioRad, Hercules, CA). The probes used were H10 52–58 (5′-d(CCCGAATTCGCAATCTA AAGGTT)-3′), complementary to H10 codons 52a–58 in CCGAATTCGCTTCTCGGAGGACAC (Fig. 1), and HnRI cut DNA. Similarly, to H10 codons 30–36 in CCGAATTCGCTTCTCGGAGGACAC (CCGGTACAGTATCCTTGAAGACAGAGGACAC)-3′, complementary to H10 codons 62–68 in CDRII, and H4/2 CDRII (5′-d(CACCGTGATATCGTGACATATGTT)-3′), complementary to CDRII codons 30–36, identical in the H4a and H2b genes (see Fig. 1, A and B). The H4/2 CDRII (5′-d(ATTTCCTCGATATCGTGACATATGTT)-3′) probe, which detects CDRII codons 49–55 is identical in the H4a and H2b genes, was used occasionally. The probe HX CDRII (5′-d(ATTTCCTCGACATGTT)-3′) is complementary to...
FIGURE 1.
A. Sequences of the germline $V_H$ genes H10, H4a, H2b (36), and JV9 (45) in the $V_{\text{H}}$ SM7 family. The HX sequence is derived from the neonatal clone 8.1.c and may be a new germline gene in the $V_{\text{H}}$ SM7 family. Underlined bases correspond to the CDR oligonucleotide probes used to screen for mutations.

B. The hybridization patterns obtained when the unmutated V(D)J rearrangements of H10, H2b, and HX are probed with the designated oligonucleotide probes, using the temperature-melt hybridization assay described in Materials and Methods. The H4a V(D)J rearrangement used as a control has a 1-bp change in codon 5, which is not detected by these probes. The (±) designation for HX with the H10 CDRI probe indicates that a hybridization band was seen occasionally, even though H10 and HX differ by 1 bp in this region (codons 30–36).

C. A representative Southern blot of the H10, H2b, H4a, and HX V(D)J rearrangements (lower band) described in B. Lane 1 is the RT-PCR product (39 Cm primer) of an unmutated H10 rearrangement. Lane 2 is the genomic DNA PCR product (39 JH 4 primer) of an H4a rearrangement with a 1-bp change in codon 5. Lane 3 is the RT-PCR product (39 Cm primer) of an unmutated H2b rearrangement. Lane 4 is the genomic DNA PCR product (39 JH 4 primer) of HX. The PCR products were cloned into pUC18, and the EcoRI digests of these plasmids were analyzed on Southern blots. Panel a is probed with the H10 CDRI (codons 30–36) oligonucleotide; panel b is probed with the H10 CDRII (codons 53–59) oligonucleotide; panel c is probed with the H4/2 CDRI (codons 30–36) oligonucleotide; panel d is probed with the HX CDRI (codons 33–39) oligonucleotide; and panel e is probed with the H10 CDRII (codons 62–68) oligonucleotide. When these V(D)J rearrangements were probed with an H4/2 CDRII (codons 49–55) oligonucleotide, the hybridization pattern was the same as that in panel c (data not shown).
CDRI codons 33–39 in the rearranged heavy chain gene 8.1.c (Table II), obtained from neonatal DNA. The 8.1.c neonatal sequence is designated HX in Fig. 1A. Southern blots of clones from Np-CCG-immunized neonates N2.1 and N2.2 were also screened with a probe for Dsp2.10 (5’- d(TAAGTACGACTA)-3’).

Hybridization conditions were as described by Brodeur (45, 46). The following conditions were used for all probes except Dsp2.10. Blots were prehybridized in 5x SSC, 1x Denhardt’s solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA for 3 h at 42°C, then hybridized overnight with radioactive probe in the same solution at 42°C. Blots were washed first with 2x SSC, 1x Biotto, and 0.1% SDS at room temperature. The second wash, in 1x SSC/0.1% SDS, was conducted at a temperature, determined for each oligonucleotide probe, that gave a hybridization signal for the specific germline gene but not for other germline genes in the family, whose sequences differed in the region probed (see Fig. 1C). The melting temperatures used ranged from 50–60°C. For the Dsp2.10 probe, the salt conditions were the same as above; however, blots were hybridized at 37°C and washed at 42°C.

CDR mutation screen

The hybridization profiles expected for V(D)J rearrangements using different Vg7SM7 family genes are summarized in Fig. 1B, and a representative Southern blot illustrating those profiles is shown in Fig. 1C. The JV9 gene (47) is not included, as we did not have a rearrangement of this gene; however, its published sequence is identical with H10 in the region detected by H10 probes CDRII (codons 30–36) and CDRII (codons 53–59) and differs by 1 bp in the region detected by H10 CDRIII (codons 62–68). This Southern blot includes the HX V(D)J rearrangement, unmapped V(D)J rearrangements of the H10 and H2b germline genes, and a 1-bp mutation (codon 5) of an H4a V(D)J rearrangement that is not detected by the probes used for hybridization. In Fig. 1C, panel a, the H10 CDRII (codons 30–36) probe detects a V(D)J rearrangement of the H10 gene, but not V(D)J rearrangements of the H4a or H2b genes, which differ from H10 by two nucleotides in this region (Fig. 1A). In some cases, the probe detects the HX V(D)J rearrangement, which differs by one nucleotide in this region. By analogy, if a V(D)J rearrangement of H10 had one or more differences (mutations) in CDRII codons 30–36, there would be no hybridization signal with the H10 CDRII (codons 30–36) probe after washing at the “melting” temperature (for this blot, 50°C). Similarly, the H10 CDRIII (codons 53–59) probe detects both H10 and HX rearrangements (no nucleotide differences), but not H4a or H2b (two or three differences, respectively; Fig. 1C, panel b). The H4a/2 CDRII probe detects both H4a and H2b rearrangements, but neither H10 nor HX rearrangements (Fig. 1C, panel c); similar results were obtained with a probe detecting H4a/2 CDRII codons 49–55 (data not shown). The HX CDRI probe detects only HX (Fig. 1C, panel d), whereas the HX CDRII (codons 62–68) probe detects all four Vg7SM7 genes tested (Fig. 1C, panel e). The clones whose hybridization profiles were different from those in Fig. 1B indicated possible mutations, and those V(D)J rearrangements were selected for sequencing.

Controls for polymerase error and mutation screen

As a control for the mutation screen, PCR with the 5’ H10 and 3’ Jg4 primers was performed using Taq polymerase and genomic DNA from the hybridoma C16-15F6, which expresses an unmutated rearrangement of H10 (32, 34). The PCR EDNA was cloned into pUC18, and individual clones were analyzed in the CDR mutation screen using the oligonucleotides described in Fig. 1. Of the 143 clones analyzed, only two had a mutation profile, that is, they gave a weak signal with the H10 CDRI probe (codons 30–36). Both clones were sequenced from +1 into or through Jg4. One clone had a 1-bp mistake in codon 36 (TGG→TAG), and the other clone had a 1-bp mistake in codon 31 (GAC→GAT); these mistakes reflect polymerase error.

We have used Taq polymerase in RT-PCR reactions of ≥40 different heavy and light chain rearrangements from hybridomas (Refs.6 and 35; J. Press, unpublished data), and sequenced replicate clones of each rearrangement. To determine the error rate for Taq, we summed the number of mistakes among the number of bases sequenced. There were 41 errors in 55,889 bp, or 1 mistake/1363 bp. This Taq error rate is similar to that of Berek et al. (24). Among the 169 RT-PCR hybridoma sequences we analyzed, 135 had no error, 27 had one error, and seven had two errors. None of these sequences had more than two errors.

Sequence analysis

Selected clones were sequenced to determine the identity (use of the H10 gene or another gene in the Vg7SM7 family) and the presence or the absence of mutations. The Sequenase kit (U.S. Biochemical, Cleveland, OH) and the SequiTherm EXCEL II kit (Epitect Technologies, Madison, WI) were used with M13 universal and M13 reverse primers (U.S. Biochemical) according to the manufacturers’ instructions and as described previously (6, 35). Data bases were searched for sequence comparisons using programs available on the web (the program DNAPlot at http://www. genetik.uni-koeln.de/dnaplot/vsearch_mouse.html and another program that searches the Kabat database, located at http://immucodonbiosme. nwu.edu). Both programs can assign sequences to Vg7SM7 families as well as identify similar sequences. The National Center for Biotechnology Information (NCBI) database was also searched, using the BLAST FASTA program.

Results

To determine whether neonatal B cells are capable of mutating, we examined heavy chain rearrangements after neonatal immunization with (T,G)-A-L-mBSA in adjuvant. The neonatal primary response to this Ag is dominated by TGB5 Id+ , GT+ Abs that use the H10 germline gene from the Vg7SM7 family (32, 34, 35). The Vg7SM7 family is small, consisting of four known germline genes, H10, H4a, H2b, and JV9 (36–38, 47) (see Fig. 1A), making it easier to distinguish between a germline and a somatically mutated sequence. However, it cannot be excluded that a putative mutated sequence is instead a rearrangement of an as yet unidentified, homologous germline gene. The neonates ranged in age from newborn (~24 h old) to 6 days old when immunized with (T,G)-A-L-mBSA. Spleen and lymph nodes from individual BALB.B mice were removed 8–14 days after priming for analysis, using RT-PCR or genomic PCR products of Vg7SM7 family heavy chain gene rearrangements, in particular, H10. Vg7SM7 rearrangements were amplified using 5’ H10 and 3’ Jg4 or Cμ primers and cloned into pUC18. As described in Materials and Methods (CDR mutation screen), the clones were screened for mutations in CDR1 and CDR2 using a temperature-melt hybridization assay and oligonucleotide probes for CDR1 or CDR2 sequences, which differ among the known germline genes in the Vg7SM7 family (Fig. 1). Included in Fig. 1A is the Vh11 sequence of a heavy chain rearrangement obtained from a neonatal clone (8.1.c, Table II), designated HX, which may be a new germline gene in the Vg7SM7 family (see below).

Analysis of H10 Vh gene rearrangements in neonates immunized with (T,G)-A-L-mBSA

We screened approximately 333 Vg7SM7 family rearrangements from 11 individual neonates (four litters) that were 3–6 days old when immunized, as well as approximately 631 rearrangements from 19 neonates (six litters) that were 24 h to 2 days old when immunized. The hybridization profiles of about 6% of these rearrangements were suggestive of mutation. This is not a measure of mutation frequency, since the screening technique scores only for nucleotide changes in selected regions of the Vh gene. From these selected clones, 37 H10 rearrangements were sequenced. The number of nucleotide changes in these clones is shown in Table I. Included in the data for selected clones was the 6-bp difference of a TGB5 Id+ , GT+ primary day 14 IgM hybridoma (C106) from a 5-day-old neonate, bringing the number (N) sequenced to 38. As shown in Table I, most of the selected clones had a 1-bp difference from the germline V h11 gene, H10. The remaining clones had ≥2-bp changes in Vh, and some clones also had nucleotide changes in the D (Table II) or Jh (not shown) gene. Some of the neonatal sequences were clonally related, for example, clones 9.3.a (1-bp difference) and 9.3.a’ (3-bp difference; Table II).

To examine V(D)J rearrangements for possible nucleotide changes in regions not detected by our probes, H10 rearrangements from 24-h-old to 2-day-old immunized neonates that appeared unmutated by their hybridization profile were randomly chosen for
sequence analysis. Approximately half of these were derived from neonates that had been primed for 8 days, and the other half came from neonates 14 days after priming. Of the 47 random V(D)J rearrangements sequenced, 35 (75%) were unmutated. The distribution of changes among the remaining 12 sequences is shown in Table I. When the data for the selected and random clones are combined, of the 50 sequences with differences in VH, the majority (68%) differed by only 1 bp from the germline H10 gene. In Table I, many of the neonatal PCR products were obtained using Taq polymerase, which has a lower fidelity than Pwo or Pfu (44); hence, some of the 1-bp changes could result from polymerase error. Our experimentally determined error rate for Taq after 30 h, hence, some of the 1-bp changes could result from polymerase error. Our experimentally determined error rate for Taq after 30 cycles is 1 mistake/1363 bp (see Materials and Methods), which represents less than one error per average V(D)J rearrangement (~350 bp). Thus, it is likely that the neonatal sequences with ≥2-bp differences (Table I) include some that were somatically mutated.

Clones with >2-bp differences in their total (V(D)J) sequence are listed in Table II, along with the D genes used. Included in Table II are some clones with fewer nucleotide changes that are probably clonally related, because they were derived from the same individual, and they share V(D)J joins and some sequence substitutions. The V(D)J sequences of the clones in Table II are shown in Fig. 2 and illustrate that nucleotide changes were found throughout the variable region.

As shown in Table II, five H10 rearrangements were isolated with 3-bp differences in VH (9.3.a*, 9.3.c, 9.4.a, 9.4.b, and 6.1.a). Another sequence, 8.3.a, had 6-bp differences from the H10 germ-line sequence. Database searches, including the full NCBI database, indicated that the sequence of 8.3.a is closest to genes in the VpSM7 family, and this search did not reveal a sequence with the 8.3.a set of substitutions. The NCBI database (Kabat) was also searched for only that part of the clone 8.3.a sequence that contains all the nucleotide substitutions (codons 1–27) to rule out that this portion of the 8.3.a rearrangement was contributed by a template jump from another gene (48). Thus, clone 8.3.a appears to be a bona fide 6-bp mutation of the H10 gene (Fig. 2). Overall, these data indicate that somatic mutation can occur in neonatal B cells.

### Table I. H10 mutations from (T.G)-A–L-immunized neonatal mice: distribution of changes

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>1 bp</th>
<th>2 bp</th>
<th>3 bp</th>
<th>4 bp</th>
<th>5 bp</th>
<th>6 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Selected” clones</td>
<td>38</td>
<td>25</td>
<td>7</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>“Random” clones</td>
<td>12</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total:</td>
<td>50</td>
<td>34 (68%)</td>
<td>8 (16%)</td>
<td>5 (10%)</td>
<td>1 2%</td>
<td>2 4%</td>
<td></td>
</tr>
<tr>
<td>Primary dy 8</td>
<td>20</td>
<td>14 (70%)</td>
<td>2 (10%)</td>
<td>2 (10%)</td>
<td>0 5%</td>
<td>1 1%</td>
<td>5 5%</td>
</tr>
<tr>
<td>Primary dy 10–14</td>
<td>30</td>
<td>20 (67%)</td>
<td>6 (20%)</td>
<td>3 (10%)</td>
<td>0 0%</td>
<td>1 3%</td>
<td></td>
</tr>
</tbody>
</table>

* Distribution of nucleotide changes in the H10 VH gene (codons 1–98) of V(D)J rearrangements cloned into pUC18. The V(D)J rearrangements were derived from PCR using RNA or genomic DNA, and Taq, Expand Long, Pfu, or Pwo polymerase. “Selected” clones were picked for sequence analysis based on giving a mutation profile upon Southern blot hybridization with oligonucleotides for CDR1, CDR2, or JH regions. Included in the selected clone sample is the sequence of one primary day 14 hybridoma, C106, derived from a neonate 5-days old when immunized. “Random” clones were picked for sequence even though their hybridization profiles did not give a mutation profile. This table also gives the total (selected plus random) distribution of VH mutations as well as the distribution of mutations in clones derived from neonates 8 versus 10–14 days after priming.

### Table II. H10 mutations from (T.G)-A–L-immunized neonatal mice: representative clones

<table>
<thead>
<tr>
<th>Litter Sib Age Primed Primary Day PCR Enz Clone*</th>
<th>No. of Mutations*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Day 5</td>
<td>C106*</td>
</tr>
<tr>
<td>8 1 Day 8</td>
<td>Taq*</td>
</tr>
<tr>
<td>9 3 Day 8</td>
<td>Taq*</td>
</tr>
<tr>
<td>3 24 h</td>
<td>Taq*</td>
</tr>
<tr>
<td>9 3 Day 8</td>
<td>Taq*</td>
</tr>
<tr>
<td>6 1 Day 10</td>
<td>Taq*</td>
</tr>
<tr>
<td>1 24 h</td>
<td>Taq*</td>
</tr>
</tbody>
</table>

* Representative clones of mutated V(D)J rearrangements obtained 8, 10 or 12 days after priming newborn mice (≤24 h old) with (T.G)-A–L-mBSA (see legend to Table I).

* PCR: used genomic DNA with the JH4 primer, except those marked by superscript * are RT-PCR with the 36 Ck primer. All reactions used the same S’ primer (Materials and Methods). Polymerases for these clones included Taq, Expand Long (ExpL), and Pwo.

* Clone refers to an individual V(D)J rearrangement cloned into pUC18. The nomenclature is litter.sib.individual clone, e.g., clone 9.3.a refers to a V(D)J rearrangement from litter nine, sib three. Clones from the same sib that have the same V(D)J join are marked with () after the clone letter; they may have derived from the same precursor (e.g., 9.3.a and 9.3.a*). Clones shown were selected for sequencing based on their CDR oligonucleotide screening profile, except for clones marked with an asterisk (*). Clones marked with an asterisk did not give mutation hybridization profiles but were randomly picked for sequence analysis.

* C106 is a hybridoma derived 14 days after priming a 5-day-old neonate; its μ heavy chain was sequenced by RT-PCR.

* The number of nucleotide changes is compared to the germ-line H10 VH sequence and JH sequences. Mutation in D is based on difference from the sequence of the known germline D gene. The total number of mutations is given in brackets [total]. D gene sequences that could originate from DSp2.5 or Sp2.7 or Sp2.8 are designated Sp2.5/7/8.
A possible new gene in the V\(\gamma\)SM7 family

Two other sequences, 8.1.c and C106, differ from H10 by 5 and 6 bp, respectively (Table II). Database searches (DNAplot, Kabat programs) using the 8.1.c sequence indicated closest homology to the V\(\gamma\)SM7 family, and no other sequence was identified with the set of substitutions found in 8.1.c. Although 8.1.c and C106 have different V(D)J joins, they differ from each other by only 1 bp (Fig. 2). It is possible that both 8.1.c and C106 are mutated rearrangements of H10 with shared sequence changes in five codons, all amino acid replacements (49). It is also possible that they are rearrangements of a previously unidentified member of the V\(\gamma\)SM7 family, where the 8.1.c sequence is this putative new germline gene (designated HX in Fig. 1), and C106 is a 1-bp mutation of it.

We sequenced the RT-PCR product of the C106-expressed light chain (V\(\kappa\)1AJ\(\kappa\)1) and found two changes (one silent, codon 86, TAT\(\rightarrow\)TAC; and one replacement, codon 91, AGT\(\rightarrow\)AGG) from the germline sequence (data not shown), suggesting that the B cell from which the C106 hybridoma was derived did somatically mutate.

To determine whether the 8.1.c V\(\gamma\) sequence is a rearrangement of a new germline gene, we analyzed V\(\gamma\)SM7 PCR products made from kidney DNA. The 5' primer was the same as that used for 8.1.c and C106. The 3' primer used (.C TAT TAC TGT GCT AGT) spans the last base of codon 93 through the first two bases of codon 98 in the germline sequence of H10, and this sequence is shared by H4a and the 8.1.c rearrangement (Figs. 1 and 2). More than 700 kidney PCR products were screened by hybridization with the oligonucleotides described in Fig. 1, including the HX CDRI oligonucleotide. Most of the PCR products appeared to be H10 or H4a by this screening method. Only three clones were detected with the HX oligonucleotide. These clones did have the HX VH sequence (8.1.c) shown in Fig. 1 (data not shown); however, the frequency of HX clones in this sample seems low. The C106 and 8.1.c sequences are identical in the region spanned by the 3' primer, except that the C106 V(D)J rearrangement lacks codon 98. If the putative germline HX gene and 8.1.c are not identical in codon 98, then a mismatch in the 3' primer could result in a lower PCR efficiency. It remains unresolved whether the C106 and 8.1.c sequences are rearrangements of a new gene in the VH SM7 family (HX) or are mutations of the H10 germline gene.

Many of the neonatal sequences have N regions

Among the 50 sequences with \(\pm 1\)-bp differences (Table I), D gene usage was heterogeneous and included FL16.1, FL16.2, Sp2.2, Sp2.7, Sp2.9, Sp2.10, and DQ52-C. Twenty-five different V(D)J joins were found, 15 of which were unique (not shared by clones from other individuals). Ten V(D)J joins were not unique, i.e., more than one individual had the same join. Among the 25 different V(D)J joins, three had no N region, and three had a 1-bp N region. The remaining joins had N regions varying in length.
The V_{H} SM7 sequences from Np-CGG-immunized neonates are mutated

It is evident from the data in Table I that although somatic mutations were found in H10 V(D)J rearrangements after immunization with (T.G)-A–L-mBSA, only a third of the sequences had more than a 1-bp difference. This was observed even for V(D)J rearrangements obtained 10–14 days after priming the neonates (Table I). Because it is possible that the type of Ag used for immunization influences the number of mutations per sequence generated in the early primary response (50), we conducted a similar analysis using neonatal mice immunized with Np-CGG. We chose this Ag because other investigators have shown that in adult mice of the Igh^{b} allotype, the primary as well as the memory response to Np is highly mutated (25, 26, 29, 30, 40).

We first asked whether neonatal mice can respond to in vivo immunization with Np-CGG. Adult mice and 1- to 2-day-old neonatal (BALB.K × BALB.B)F_{1} mice were primed with Np-CGG in adjuvant and bled 14 and 28 days after priming; they were then boosted with Np-CGG in aqueous solution and bled 7 days later. In adult Igh^{a} allotype mice, approximately 50% of the primary anti-Np Abs use \lambda light chains, and these Abs are usually heteroclitic, i.e., bind the related hapten Nip better than Np (41). Therefore, we assayed the neonatal sera on Nip-BSA and used both an anti-mouse F(ab')_{2} Ab, which detects primarily Abs using the \kappa light chain (5), and an anti-mouse \lambda Ab. As shown in Fig. 3, (BALB.K × BALB.B)F_{1} mice immunized when less than 2 days old had a primary day 14 anti-Np Ab response, although the response was lesser in magnitude than that of adults. Both the neonatal and adult responses increased by 28 days. Moreover, both the adult and neonatal mice isotype switched (IgG) during their primary response, and they generated IgG memory that could be
The results, shown in Table III, illustrate several points. First, the neonatal H4a and H10 heavy chains are somatically mutated. Database searches showed that these heavy chains are most homologous to \( V_H \) SM7 family genes, and no sequence in the database was identified with the same set of substitutions. Second, the number of mutations per rearrangement in the neonates is similar to that in the adult. In addition, there were more nucleotide changes per \( V_H \) sequence in clones isolated from neonates immunized with Np-CGG than with (T,G)-A–L-mBSA. Mutations were not restricted to CDR regions (Table III and Fig. 4), consistent with studies showing mutations in framework as well as in hypervariable regions (22, 24–26, 29, 40). In the well-characterized C57BL/6 anti-Np response, most secondary Abs have a common mutation indicative of affinity maturation, namely, the canonical tryptophan to leucine change at codon 33 (28, 29). However, not all primary anti-Np sequences from C57BL/6 mice have this change, e.g., primary day 10 and day 16 V(D)J rearrangements from germinal center cells have been isolated that are mutated yet do not contain this characteristic substitution (25, 26). While there are no published studies on whether Np-specific B cells in Igha allotype mice can initiate affinity maturation, the finding of mutations in neonatal sequences lacking N regions suggests that cells in the putative fetal B cell lineage (see “Discussion”) can activate the somatic mutation machinery.

We rescreened the 94 RT-PCR clones derived from the N2.1 sibling with a DSp2.10 probe to look for V(D)J rearrangements that might be clonally related to the mutated N2.1.a sequence. Five clones were identified and sequenced; all five were identical and had the same V(D)J join as N2.1.a (join G) is not identifiable as there is only 1 bp in this region.

### Table III. Mutations from Np-CGG-immunized mice

<table>
<thead>
<tr>
<th>Age</th>
<th>Litter/Primed</th>
<th>Clone</th>
<th>( V_H )SM7 Gene</th>
<th>( V_H ) D; JH [total]</th>
<th>D Gene</th>
<th>Join^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>1</td>
<td>A1.a</td>
<td>H4a</td>
<td>1</td>
<td>0; 0/JH [1]</td>
<td>Sp.2.5/7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>A2.a</td>
<td>H10</td>
<td>5</td>
<td>0; 0/JH [2]</td>
<td>Sp/FL</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>A3.a</td>
<td>H10</td>
<td>8</td>
<td>1; 0/JH [9]</td>
<td>Sp.2.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>A3.b</td>
<td>H4a</td>
<td>4</td>
<td>0; 0/JH [2]</td>
<td>FL.16.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>A3.c</td>
<td>H10</td>
<td>5</td>
<td>0; 2/JH [7]</td>
<td>Sp.2.5</td>
</tr>
<tr>
<td>Neonate</td>
<td>Day 0–2</td>
<td>1/1</td>
<td>N1.1.a</td>
<td>H10</td>
<td>8</td>
<td>0; 3/JH [11]</td>
</tr>
<tr>
<td></td>
<td>Day 0–2</td>
<td>1/2</td>
<td>N1.1.b</td>
<td>H4a</td>
<td>2</td>
<td>0; 0/JH [2]</td>
</tr>
<tr>
<td></td>
<td>Day 0–2</td>
<td>2/1</td>
<td>N1.2.a</td>
<td>H10</td>
<td>6</td>
<td>2; 1/JH [9]</td>
</tr>
<tr>
<td></td>
<td>Day 0–2</td>
<td>2/1</td>
<td>N1.2.b</td>
<td>H10</td>
<td>4</td>
<td>1; 1/JH [6]</td>
</tr>
<tr>
<td></td>
<td>Day 0–1</td>
<td>2/1</td>
<td>N2.1.a</td>
<td>H10</td>
<td>6</td>
<td>0; 3/JH [9]</td>
</tr>
<tr>
<td></td>
<td>Day 0–1</td>
<td>2/2</td>
<td>N2.2.b</td>
<td>H4a</td>
<td>5</td>
<td>0; 0/JH [5]</td>
</tr>
<tr>
<td></td>
<td>Day 0–1</td>
<td>2/3</td>
<td>N2.3.a</td>
<td>H10</td>
<td>1</td>
<td>0; 0/JH [1]</td>
</tr>
</tbody>
</table>

^a BALB·K mice were immunized as adults or as neonates that were 0–2 days old (litter 1) or 0–1 day old (litter 2). Adults and litter one neonates were immunized with 50 μg, and litter two neonates with 25 μg of Np-CGG, respectively, in CFA. Spleens and lymph nodes were removed 14 days after priming. PCRs for the adult and neonatal litter one were done with Pwo polymerase, the 3′ JH1 primers, and genomic DNA from individual donors. For neonatal litter two, RT-PCRs were performed with the 3′ Cα primers and Pwo polymerase. All reactions used the same 5′ primer as in Tables I, II.

^b Clone refers to individual, cloned V(D)J rearrangements that were sequenced. The nomenclature is adult or neonate [A or N], individual or sib, individual clone; e.g., N1.2.a refers to a cloned V(D)J rearrangement from neonatal litter 1, sib no. 2.

^c Mutations in rearrangements of H4a as well as H10 germline genes from the \( V_H \) SM7 family are given. The number of nucleotide changes is compared to germline H10 and H4a \( V_H \) sequences and JH sequences. Mutation in D is inferred, based on difference from known germline D gene. The total number of mutations is given in brackets [total]. Clones shown were selected for sequencing based on their CDR oligonucleotide screening profile.

^d Letters (A, B, C) refer to specific V(D)J join sequences (shown in Fig. 5). The B join could originate from either the DSp2 family or DFL-FL16.1. The A join could originate from DSp2.5 or DSp2.7. The C join is not described above for the (T,G)-A–L analysis. Those V(D)J rearrangements shown in Fig. 4 have the same substitution in codon 34, leading to an amino acid replacement (Met→Ile).

The D genes used included members of the Sp2 and FL families, as well as Q52 (Table III), and all the joins were unique (Fig. 5). Although the adult sequences had N regions, most of the neonatal sequences did not (unlike the joins in sequences derived from (T,G)-A–L-mBSA-immunized neonates). The finding of mutations in neonatal sequences lacking N regions suggests that cells in the putative fetal B cell lineage (see “Discussion”) can activate the somatic mutation machinery.

Recalled upon secondary immunization with Np-CGG. Thus, 2-day-old neonatal mice could respond to in vivo immunization with Np-CGG, indicating that it was feasible to examine their heavy chain gene rearrangements for somatic mutations.

In C57BL/6 mice (Igh\(^b\) allotype), the primary anti-Np response is dominated by Abs using the \( V_H \)186.2 gene (J558 family) with the \( \lambda \) light chain (28, 29, 42). The anti-Np response in Igh\(^b\) allotype mice is not as well characterized at the molecular level; however, primary anti-Np Abs using V(D)J rearrangements of the H10 \( V_H \) gene are elicited in adult Igh\(^b\) allotype mice after immunization with Np-CGG (42, 43). Therefore, we immunized adult and neonatal BALB·K mice with Np-CGG and examined their \( V_H \) SM7 heavy chain rearrangements for somatic mutations 14 days after priming. Genomic DNA was isolated from four individual adult mice and from four individual siblings of one neonatal litter that was 0–2 days old when primed. Approximately 248 and 293 genomic clones were screened from the adults and neonates, respectively (adults and neonatal litter 1, Table III), using the temperature-melt hybridization assay and the oligonucleotide probes described above for the (T,G)-A–L analysis. Those V(D)J rearrangements giving mutation hybridization profiles were sequenced. We also examined the RT-PCR products from four individual siblings of a second litter that was 0–1 days old when primed. Approximately 417 neonatal clones (litter 2, Table III) were screened. We noticed that the proportion of clones detected with the H4/2 probe (V(D)J rearrangements of either H4a or H2b) appeared to be higher in the Np-CGG-immunized mice than in the (T,G)-A–L-immunized mice (data not shown). Consequently, we sequenced H4a clones as well as H10 clones that gave a mutation profile after hybridization with the relevant CDR1 and CDR2 oligonucleotides.

The heavy chain rearrangements shown in Table III illustrate several points. First, the neonatal H4a and H10 heavy chains are somatically mutated. Database searches showed that these heavy chains are most homologous to \( V_H \) SM7 family genes, and no sequence in the database was identified with the same set of substitutions. Second, the number of mutations per rearrangement in the neonates is similar to that in the adult. In addition, there were more nucleotide changes per \( V_H \) sequence in clones isolated from neonates immunized with Np-CGG than with (T,G)-A–L-mBSA. Mutations were not restricted to CDR regions (Table III and Fig. 4), consistent with studies showing mutations in framework as well as in hypervariable regions (22, 24–26, 29, 40). In the well-characterized C57BL/6 anti-Np response, most secondary Abs have a common mutation indicative of affinity maturation, namely, the canonical tryptophan to leucine change at codon 33 (28, 29). However, not all primary anti-Np sequences from C57BL/6 mice have this change, e.g., primary day 10 and day 16 V(D)J rearrangements from germinal center cells have been isolated that are mutated yet do not contain this characteristic substitution (25, 26). While there are no published studies on whether Np-specific B cells in Igh\(^b\) allotype mice generate shared or dominant mutations that correlate with Ag selection, it is interesting that four of the V(D)J rearrangements shown in Fig. 4 have the same substitution in codon 34, leading to an amino acid replacement (Met→Ile).

The D genes used included members of the Sp2 and FL families, as well as Q52 (Table III), and all the joins were unique (Fig. 5). Although the adult sequences had N regions, most of the neonatal sequences did not (unlike the joins in sequences derived from (T,G)-A–L-mBSA-immunized neonates). The finding of mutations in neonatal sequences lacking N regions suggests that cells in the putative fetal B cell lineage (see “Discussion”) can activate the somatic mutation machinery.

We rescreened the 94 RT-PCR clones derived from the N2.1 sibling with a DSp2.10 probe to look for V(D)J rearrangements that might be clonally related to the mutated N2.1.a sequence. Five clones were identified and sequenced; all five were identical and had the same V(D)J join as N2.1.a. Since these five sequences
were unmutated (data not shown), including the positions for the six nucleotides in V\(_H\) and the three nucleotides in J\(_H\) that are mutated in clone N2.1.a, they may represent the clonal precursor of N2.1.a. We also sequenced a small sample of clones from among those designated unmutated by our hybridization screen. We randomly chose five H10 clones from neonatal sibling N2.1 and five H4/2 clones from neonatal sibling N2.2 and found that the V(D)J rearrangements of all 10 of these clones were unmutated.

The results of our analysis of V(D)J rearrangements from Np-CGG-immunized neonates strengthen the conclusions reached from our (T,G)-A–L-mBSA data. Taken together, our results show that B cells in very young mice can somatically mutate their heavy chain genes.

Discussion

The process of somatic mutation that is initiated by first encounter with TD Ag gives rise to Abs with higher affinity and is coincident with the generation of B cell memory (22–30). As a consequence, second encounter with Ag elicits a rapid, high affinity Ab response from an expanded pool of mutated memory B cells that were generated after priming. Thus, the ability of B cells to somatically mutate has important biologic ramifications for protective immunity. Since a precedent exists for a difference in the molecular mechanisms active in adult vs fetal and early neonatal B cell populations, notably, TdT expression (13–16), we asked whether the somatic mutation machinery can be activated in neonatal B cells. The data presented in this paper demonstrate that B cells in neonatal mice immunized within 1 or 2 days after birth are indeed capable of somatically mutating their Ig heavy chain genes. Because the majority of V(D)J rearrangements analyzed in our study were screened to preselect those with possible mutations in CDRs, a mutation frequency cannot be determined. Nonetheless, it is possible to compare the adult and neonatal responses in terms of the number of mutations observed per sequence. By this criterion, the range of total mutations in the primary day 14 sequences from Np-CGG immunized adult and neonatal mice is similar, suggesting that once activated, the somatic mutation machinery gives comparable mutation frequencies in both neonate and adult. Our study indicates that the neonate can use somatic mutation to further diversify an Ig repertoire that is initially limited in VH gene usage and junctional diversity due to lack of N region addition (17–21).

The B cell population early in neonatal ontogeny is very heterogeneous by a variety of criteria, including surface phenotype and gene expression (51–56). Several investigators have proposed that this heterogeneity reflects a transition in B lymphopoiesis during mouse ontogeny, i.e., a switch occurs between two discrete developmental pathways for B lymphopoiesis (fetal vs adult type) (51–54). For example, most splenic Ig\(_1\) cells are class II negative in the 1-day-old neonatal mouse; however, in the 4-day-old neonate, approximately 43% of Ig\(_1\) cells express class II (54). The switch or transition from fetal- to adult-type B cell lymphopoiesis appears to begin after birth and finishes 2 or 3 mo later (54, 56). Thus, according to this model, B cells from both the fetal and adult pathways would be present during the ontogenetic period when the
neonates in our study were immunized. The dogma that the presence or the absence of N regions can distinguish fetal vs adult B cells is not absolute, as V(D)J sequences with N regions can be found in young neonates, and sequences without N regions have been isolated from adults (14, 15, 17, 19, 57). Nonetheless, the finding that Np-CGG-immunized neonates have mutations in heavy chain rearrangements lacking N regions suggests that somatic mutation can occur in B cells derived from the putative fetal lineage.

The number of mutations per V_H gene sequence seemed dependent on which Ag was used for immunization. The majority of mutated V(D)J sequences from (T,G)-A–L-mBSA immunized neonates had only one or a few nucleotide changes from the germline H10 gene. In contrast, most of the mutated V_H sequences from Np-CGG-immunized neonates had more than two changes from the germline sequence. Thus, even though the same V_H genes in the V_H SM7 gene family were analyzed for mutations, the level of mutation (the number of changes per sequence) varied depending on the Ag used. We do not think that this differential response is intrinsic to the neonate. Rather, our findings in the neonate are consistent with an earlier observation made in adult mice comparing the responses to Np and to the hapten phosphorylcholine, where the number of mutations per sequence found in the adult primary response (days 10–14) to phosphorylcholine was much lower than that to Np (50). Whether the observed variability in the level of somatic mutation in both adult and neonatal B cells is a function of the particular V_H/V_L combinations used, the nature of the Ag, and/or selection events in the germinal center is not understood. One possibility is that the H10/V_k1 germline V gene combination that dominates the primary Ab response to (T,G)-A–L (5, 32) provides sufficient binding affinity to reduce the need for generation and selection of B cell variants with higher affinity for the epitope(s) recognized on this polypeptide Ag, perhaps similar to the unmutated, high affinity, early primary IgG response of mice to the glycoprotein of vesicular stomatitis virus and the concomitant lack of affinity maturation in the memory response to this Ag (58–60). Analysis of affinity maturation and somatic mutation in the H10/V_k1-memory Ab response to (T,G)-A–L should help clarify this.

Several studies have indicated that mouse neonatal and adult B cells do not give equivalent responses after ligation of their B cell receptors (surface Ig). For example, compared with adult B cells, neonatal B cells do not proliferate or up-regulate levels of class II after surface Ig ligation (61, 62). Adult vs neonatal B cell differences in response to CD38 ligation have also been reported (63). Our data indicate that even though certain signaling differences may exist, neonatal mouse B cells, like adult B cells, can activate the mechanism for somatic mutation. Our studies thus add another dimension to the capabilities of the neonatal B cell population. It is now recognized that exposure of neonatal lymphocytes to Ag

FIGURE 5. The V(D)J joins of the clones from Np-CGG-immunized adult and neonatal mice. Joins are designated by alphabetical letters and correspond to the clones listed in Table III. PV, PD, and PJ refer to templated P nucleotides from the V_H, D, or J_H genes, respectively. N refers to nontemplated nucleotides. Mutations are bolded. The D gene used in the B join of clone A2.a could derive either from Sp2.5-C or Sp2.8-C (TTACC) or from FL16.1 (TATTAC); the latter is used in this figure. The G join of clone A2.a has a 1-bp mutation in J_H1 (TAC GGG TAC TTC...). The D join of clone A3.a has a 1-bp mutation in the DSp2.2 gene (.TAT GGT GAC..). The F join of clone A3.c has a 2-bp mutation in J_H4 (.TAC TAT TCT TCT GAC...). The D gene used by the G join of clone N1.1.a could not be determined: it is assumed that the T in codon 98 (AGT) derives from a D gene and not from an N nucleotide or from J_H4. The G join of clone N1.1.a has a 3-bp mutation in the J_H4 gene (GCT CTG GAC TCC TGG GGT CCA GGA...). The underlined bases in codon 97 of join H could have derived from either the V_H gene or the D gene; the underlined base in the PD of join H could also derive from J_H4. The I join of clone N2.1.a has a 2-bp mutation in the J_H4 gene (.TAC TAT TCT GAC..). The J join of clone N1.2.b has a 1-bp mutation in the DFL16.1 gene and a 1-bp mutation in the J_H4 gene (.TAC TAT TCT GAC..). The underlined base(s) in the D genes of joins J and M could also derive from J_H4 and J_H2, respectively. The J join of clone N1.2.b has a 1-bp mutation in the DFL16.1 gene and a 1-bp mutation in the J_H4 gene (.TAC TAT TCT GAC..). In joins K, L, the underlined AG in codon 98 could also come from the D gene (DSp2.10). Joins K and L have a mutation in codon 97 (TCT for join K, ACT for join L). The K join of clone N2.1.a has a 3-bp mutation in J_H4 (TAT TCT ATG AAC TAC..).
neel not lead to tolerance induction. Recent studies have illustrated that the neonatal mouse can mount the cellular and humoral immune responses generally regarded as important and necessary for protective immunity to infection. Mice immunized at birth can generate functional T cell responses, both helper and cytotoxic (7–9, 64–70). In some cases, responses appear to be Ag dose dependent (7–9), perhaps reflecting a lower number of T cells or decreased activity of APCs in the neonate (8, 9, 71). Although some studies have shown a skew toward Th2 cells after in vivo immunization of the newborn, which may be a function of Ag dose or mode of immunization, both Th1 and Th2 effector cells can be generated during the first week of life after Ag priming (7, 9, 65–70). It is also evident that the B cell population in the newborn has the functional capabilities of the adult in mounting responses to both conventional Ags and DNA vaccines; in vivo immunization of the newborn mouse can lead not only to Ab secretion, isotype switching (IgG2a, IgG2b, IgG3, as well as IgG1), and memory cell generation (6, 7, 64–70), but also to somatic mutation, as we have shown here.

In many countries, it is customary to vaccinate against several commonly occurring childhood diseases to induce immunity and ameliorate disease. If our finding that somatic mutation occurs in mice immunized early after birth can be extrapolated to human ontogeny, it has important implications for vaccine immunity in infants: immunization early in childhood should generate both memory cells and Abs of higher affinity, due to mutation and selection. This may be of particular relevance in circumstances where the infant has not received protective immunity via maternal Abs. The identification of heavy chain transcripts with JH mutation, as we have shown here.

References

44. Cline, J. E. C. Braman, and H. H. Hogrefe. 1996. PCR fidelity of phu DNA poly-
merase and other thermostable DNA polymerases. Nucleic Acids Res. 15: 3546.
46. Shreahm, K. M., C. A. Mainville, S. Willert, and P. H. Brodeur. 1993. The uti-
lization of individual V\textsubscript{H} exons in the primary repertoire of adult BALB/c mice. J. Immunol. 151:5364.
47. Shlomchik, M. D., N. Nemaze, J. Van Snick, and M. Weigert. 1987. Variable region sequences of murine IgM anti-IgG monoclonal autoantibodies (thema-
toid factors). II. Comparison of hybridomas derived by lipopolysaccharide stim-
eecules created by gene amplification interfere with the analysis of somatic hy-
terations in V\textsubscript{H} genes expressed by independently generated hybridomas appar-
54. Lam, K.-P., and A. M. Stall. 1994. Major histocompatibility complex class II expression distinguishes two distinct B cell developmental pathways during on-
64. Singh, R. R., B. H. Hahn, and E. E. Sercarz. 1996. Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune devia-
72. Mortari, F., J.-Y. Wang, and H. W. Schroeder, Jr. 1993. Human cord blood antibody repertoire: mixed populations of V\textsubscript{H} gene segments and CDR3 distri-